*In vitro* study of bone marrow derived progenitor
cells in liver-like microenvironments

By

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A thesis submitted in conformity with the requirements for the degree of
Masters of Applied Sciences and Engineering

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"In vitro study of bone marrow derived progenitor cells in hepatic microenvironments"

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Abstract

Published observations of the in vivo differentiation of bone marrow (BM) derived cells into hepatocytes led us to study the mechanisms that regulate this differentiation in vitro. Phenotypically defined (Lin-) BM populations were cultured in microenvironments that mimic parenchymal and nonparenchymal liver components. A significant difference in the spectrum of cells generated from BM under various conditions was observed. Specifically, enriched BM cells gave rise to endoderm-like cells in co-culture with fibroblasts.

Lin- cells were shown to generate liver-like cells that expressed CK8 and albumin upon exposure to high HGF concentrations. Also, individual hematopoietic stem cells (HSC - c-kit+ Lin- Sca-1+) were shown to give rise to albumin-secreting cells, in presence of HGF while the addition of hematopoietic cytokines decreased the frequency of hepatic progeny. These results suggest that HGF is an important regulator of HSC, which may act in inducing HSC to give rise to hematopoietic or hepatic progeny.
"If error is corrected whenever it is recognized as such, the path of error is the path of the truth."
- Hans Reichenbach (1891-1953)

"And ye shall know the truth, and the truth shall make you free."
- The Bible John 8:32

“What is now proved was once only imagined”
William Blake (1751-1827)

"The tragedy of the world is that those who are imaginative have but slight experience, and those who are experienced have feeble imagination. Fools act on imagination without knowledge, pedants act on knowledge without imagination. The task of a university is to weld together imagination and experience."
Alfred North Whitehead (1861-1947)
"When I was a boy of 14 my father was so ignorant I could hardly stand to have the old man around. But when I got to be 21, I was astonished by how much he had learnt in 7 years". This quotation by Mark Twain seems accurate in describing my maturation and development as an undergraduate and graduate student at U of T.

For these experiences I am indebted to my supervisors Prof. P.W. Zandstra and Prof. M.V. Sefton. I would like to thank Peter for taking on an arrogant and naive student and turning him into a more knowledgeable and mature (and still somewhat arrogant) researcher. I also would like to thank Peter for allowing me to work on such a fascinating scientific area as stem cell bioengineering. Furthermore, I would like to thank my original mentor, Prof. M.V. Sefton for his guidance and teachings over the past few years as well as providing me with resources to conduct independent research, and freedom to explore my ideas and broaden my imagination. I have been blessed with excellent teachers and the goal of having the same positive impact on my students will be a lifelong challenge.

I would like to thank Dr. Bill Stanford for his guidance, and for giving me access to "glowing mice", which was a key component of this project. Also, I am grateful to my thesis committee members Drs. Grant Allen and Jane Aubin for their time and critical assessment of this work.

I have benefited greatly from my association with all past and present members of Sefton and Zandstra lab, in particular Shahab Lahooti, Michael May, Kim Jones, Jennifer Vallabacka, Alison McGuigan and Eric Tsang (in Sefton’s lab) as well as Gerard Madlambayan, Sowmya Viswanathan, Karen Chang, Celine Bauwens, Elaine Fok and Roni Dattani (in Zandstra’s lab). A simple thank you is insufficient for Ting Yin for her technical assistance throughout my stay at Zandstra’s laboratory.

My graduate work has also been a learning experience in teamwork and leadership in organizations such as BESA, CEGSA and tissueeng.net. I would like to thank all staff and students whose interactions aided in my development into becoming a more complete individual. In particular, I would like to acknowledge Nanthan Yogachandran, Jeff Karp, Paul Dalton, and XuDong Cao for their support.

I also would like to acknowledge the funding sources for Prof. Sefton and Prof. Zandstra as well as NSERC-PGSA for providing the support for my graduate studies. Finally, I would like to thank my family, friends and Yu San for their unconditional support and love.
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<th>Description</th>
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<tbody>
<tr>
<td>2-AAF</td>
<td>2-acetylaminofluorene</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>AFP</td>
<td>alpha-fetoprotein</td>
</tr>
<tr>
<td>AS cells</td>
<td>adult stem cells</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>BEC</td>
<td>biliary epithelial cell</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>CFC</td>
<td>colony forming cell</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFU-E</td>
<td>colony forming unit - erythroid</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>colony forming unit - granulocyte, erythroid, monocyte, megakaryocyte</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony forming unit - granulocyte, monocytes</td>
</tr>
<tr>
<td>CFU-S</td>
<td>colony forming unit - spleen</td>
</tr>
<tr>
<td>CK</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>D</td>
<td>daltons</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunoassay</td>
</tr>
<tr>
<td>Epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FAH</td>
<td>fumarylacetoacetate hydrolase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FITC</td>
<td>fluorescein</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FL</td>
<td>FLT-3 ligand</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transpeptidase</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>HM</td>
<td>hematopoietic medium</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
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<tr>
<td>IF</td>
<td>intermediate Filament</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KLS</td>
<td>Lin’ Sca-1” c-kit”</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>Lin’</td>
<td>Lineage depleted</td>
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<tr>
<td>LT-HSC</td>
<td>long-term hematopoietic stem cells</td>
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<tr>
<td>LTBMC</td>
<td>long-term bone marrow culture</td>
</tr>
<tr>
<td>LTC</td>
<td>long-term culture</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>long-term culture initiating cell</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>mL, ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NSC</td>
<td>neural stem cells</td>
</tr>
<tr>
<td>OSM</td>
<td>oncostatin M</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>cyanine 5</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PH</td>
<td>partial hepatectomy</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>rh</td>
<td>recombinant human</td>
</tr>
<tr>
<td>rm</td>
<td>recombinant murine</td>
</tr>
<tr>
<td>Sca-1</td>
<td>stem cell antigen - 1</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor (a.k.a. steel factor or c-kit ligand)</td>
</tr>
<tr>
<td>SP cells</td>
<td>side population cells</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short-term hematopoietic stem cells</td>
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<td>TPO</td>
<td>thrombopoietin</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethyl benzidine</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WBM</td>
<td>whole bone marrow</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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The behavior of bone marrow (BM) derived progenitor cells in hepatic microenvironments was investigated. With the underlying premise that BM derived cells exist in liver and have the capability to reconstitute hematopoiesis and generate functional liver cells, the objective of this project was to develop a culture system to investigate the effects of various microenvironmental parameters on hematopoietic and hepatic differentiation of BM derived progenitors under controlled conditions. As a first approach to study this behavior, liver-like microenvironments were developed using primary hepatocytes as well as hepatocyte and fibroblastic cell lines. The ability of various BM populations in these cultures was evaluated in terms of their viability, proliferation, and hematopoietic and hepatic gene expression. The results from these experiments provided the basis for a more mechanistic study of specific signals which regulate BM derived cell fate in liver microenvironments. More specifically, the effects of TPO and HGF were investigated.

The thesis is divided into the following sections. Chapter 1 provides the background for the work and introduces the previous research regarding various aspects of the project. Chapter 2 outlines the hypotheses and objectives of this thesis. Chapter 3 provides the rationale and the approach taken in order to achieve the objectives of the work. Chapter 4 provides a detailed account of the procedures and methods used in developing the co-culture system. In addition, a full description of the analytical procedures is provided. These detailed instructions are intended to assist readers in performing the experiments described in this thesis. Chapter 5 outlines the results obtained in this work in four parts. In part 1, different populations of BM derived progenitors are characterized. In part 2, the results regarding the development of the culture system and the difficulties encountered with their setup are explained. In part 3, the results regarding the hematopoietic maintenance in various cultures are analyzed, while in part 4, the endoderm differentiation of BM derived cells is studied. Chapter 6 provides a discussion of the results of the project. Chapter 7 provides an outline of the conclusions and recommends directions for future work.
1 INTRODUCTION AND BACKGROUND

1.1 Stem cell therapy in tissue engineering

Despite attempts to encourage organ donations\(^1\)\(^2\), there is a shortage of transplantable human tissues such as bone marrow (BM), hearts, kidneys, livers and lungs. For example, currently more than 74000 patients in the United States (USA) are awaiting organ transplantation, while only 21000 people receive transplants annually\(^3\). This gap between eligible patients and available organs is growing constantly\(^4\). It is from these motivations that tissue engineering has emerged.

Tissue engineering is an interdisciplinary field that applies the principles of biology and engineering to the development of viable substitutes, typically composed of biological and synthetic components which restore, maintain, or improve the function of human tissues\(^5\)\(^6\). This generally differs from standard drug therapy in that the engineered tissue becomes integrated within the patient, providing a potentially permanent and specific cure for the diseased state.

One approach currently under development to alleviate human tissue shortage is xenotransplantation. Xenotransplantation, despite its ethical and immunological considerations\(^2\) may overcome the current shortage of organ donors. To prevent rejection of xenotransplants, immune reaction to transplanted organs has been minimized using immunosuppressive drugs (i.e. cyclosporine) or genetic modification of donor specific antigens. However, significant challenges such as carcinogenic and toxic effects of immunosuppressive drugs\(^7\) and cross-species viral infections\(^8\) still remain. These side effects have encouraged researchers to search for alternative methods of cell delivery.

An emerging therapeutic approach to overcome the short supply of transplantable cells is the use of stem cells. Stem cells are cells that are capable of extended self-renewal as well as the ability to give rise to many functional tissue types (i.e. differentiate into multiple lineages). Because stem cells can differentiate and give rise, typically in a cascade of progressive maturation steps to progeny with the functional and genetic properties of mature cells, they represent a potential source for tissue and cellular engineering. For example, adult or embryonic stem cells may one day be used to generate cardiomyocytes for cardiac muscle regeneration, pancreatic islet cells for diabetes, liver cells for hepatitis, and neural cells for Parkinson's or Alzheimer's diseases.

It is known that highly differentiated cells can only divide a limited number of times\(^9\)\(^,\)\(^10\). Stem cells are advantageous in regenerative medicine because of their capability to self-renew (i.e. give rise to cells with the same potential and genetic expression as the original cells). As evident by the developmental potential of embryo-derived cells or by the ability of hematopoietic stem cells (HSC) to
reconstitute primary and secondary hosts' hematopoietic system in BM transplants, a small number of stem cells can produce a therapeutically significant amount of tissue. Due to these properties, stem cells can be applied to regenerate tissues directly, such as in BM transplantation, or in conjunction with tissue engineering constructs, thus providing a method for in vivo tissue engineering. Furthermore, stem cells are suitable gene therapy vehicles (since the gene product may be expressed in all their progeny), making it possible to achieve a life long correction for a specific disorder.

It has been estimated that in the USA alone, over 128 million people can be helped by the resulting benefits from stem cell research. However, despite this promise, therapeutic use of stem cells has been limited because of our limited knowledge of the microenvironmental factors which affect stem cell differentiation and self-renewal. Clearly, in order to utilize stem cells in clinically relevant applications, an improved understanding of the microenvironmental cues that govern their behavior is required. Therefore, further research into understanding stem cell microenvironments will be of great therapeutic value by providing an ample supply of cells for transplantation.

1.2 Sources of stem cells

Stem cells can be divided into two distinct groups: embryonic stem (ES) cells and adult stem (AS) cells. ES cells are pluripotent (i.e. capable of generating all differentiated cells of the body) cells that can be derived from the inner cell mass of the developing blastocysts. In contrast, AS cells are multipotent cells that reside in adult tissues and are believed to be important in tissue regeneration and maintenance.

1.2.1 Embryonic stem cells

Although rodent ES cells have existed for many years, the interest in the clinical use of embryo-derived cells was ignited by the derivation of human ES cells. ES cells can be genetically modified, produced in large numbers, and differentiated prior to use in regenerative medicine.

Excitement over the use of ES cells has increased with their demonstrated ability to give rise to hematopoietic, endothelial, cardiac, neural, osteogenic, hepatic, and pancreatic tissues in vitro. However, despite their therapeutic potential, ES cells present a number of challenges associated with their clinical application. For example, ES cells may have immunological incompatibility with recipients. To overcome immune rejection, ES cells would require a cloning step in which the nucleus of a somatic cell from the recipient is transferred to the donor cells in order to overcome the possibility of tissue rejection. However, this step may be limited by the availability of human eggs and the possible generation of abnormal embryos from the intrinsic genetic instability of
cloned cells\textsuperscript{29}. Furthermore, transplanted ES cells may form tumors; thus, great care must be taken to ensure that all cells are fully differentiated. Also, more research is required to understand the signals that direct ES cell differentiation into various lineages.

One of the major challenges in the widespread use of human ES cells is due to bio-ethical controversy in using cells that require the destruction of embryos. Furthermore, despite enormous patient support\textsuperscript{12}, ES cell research has encountered significant resistance\textsuperscript{30,31} by people who oppose their derivation from human embryos for fear that their use will lead to the creation of genetically engineered human beings\textsuperscript{32}. Due to these difficulties, along with the increased potential of AS cell research described in sections 1.4.2 and 1.2.2, some legislators oppose ES cell research. However, the majority of scientists advocate ES cell as well as AS cell research\textsuperscript{33}.

1.2.2 Adult stem cells and plasticity

In contrast to their embryonic counterparts, AS cell therapy has been used in the form of BM transplantation for many years. AS cell research does not have the ethical issues associated with the use of embryos since they can be derived from the patient and thus have the potential to be histocompatible with the patient. Also, AS cells may not form tumors as readily in patients, as BM transplantations have demonstrated. However, the use of AS cells was thought to be limited since they have been isolated from relatively few tissues and have been considered to generate only a narrow spectrum of cell types.

Traditional dogmas regarding stem cell differentiation held that organ specific stem cells are restricted to generate differentiated cell types from the tissues in which they reside. Thus, they were thought to be much more restricted in their developmental capabilities than ES cells. This dogma was supported by embryological experiments in which cells of undifferentiated tissue transplanted from one region of the embryo to another, quickly lost their ability to become part of the new tissue\textsuperscript{13}. However, new evidence suggests that AS cells have much greater regenerative capability than previously believed.

The first demonstration that AS cells may be less lineage restricted than previously thought was shown with the \textit{in vivo} derivation of myogenic progenitors from whole bone marrow (WBM)\textsuperscript{34} cells; as well as the \textit{in vitro} differentiation of BM stroma cells into contractile myotubes\textsuperscript{35} and cardiomyocytes\textsuperscript{36}. In addition, muscle cells were shown to give rise to hematopoietic cells\textsuperscript{37}. Further experiments revealed that enriched HSC were capable of migrating to muscle\textsuperscript{38} and differentiating into muscle-like cells\textsuperscript{39} (however, despite initial optimism\textsuperscript{40}, recent results have questioned the functional capability of BM derived muscle fibers\textsuperscript{41}). Furthermore, Bjornson et al demonstrated that neural-cell
cultures containing neural stem cells, which were thought to be limited in potential to the development of neurons, oligodendrocytes and astrocytes, could differentiate into blood cells. Moreover, BM derived cells differentiate into astroglia and microglia following injection into the brain and adherent BM stroma have been shown to give rise to neurons and glia.

The most convincing evidence regarding the multi-tissue regenerating potential of AS cells is in the ability of HSC to give rise to cells of other tissues such as hepatocytes or other epithelial cells (see Section 1.4.4.3). Definitive proof of the multi-organ generating capability of BM derived stem cells was recently demonstrated by Krause et al, who showed that a single cell, which homed to the BM, could reconstitute hematopoiesis in primary and secondary recipients as well as differentiate into epithelial cells of the liver, lung, intestine and skin.

Researchers have thus begun to question the classical germ layer origin of tissues. An emerging model for the hierarchal structure of stem cells is shown in Figure 1. In this model, early embryonic development is initiated from undifferentiated totipotent cells. These totipotent stem cells give rise to pluripotent ES cells, which generate the three germ layers and perhaps putative somatic stem cells. The somatic stem cells are multipotent stem cells, and tissue-restricted stem cells may derive from multipotent stem cells. Hematopoietic stem cells (HSC), muscle stem cells (Muscle-SC), and neural stem (CNS-SC) cells may be multipotent stem cells or tissue-restricted stem cells that dedifferentiate into multipotent stem cells.

Despite numerous scientific papers on the subject, AS cell transdifferentiation or multi-organ regenerating capability has not yet been clearly understood. Most papers have failed to quantify the

Figure 1-1 A model for stem cell differentiation
degree of transdifferentiation or to evaluate functionality of the transplanted cells in the host. Furthermore, most literature has failed to identify the progenitor or the importance of in vitro culturing prior to transplantation. It is not clear whether AS cells transdifferentiate through gene reprogramming when exposed to the novel microenvironment or if there exists a primitive adult cell that has significant multi-tissue regenerating capability. Studies that demonstrate transdifferentiation without culture manipulation suggest that the tissue environment is a critical factor. Therefore, the reason for the inability of specific AS cells to give rise to certain tissues may be because of their failure to access them in vivo\textsuperscript{47}. If this is the case, then in vitro culture systems which mimic in vivo conditions (as developed in this thesis) as well as genetic modifications leading to receptor expression which preferentially lead to localization in a specific microenvironment may be critical in developing future cell based regenerative therapies.

However, despite uncertainties regarding the intrinsic potential of AS cells, the ability of cells of one tissue to give rise to differentiated cells of another tissue will be of great clinical significance. This ability may allow for the expansion of AS cells of one tissue prior to differentiation into cells of a different tissue, which may overcome much of the shortcoming and criticism associated with difficulties in expansion of certain AS cells in vitro. Furthermore, this ability may provide alternative AS cell sources that are easier to access (i.e. peripheral blood or fat-derived stem cells) as substitutes for traditional sources (i.e. BM derived).

1.3 Motivations for the use of cell therapy in liver tissue engineering

The physiological functions of the liver include bile production, deamination of amino acids and maintenance of constant blood glucose concentration as well as metabolism of proteins (such as urea). The liver also synthesizes a number of plasma proteins such as albumin and coagulation factors; it detoxifies toxic substances using enzymes (such as alcohol dehydrogenase or cytochrome P (CYP) enzymes) and provides storage for iron and vitamins A, D and B12. In addition, Kupffer cells (liver macrophages) eliminate damaged erythrocytes. With these properties, the liver is critical to health and its functional loss will quickly lead to multiple organ failure.

There are over 200,000 patients in the US who are suffering from liver diseases such as liver cancer, cirrhosis and metabolic disorders\textsuperscript{6}. Historically, technologies that have aimed to replace liver function have focused on removal of toxins through means such as dialysis, immobilized enzymes, or exchange transfusions\textsuperscript{48}. However, these methods do not provide the patient with the full functionality of a healthy liver. In order to regain the full potential of a functional liver, hepatocytes must replace the lost organ. Although a number of cell transplantation devices have been developed to treat liver failure, such as hepatocyte microencapsulation and artificial liver devices\textsuperscript{6,48-51}, the shortage of transplantable
hepatocytes remains a significant barrier to the therapeutic applications of liver tissue engineered devices.

The difficulty in obtaining transplantable hepatocytes has been enhanced by the obstacles encountered in expanding hepatocytes in vitro. Stem cells have been proposed as an ideal source for generating functional hepatocytes. The use of AS cells is particularly inviting for liver therapy since, for chronic or acute liver failure, a temporary treatment with artificial liver devices provides sufficient lag time to grow transplantable liver tissue in vitro.

1.4 Liver architecture, function and regeneration

1.4.1 Liver function and architecture

The liver, which lies in the upper right abdominal cavity beneath the diaphragm, is the largest visceral organ and the largest gland in the body. It weighs approximately 1.5 kg in humans and 1-2 g in mice. Because it is functionally located between the gastrointestinal (GI) tract and the heart, the liver has a dual blood supply. It receives oxygenated blood from the general circulation via the hepatic artery (25%) and a larger volume of poorly oxygenated blood draining the GI tract, stomach, pancreas, spleen, and small and large intestines via the portal vein (75%). The hepatic tissue is a highly vascularized tissue in which hepatocytes (which make up the parenchymal portion of the liver) provide the body with diverse metabolic functions, while nonparenchymal cells usually serve structural and regulatory purposes. The liver is comprised of 3 major lobes (see Figure 1-252), which consist of hexagonal columns of hepatocytes arranged radially from the central vein (see Figure 1-352). The hepatocyte columns are covered by sinusoids on each side.

Figure 1-2 The surface of the liver

Figure 1-3 A single liver lobule
The liver contains two major differentiated endoderm cell types: hepatocytes located in the hepatic parenchymal plates and biliary epithelial cells located in the bile ducts. The most abundant and functionally important cells within the liver are the hepatocytes, which account for 95% of the liver mass and 60% of the total liver cell numbers. Hepatocytes are large (18-30 μm), polyhedral cells that are rich in organelles such as rough endoplasmic reticulum, smooth endoplasmic reticulum, mitochondria and Golgi complexes which are reflective of their functional capabilities.

Besides hepatocytes, the mammalian liver is composed of biliary epithelial cells (BEC or cholangiocytes), as well as several cell types presumed to be of mesenchymal origin including endothelial cells, Kupffer cells (macrophages in hepatic sinusoids) and stellate cells (or Ito cells – unique to the liver and located under the sinusoids where they store vitamin A, synthesize connective tissue proteins and secrete several growth factors). The liver also contains nerve cells and cells entrained in perfusing blood. The majority of these cells are associated with hepatic sinusoids, which are large, irregular shaped capillaries. These cells are separated from the hepatocytes by a narrow space within which stellate cells lie (thus controlling sinusoid lumen aperture). Cholangiocytes are the most abundant cells next to hepatocytes. They constitute about 5% of the normal liver population and occupy no more than 1% of the total liver mass and are typically much smaller than hepatocytes (~10 μm). Kupffer cells reside within the lumen of the sinusoids. The sinusoidal lining is comprised of endothelial cells (~48% of total sinusoid cells), Kupffer cells (~29%) and stellate cells (~20%).

1.4.2 Liver and bone marrow interactions

Throughout embryonic development, there is a very close association between the fetal liver and hematopoiesis (see Figure 1.4). In mice, primitive hematopoiesis occurs within the blood islands of the yolk sac at embryonic day 7 (E7). Blood islands fuse and establish blood vessels and circulation, and with it hematopoiesis shifts from embryonic yolk sac to fetal liver at E11 after the emergence of the liver bud from the endoderm lining of the ventral foregut. The fetal liver contains hepatoblasts, the bipotential progenitors that express alpha-fetoprotein (AFP), cytokeratin 8 (CK8), cytokeratin 18 (CK18) and gamma-glutamyl transpeptidase (GGT – a protein associated with most fetal epithelial cells in the liver), and subsequently become restricted to cholangiocytes or hepatocytes postnatally.
In fetal liver development, hematopoietic cells induce the differentiation of hepatoblasts into hepatocytes by secreting Oncostatin M (OSM)\textsuperscript{63}. Also, Fibroblast growth factor 8 (FGF8), produced by the liver mesoderm tissue, contributes to the morphogenic outgrowth of the hepatic endoderm\textsuperscript{64}. The fetal liver remains the major hematopoietic organ during mid and late gestation. At E15, shortly prior to birth, hematopoiesis begins to shift to spleen and BM. The hepatocytes assume their polarized definitive differentiation only just before birth\textsuperscript{65}.

The liver and BM interaction continues throughout adulthood. It has been shown that rare Lin\textsuperscript{−} c-kit\textsuperscript{−} Sca-1\textsuperscript{−} murine cells with hematopoietic repopulating ability reside in the liver\textsuperscript{66,67}. Furthermore, adult liver can become the site of hematopoiesis during myeloproliferative disease such as myelofibrosis\textsuperscript{68}.

1.4.3 Liver regeneration

In the process of normal tissue renewal in the liver, only one in 10000-40000 hepatocytes are dividing at any one time\textsuperscript{69-71}. Although the liver is a quiescent organ, terminally differentiated hepatocytes are able to undergo active proliferation after cell loss\textsuperscript{72}. In fact, division of mature cells is the most common pathway utilized in the ‘neoformation’ of liver parenchymal cells\textsuperscript{73}. For example in 2/3 partial hepatectomy, a major portion of the liver mass can be surgically removed without permanent damage. In this process, the mature hepatocytes are first activated, followed by a delayed proliferation of cholangiocytes and sinusoidal cells. This regenerative capability has even been mentioned in Greek mythology in which Prometheus’s liver was subjected to daily partial hepatectomy by a bird after he stole the secret of fire from the gods and passed it to humans\textsuperscript{74,75}.

In normal liver regeneration, “unipotential” hepatocytes as well as all other cellular populations within the liver can proliferate to replenish the lost tissue mass\textsuperscript{72,76}. Usually, these cells have the capability to regenerate the liver. For example, hepatocytes have been shown to be capable of
undergoing 12-16 cell divisions, which would normally suffice for regenerating the lost tissue. However, under conditions of severe damage or impaired hepatocyte division due to toxic injury (e.g. chemical carcinogenesis), the stem cell compartment of the liver is activated.

1.4.4 Liver progenitor hierarchy

The hematopoietic cells, or other cells such as skin, rely on the participation of stem cells in their renewal process. Although the liver is different from these systems, in that it is composed of highly differentiated cells which retain replicative potential, the existence of liver stem cells has been reported. However, until recently, the purification of liver stem cells capable of multilineage differentiation into hepatic and biliary cells had eluded researchers. With recent evidence, a picture of liver stem cell hierarchy is emerging, comprised of BM derived stem cells, oval cells and small hepatocytes.

Figure 1-5 Interactions between hepatic stem cells.

Pathways for derivation of various hepatic cells are indicated in embryonic (blue arrows) and adult tissues (orange), (dotted arrows indicate pathways which have not been conclusively proven). H-CFU-C (c-kit/CD45/TER119) are cells isolated from murine fetal liver which express hepatic and bile duct markers and were recently introduced as hepatic stem cells.

1.4.4.1 Small hepatocytes

Small hepatocytes are a population of cells initially isolated from rat liver and subsequently shown to cause clonal expansion of hepatocytes in humans. These cells comprise 1-2% of hepatocytes and are able to undergo 5-6 cell divisions within a few days and yet retain hepatocyte
phenotype in vitro. The precise origin or location within the liver of these cells has not been defined. It has been proposed that oval cells (the more primitive liver cells) give rise to small hepatocytes. Furthermore, since these cells have not been shown to give rise to cholangiocytes in vivo, they seem to be the direct precursors of hepatocytes. However, a cell fraction of small hepatocytes, grown clonally in culture, has been shown to give rise to biliary epithelial cells, which leaves the possibility that these cells may be able to give rise to cholangiocytes under certain conditions.

1.4.4.2 Oval cells

Until recently, oval cells were thought to be the most likely candidate for liver stem cells. Oval cells appear in the liver of adult animals when the liver damage is so severe that hepatocytes cannot proliferate or are somehow prevented from proliferating (by either exposure to hepatotoxins or carcinogens alone or combined with other surgical or dietary regimens). They reside in a heterogeneous population of nonparenchymal cells and are known by the morphological descriptor "oval cells". Oval cells are believed to originate from the canals of Herring (that is the region where cells are transitional between the periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts) or blast like cells located next to bile ducts.

Oval cells are thought to have both clonogenic and bipotential capacity to proliferate and differentiate into both hepatocytes and biliary epithelial cells. Interestingly, there is evidence that under certain conditions oval cells can be induced to differentiate into non-hepatic lineages including intestinal and pancreatic epithelium and mature cardiomyocytes in adult heart tissue.

Oval cells share numerous markers with fetal hepatocytes, including the expression of AFP, albumin, GGT, and the pattern of cytokeratin expression (CK8, CK18, CK19). Oval cells also express many phenotypical markers that are usually associated with HSC. In rodents, oval cells express CD34, Thy-1 (a marker that is present on rat HSC) as well as c-kit mRNA and proteins. In humans, oval cells and HSC share CD34 and c-kit. The presence of such markers was the first evidence that hepatic stem cells may in fact originate from the BM.

1.4.4.3 Bone marrow derived stem cells

Recent papers offer a new explanation for the location of liver stem cells. Based on evidence that a number of surface markers are shared between HSC and oval cells, Peterson et al demonstrated that BM derived cells have the potential to give rise to oval cells and to further differentiate into hepatocytes and/or cholangiocytes. By transplanting rat BM into lethally irradiated recipients and following the fate of syngeneic cells using various markers, they showed striking changes in the livers induced to
regenerate using 2-acetylaminofluorene (2-AAF) treatment (to block hepatocyte differentiation) followed by partial heptectomy (to induce oval cell regeneration). In these livers, evidence was presented to suggest that the donor cells migrated into the liver of recipient animals and subsequently underwent differentiation to become hepatocytes, though it was less clear whether bile duct cells developed.

A second published study used a similar approach but without a liver injury step\textsuperscript{97}. They observed that after lethally irradiating female mice and transplanting male donor BM cells, up to 2.2% of total hepatocytes were identified, (by simultaneously expressing Y chromosome and albumin mRNA) as donor derived. Since these irradiated mice had no demonstrable acute hepatic injury such as inflammation, necrosis, oval cell proliferation or scarring, it was concluded that BM derived hepatic differentiation could occur in absence of liver injury. In both studies, the number of cells which undergo the transition appears to be relatively small. In addition to rats and mice, generation of BM derived hepatocytes has also been demonstrated in human patients. In these results, the number of BM derived hepatocytes and cholangiocytes ranges from 4-43\% and 4-48\% respectively\textsuperscript{98,99}.

The experiments mentioned above mostly used WBM populations and left many questions unanswered regarding the identity of the BM population capable of differentiating into hepatic lineages. The identity of this cell was revealed in a series of experiments. These et al demonstrated that 200 CD34\(^{+}\)Lin\(^{-}\) cells were capable of giving rise to functional hepatocytes (~0.2-0.5\% of total hepatocytes) after 8 months\textsuperscript{97}. However, the most definitive demonstration of BM to liver transplantation was performed by Lagasse et al who showed that when a small number of c-kit\(^{+}\) thy\(^{-}\) Lin\(^{-}\) Sca-1\(^{+}\) cells (believed to be greatly enriched in HSC) was injected intravenously into lethally irradiated fumarylacetoacetate hydrolase (FAH\(^{-}\)) deficient mice (a model animal which shows progressive liver failure), the mice were rescued and the biochemical function of liver was restored. At seven months post transplantation, more than 30\% of the hepatocytes were shown to be donor derived\textsuperscript{100}. Furthermore, the ability of cells to perform non-hematopoietic reconstitution was limited to HSC. In experiments in which a single HSC was transplanted into irradiated hosts, donor derived cells which morphologically resembled hepatocytes and cholangiocytes\textsuperscript{45} have been observed. Unfortunately, the antibodies that were used do not stain liver cytokeratins\textsuperscript{101}, thus a definitive demonstration of this differentiation was not possible.

It is not yet determined whether HSC or their progeny seed and engraft to the liver\textsuperscript{100}. \textit{In vivo} experiments have demonstrated two distinct modes of hepatocyte engraftment\textsuperscript{99}, which may suggest distinct mechanisms of growth. In the first, BM derived cells dispersed throughout the liver, suggesting random integration into the parenchyma followed by clonal growth and differentiation, in a manner
similar to other liver cell transplantation studies, resulting in a cluster of hepatocytes. However, in the presence of liver injury, BM derived stem cells appear to become an oval cell-like intermediate with subsequent expansion and differentiation. Therefore based on these results, oval cells are derived from HSC, which then differentiate to small hepatocytes before fully differentiating into functional hepatocytes.

Evidence regarding engraftment of HSC into hepatocytes indicates that liver regenerative environments have higher frequencies of BM derived hepatocytes. In addition to demonstrating the importance of the liver microenvironment, this observation may provide important insight into generating in vitro conditions that allow for expansion and clinical use of such cells in regenerative medicine.

1.4.5 Liver growth factors

The mechanism by which hepatocytes and their progenitors exit from quiescence (G0) and enter into the proliferation cycle (G1-S) is triggered by the surrounding extracellular matrix (ECM), and growth factors such as hepatocyte growth factor (HGF), transforming growth factor - α (TGF-α), epidermal growth factor (EGF), tumor necrosis factor - α (TNF-α) and Interleukin 6 (IL-6) derived from non-parenchymal cells acting in paracrine or juxtacrine fashion. The results here focused on three cytokines, two of which (HGF and TGF-α) are directly involved in hepatic regeneration and one that is important in hematopoiesis (TPO).

1.4.5.1 Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a 90kD heparin binding glycoprotein that is secreted as a single polypeptide chain and cleaved extracellularly into a biologically active heterodimer. HGF is also called scatter factor because of its ability to spread coherently growing epithelial cells, a property which is attributed to the disruption of cell-cell junctions. Furthermore, HGF induces variety of other responses in a variety of cells ranging from mitogenesis, cell motility and matrix invasion. HGF activates a transmembrane tyrosine kinase receptor encoded by the Met proto-oncogene (c-Met). c-Met receptor is expressed in adult epithelial tissues including liver, intestine, kidney, hematopoietic precursors, endothelial cells, BM stroma, osteogenic and osteosarcomas. A similar expression pattern is found during development, where c-Met is expressed on embryonic erythroblasts, pancreatic cells, human and rat fetal liver and mouse yolk sac.
HGF is produced by a number of cells, including adult and embryonic fibroblasts, osteoblasts, monocytes/macrophages, B-cells, and smooth muscle cells. In the liver, HGF is produced by the non-parenchymal compartment in particular by stellate cells. In vivo extracts from pancreas, brain, thyroid, salivary gland and small intestine have been shown to contain HGF. Amounts per pancreas, brain, thyroid and small intestine are shown to be 300, 30, 76 and 250 ng/g tissue respectively.

HGF is essential for normal liver development. HGF is expressed in murine fetal liver through 2 weeks neonatal after which time its expression is no longer detected. HGF or c-Met knockout mice die in utero between E13-E16.5. In these animals the livers show retarded growth and contain large 'empty' sinusoidal spaces and the parenchymal cells appear dissociated and apoptotic. HGF also mediates mesenchymal-epithelial cell interactions in vivo and plays an important role in organ regeneration. HGF has potent mitogenic effects on hepatocytes and can rapidly induce the proliferation of adult rat hepatocytes in primary cultures. Although little HGF is present in adult liver, during liver regeneration pancreatic HGF can be directly transported to the liver through the portal circulation. It has also been proposed that HGF is constantly made available to the liver from exocrine pancreas and small intestine, and its continual mitogenic stimulation is balanced by mitotic inhibitors such as TGF-β. Very small amounts of TGF-β mRNA are present in the normal liver. TGF-β, an inhibitor of hepatocyte proliferation both in vivo and in vitro, inhibits hepatocyte proliferation induced by HGF in culture as well as HGF expression in stellate cells, thus regulating in part the balance between liver fibrosis and regeneration.

HGF is an important regulator of hematopoietic progenitor cells, derived from CD34⁺ cells in human BM, peripheral blood and umbilical cord blood. HGF has a stimulatory effect on colony forming cells (CFCs) and its addition to BM cultures enhances non-adherent cell numbers. In particular, erythroid progenitors are stimulated to grow and differentiate by the release of this growth factor in BM cultures. These effects are synergistic with the activity of granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), Interleukin-3 (IL-3) and SCF.

1.4.5.2 Transforming growth factor-α (TGF-α)

Transforming growth factor – α (TGF-α), is a ligand that binds with the epidermal growth factor (EGF) receptor. TGF-α is believed to regulate normal growth in epithelial tissues, and its overproduction in cells possessing EGF receptor is often correlated with malignant transformation.
TGF-α is also involved in cell growth accompanying the healing process in multiple organ systems, and it influences fetal liver development as well as liver repair following hepatotoxic damage or regeneration following partial hepatectomy. TGF-α is shown to be particularly important for early stages of liver regeneration, at which time hepatocytes are signaled through TNF-α receptor\textsuperscript{145} to produce and respond to TGF-α\textsuperscript{146} in an autocrine manner.

1.4.5.3 Thrombopoietin (TPO)

Thrombopoietin (TPO) (a.k.a. megakaryocyte colony stimulating factor) and its receptor c-mpl proto-oncogene act as stimulators of megakaryocyte differentiation\textsuperscript{147-150}. TPO is produced mainly by hepatocytes\textsuperscript{151,152} but also by kidney cells. However, factors regulating TPO expression are not well understood and do not seem to change with respect to liver injury\textsuperscript{152}.

The TPO receptor (c-mpl) is found on a large proportion of HSC from fetal liver as well as BM\textsuperscript{153,154} from which TPO enhances megakaryocyte formation\textsuperscript{155}. In the liver, the TPO receptor is found in endothelial cells and has been shown to induce the proliferation and cytokine regulation of these cells\textsuperscript{155}. No study of their effect on liver resident HSC has been performed.

TPO modulates the biological response of HSC both \textit{in vitro} and \textit{in vivo}. The addition of TPO to BM cultures has been shown to result in the generation of both long and short-term repopulating HSC, as detected by \textit{in vivo} competitive repopulating assays\textsuperscript{156}. Furthermore, it has been shown that targeted disruption of the mpl gene greatly reduces the number of spleen colony forming units after 12 days (CFU-S\textsubscript{S12})\textsuperscript{157}. It has been observed that TPO alone supports survival and modest proliferation of highly enriched HSC \textit{in vitro}\textsuperscript{158,159}. However, the direct effect of TPO on HSC expansion is under debate with some views expressing that TPO induces megakaryocyte differentiation, which in turn secretes other factors that alter the fate of HSC.

1.5 Hematopoiesis and hematopoietic stem cells

1.5.1 Hematopoiesis

Hematopoiesis is an active process in which blood cells are regenerated. For example, in adult humans, the red blood cell (RBC) pool of approximately 25 trillion cells is turned over every 120 days, which necessitates the production of approximately 200 billion cells per day to maintain hematopoiesis. This production is maintained by the continuous production of a large number of blood cells from a relatively small number of HSC that reside in the BM.
Great progress has been made over the past four decades to understand blood cell hierarchy since Till and McCulloch first reported the ability of the injected BM cells to form macroscopic colonies in the spleen of mice\textsuperscript{160}. It is believed that the process of blood production is arranged in an irreversible descending hierarchy (see Figure 1-6\textsuperscript{46}). In this scheme, a hematopoietic stem cell passes through several stages of differentiation to produce functional blood cells. Primitive HSC are divided into long-term (LT) and short-term (ST) HSC based on their functional capability to reconstitute hematopoiesis in mice. LT-HSC are slow cycling yet highly self-renewing cells that have the potential to reconstitute hematopoiesis in primary and secondary recipients. As these cells become more differentiated they give rise to ST-HSC and multipotent progenitors. These progenitors then become committed to be either common lymphoid or myeloid precursors (CLPs and CMPs respectively). CLPs migrate to lymphoid tissue where they divide and differentiate into B and T lymphocytes. CMPs remain in the BM and divide further until their progeny becomes committed to producing one type of blood cell. These committed unipotent progenitors proliferate significantly as they differentiate towards the mature form of the blood cell. Upon differentiation, these restricted progenitors give rise to erythrocytes, megakaryocytes, granulocytes and monocytes/macrophages (see Figure 1-6).

Figure 1-6 Hematopoietic differentiation hierarchy
1.5.2 Hematopoietic cell culture

In vivo, hematopoiesis resides in a well-defined microenvironment characterized by local geometry (structural and vasculature), by stromal cells (accessory cells of mixed origin), and by an extracellular matrix (ECM) composed of collagen-like molecules and proteoglycans (produced by stromal cells). Stromal cells and the ECM also provide surfaces for the presentation of adhesion molecules and cytokines (bound cytokines, which cannot be internalized by the recipient cells, act different than soluble cytokines). Previous studies into the maintenance of BM derived hematopoietic cultures have involved the use of numerous methods in order to mimic the in vivo microenvironment. However, despite advancements in the long-term culture (LTC) of hematopoietic cells in vitro, cultures of HSC usually result in dramatic proliferation coupled with a significant functional loss of HSC. This suggests that HS cell differentiation (not self-renewal) is the predominant fate of HSC in vitro.

1.5.2.1 Stroma mediated long-term cultures

One of the best in vitro models of hematopoiesis is the culture system developed by Dexter and colleagues. In these cultures, hematopoiesis is maintained for periods of several months in the form of nonadherent hematopoietic cells generated from initial cultures of whole bone marrow (WBM), which develop an adherent stroma layer containing a large variety of cell types including fibroblast, endothelial cells and adipocytes. The use of such stroma layers and the signals they provide to the HSC has been used as one method of initiating long-term bone marrow cultures (LTBMC).

The role of BM stroma is believed to be the creation of a microenvironment that is suitable for hematopoietic cell growth. In particular, the use of fibroblast feeder cells has been developed as a method of maintaining HSC cultures and measuring the frequency of long-term culture initiating cells (LTC-ICs – the LTC-IC assay tests for the presence of cells capable of initiating LTBMC and giving rise to progenitors that can be detected by replating into CFC assay after 5 to 8 weeks). Stromal cells condition the media by secreting cytokines and extracellular matrix molecules. Studies have shown that direct contact between stroma cells and the HSC is not required. By using cultures in which HSC are separated by a membrane from stroma cells, it has been demonstrated that LTC-ICs are better maintained in non-contact conditions. This indicates that the hematopoiesis obtained in these cultures is mediated by soluble factors and not direct cell contact.

1.5.2.2 Alternative cultures

With the increased knowledge of the molecules that are deemed as essential in regulating HSC fate, stroma free culture systems have been developed. These cultures provide more control in studying
the effects of specific signals on HSC behavior, and they may be desirable in clinical applications of ex vivo HSC expansion. Through research, a "cocktail" of cytokines has been developed which contains SCF, FL and IL-11 family of cytokines\textsuperscript{172}. 

Stem cell factor (SCF - a.k.a. Steel factor and c-kit ligand) is a hematopoietic growth factor that is important in regulating early stages of hematopoiesis. SCF has been shown to be critical in \textit{in vitro} maintenance and expansion of HSC. SCF or c-kit elimination in mice, leads to a profound reduction in the number of repopulating HSC\textsuperscript{173}. SCF administration has also been shown to expand the number of transplantable HSC\textsuperscript{174}. However, SCF alone cannot maintain HSC \textit{in vitro}\textsuperscript{175}. Thus, the interaction of other cytokines is important in maintaining undifferentiated HSC phenotype in culture. SCF has been shown to act synergistically with various growth factors including TPO (described previously), FL and IL-11 to induce proliferation and maintenance of myeloid, erythroid and lymphoid progenitors.

Flt-3 ligand (FL) is a hematopoietic cytokine that binds and signals through the transmembrane receptor Flt-3/Flik-2. Flt-3 receptor is expressed on CD34\textsuperscript{+}\textsuperscript{176} and c-kit\textsuperscript{high}\textsuperscript{177} fetal liver and BM HSC. FL is very important in hematopoiesis and Flt-3 receptor knockouts have a five-fold reduction in the number of long term repopulating stem cells\textsuperscript{178}. \textit{In vitro}, FL has been shown to synergize with a wide variety of hematopoietic cytokines (in particular SCF and the IL-11 family of cytokines\textsuperscript{179}) to stimulate the proliferation, self-renewal and differentiation of HSC\textsuperscript{180}. 

IL-11 and other family members such as IL-3, IL-6 and hyper IL-6 (HIL-6) are pleiotropic cytokines that have multiple effects on HSC. Although IL-11 does not seem to be necessary \textit{in vivo}, as demonstrated by the lack of hematopoietic defects in IL-11 knockouts mice\textsuperscript{181}, its administration \textit{in vitro} in combination with SCF and FL are sufficient for maintaining the number of HSC in serum free cultures\textsuperscript{182,183}. In our experiments, HIL-6 was used as a substitute for IL-11. Hyper IL-6 also interacts with GP130 receptor and has been has been shown to have the self-renewal effects on hematopoietic cultures\textsuperscript{180}. 

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2 OBJECTIVES AND HYPOTHESES

Although numerous in vivo studies have suggested the existence of BM derived cells capable of hematopoietic and endoderm differentiation, the mechanisms that govern this behavior have not been elucidated. The overall goal of this work was to develop an in vitro culture system to investigate the hepatic differentiation from BM derived progenitors. The objective of the thesis was to study the effects of cellular and microenvironmental parameters that mimic the regenerating liver on hematopoietic and hepatic differentiation of BM derived cells. In particular, the endoderm differentiation of BM derived cells into hepatic lineages was studied.

It was hypothesized that culture conditions designed to mimic liver regeneration will allow for the expression of markers and function associated with hepatic derived cells by hematopoietic stem cells.

![Figure 2-1 Possible factors that regulate BM derived cell fate in hepatic microenvironments](image)

Figure 2-1 Possible factors that regulate BM derived cell fate in hepatic microenvironments
3 APPROACH, AND DEVELOPMENT OF METHODOLOGIES

3.1 Creation of an *in vitro* liver microenvironment

The *in vivo* microenvironment is very complex and mechanisms that govern cell behavior are difficult to determine. Despite this difficulty, much progress has been made in determining important parameters that control cell fate decisions. While the specific parameters differ depending on the cell type involved, *in vitro* systems attempting to maintain or induce differentiation include extracellular matrices, appropriate cell-cell interactions and the presence of hormones, growth factors or 'differentiation inducers'.

To clarify the mechanisms of hepatic differentiation, we established culture systems in which the effects of various liver factors could be investigated. Our system was based on the co-culture of defined populations of yellow fluorescent protein (YFP) BM derived stem cells with hepatocytes, fibroblasts, soluble factors and extracellular matrices. At various times after initiation of the cultures, cells were harvested and analyzed using flow cytometry and fluorescent microscopy for the expression of endoderm intermediate filament markers cytokeratin 8 (CK8) and 18 (CK18), serum albumin as well as clonogenic CFC assays.

3.1.1 Hepatocyte isolation and culture

To establish a liver microenvironment, the feasibility of using primary hepatocyte cultures was investigated. However, culture and genetic manipulation of hepatocytes require a high degree of skill and long-term culture of hepatocytes is yet impossible.\(^{184}\)

To isolate hepatocytes, a common feature is the use of collagenase to promote tissue dissociation. The most efficient technique in isolating hepatocytes involves the use of a two-step perfusion method.\(^{185,186}\) This method involves perfusion through the hepatic portal vein by a calcium removing solution followed by collagenase containing medium. However, the use of a single step isolation method was adopted in this thesis for its ease of use as well as its relatively good yields.

Many attempts have been made to establish a primary hepatocyte culture for extended periods of time, such as the use of spheroid cultures, culture in well-defined media\(^{187}\), the addition of extracellular matrices (such as Matrigel\(^{188}\) and collagen) and co-culture with non-parenchymal cells\(^{189}\). In this study, hepatocyte cultures were established by modifying the ECM and the culture media.
3.1.2  Cell lines

Due to the difficulties in maintaining hepatocytes, the use of hepatocyte and fibroblast cell lines were considered. Fibroblast cell lines were used as a substitute for nonparenchymal cells while hepatocyte cell lines were used to test the effects of parenchymal liver cells on BM derived cells.

3.1.2.1  AML12

The cell line selected to mimic hepatocyte function in vitro was AML12 (α mouse liver 12), which is a murine derived hepatocyte cell line. The AML12 cell line was established from hepatocytes from mice (C57 strain, line MT42) transgenic for human TGF-α. Electron microscopy shows that these cells exhibit typical hepatocyte features such as peroxisomes and bile canalicular-like structure. They retain the capacity to express high levels of mRNA for serum (albumin, AFP, glyceraldehydes-3-phosphate dehydrogenase and transferrin) and gap junction (connexins 26 and 32) proteins. AML12 cells express high levels of human TGF-α and lower levels of mouse TGF-α. As previously discussed, TGF-α is an important regenerative liver growth factor and the use of this cell line was aimed to enhance the formation of a regenerating liver microenvironment in the cultures. Expression of liver specific proteins decreases with time in AML12 cultures, but it is reactivated by growing the cells in serum-free medium.

3.1.2.2  MMH and other murine liver cell lines

Other murine hepatocyte cell lines considered were the MMH, HepG2 and the L2039 cell lines. MMH cells were generated by Amicone et al. from liver explants at different developmental stages of transgenic mice expressing a truncated and constitutively active form of the c-Met. MMH cells, display characteristics typical of hepatocytes, as indicated by morphological, phenotypic and functional criteria. MMH cells fail to grow on soft agar and are unable to give rise to tumors in nude mice; they retain epithelial cell polarity, express hepatocyte enriched transcriptional factors and differentiated liver products. These cells also contain bipotential precursors that are capable of differentiating into hepatocytes and bile duct cells in response to EMC and soluble factors and do not express the liver-enriched transcription factors. However, AML12 cells were preferred because of their expression of TGF-α and the formation of conditions which mimic liver regeneration.

HepG2 cells derived from human hepatocarcinoma were also considered to mimic in vivo liver microenvironment because they express many liver proteins. However, HepG2 cells are transformed tumor cells, and for this reason they may lack characteristics typical of hepatocytes. Furthermore, they are human hepatocytes and their secreted products or cell-cell signals may not react with murine cells.
Another liver cell line considered was the L2039, which was derived from E14 fetal mouse liver after transformation with temperature sensitive SV-40 large T antigen. These cells have epithelial morphologies and express the genes of both biliary and hepatocytic genes such as albumin, AFP, CK8, CK18 and laminin. This cell line was rejected because it requires abnormal temperature (39 °C) to express hepatocyte phenotype.

3.1.2.3 NIH-3T3

The NIH-3T3 is a continuous cell line of highly contact-inhibited cells, established from NIH (National Institute of Health) Swiss mouse embryo cultures. It was used to mimic the stellate cells (which produce various growth factors including HGF\textsuperscript{110,128} and SCF\textsuperscript{196-198}) in the liver. In addition to mimicking stellate cells, NIH-3T3 cells have been shown to induce differentiation of oval cells into hepatocytes in vitro at rates superior to stellate cell lines\textsuperscript{199,200}.

NIH-3T3 cells secrete biologically active HGF as evidenced by the ability of their conditioned media to stimulate HGF receptor phosphorylation in epithelial cells\textsuperscript{201}. Autocrine-mediated Met-HGF signal transduction in met transfected 3T3 cells causes them to become tumorigenic\textsuperscript{202,203}; further evidenced in their ability to secrete HGF. NIH-3T3 cells also secrete hematopoietically important factors such as SCF\textsuperscript{20}. Due to these properties, they were chosen as the cell line representing non-parenchymal liver cells.

3.1.3 ECM Related Factors

ECM is a dynamic assembly of interacting molecules that recognizes and regulates cell function in response to endogenous and exogenous stimuli\textsuperscript{204}. ECM is produced by cells and consists of collagens, proteoglycans, adhesive glycoproteins and glycosaminoglycans and associated bound protein modulators of cell function. Along with providing a framework within which cells form tissues, ECM modulates cell attachment, shape, morphology, migration, orientation and proliferation. For example, changes in matrix and mutations in genes for ECM proteins cause connective tissue disorders. ECM also serves as a reservoir for various growth factors. It has been proposed that the existence of matrix is essential for the activity of growth factors (such as HGF, TGF-β and acidic and basic FGF) since bound growth factors are more stable\textsuperscript{205}.

ECM is very important in maintaining differentiated hepatocyte phenotype. Primary isolates of hepatocytes plated on plastic attach poorly. However, when cells are cultured on tumor derived basement matrix (i.e. Matrigel\textsuperscript{®} or Engelbreth-Holm-Swarm sarcoma matrix) that contains laminin, type IV collagen, heparan-sulfate proteoglycans and other proteins, hepatocyte morphology and liver
specific function, as measure by albumin and cytochrome P450 (CYP450) expression, are preserved.

### 3.1.4 Medium requirements

The basal nutrient medium used can greatly affect culture behavior. The medium used in the majority of the experiments was a combination of F12 and DMEM medium that has been previously optimized to maintain differentiated function of hepatocytes in culture. This medium has been found particularly useful for serum-free studies. In later experiments, a combination of SCF, TPO, FL and HIL-6 were added to regulate HS cell fate in vitro (see Section 1.5.2.2).

### 3.2 Cell tracking methods in vitro

The ability to distinguish BM derived hepatocytes from other cells in the co-culture (such as hepatocytes) requires the use of cell tracking systems. To establish such cultures, several cell tracking systems were considered.

#### 3.2.1 YFP

The green (G) and yellow (Y) fluorescent proteins (FPs) have proven to be excellent markers for tracking living cells. GFP is a small cytoplasmic protein that is cloned from jellyfish *Aequorea Victoria*. GFP molecule absorbs blue light and emits green without substrates, cofactors or other gene products. Although GFP was the original protein used for this purpose, several variants have been engineered with more efficient translation and brighter fluorescence. YFP is a modified form of GFP with a longer fluorescence lifetime and better overall expression. GFP/YFP expression on cultured cells or in tissues of transgenic mice does not cause any observable deleterious effects. In particular, no effect on the in vivo proliferation and differentiation of the hematopoietic progenitors has been observed. YFP* mice were created by germline transmission of YFP* ES cells. In these mice, the YFP gene is ubiquitously expressed in all cellular progeny throughout development and adulthood. The shift from green to yellowish green emission is not great enough to make changes in flow cytometric pattern thus YFP can be analyzed using the same filter as GFP during microscopy and FACS analysis.

#### 3.2.2 Membrane dyes and other techniques

Cells labeled with membrane bound dye have also been used to detect transplanted cells or certain populations in co-cultures. In particular, PKH-2 and CFSE dyes have been used. CFSE binds
irreversibly to the internal constituents of the cytoplasm, and it is equally partitioned to the daughter cells.\textsuperscript{221-223}

Table 1: Donor cell markers and their difficulties in detection by flow cytometry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression site</th>
<th>Difficulties</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFSE</td>
<td>Stained cells</td>
<td>Expression lost with cell division</td>
</tr>
<tr>
<td>YFP</td>
<td>Transfected cells</td>
<td>Difficult to transfect cells</td>
</tr>
<tr>
<td>Y-chromosome</td>
<td>Male cells</td>
<td>Difficult to detect</td>
</tr>
<tr>
<td>Ly-5 (CD45)</td>
<td>All leukocytes</td>
<td>Only on hematopoietic cells</td>
</tr>
<tr>
<td>PKH-2</td>
<td>Stained cells</td>
<td>Expression lost with cell division</td>
</tr>
</tbody>
</table>

Other methods of tracking cells in culture include the usage of ROSA26 mice, which have been derived from transgenic ES cell lines expressing the β-galactosidase (β-Gal) protein\textsuperscript{224}. These mice have a ubiquitous expression of β-Gal that allows for their utility in chimera, transplantation and cocultures studies. However, the flow cytometric analysis of ROSA-26 mice is considerably more difficult since it requires fluorescent staining of the β-gal molecule prior to analysis\textsuperscript{225}.

Fluorescent in situ hybridization (FISH) for Y-chromosome can also be used to track transplanted cells using Y-chromosome specific repetitive DNA probe that has been applied to implanted hepatocytes\textsuperscript{226} and BM cells\textsuperscript{227}. This method seems to have a sensitivity in the order of 0.1%. However, detection using flow cytometry is tedious due to low fluorescence emission from a single chromosome. Another widely used technique that has been used to track hematopoietic cells in BM transplant studies is the Ly5.1/5.2 system. However, this method was not an option in tracking of non-hematopoietic cells since they do not express CD45 antigen.

As Table 1 indicates, the use of YFP marker was the most attractive option. This was due to the expected difficulties associated with some techniques and the failure of other techniques that were tested (see Section 5.2.3)

3.3 Analysis

Various markers can be used to identify various hematopoietic and hepatic cell types. These markers include phenotypical markers and functional assays.

3.3.1 Identification of hematopoietic stem cells

In contrast to liver stem cells, the identity of HSC has been much more rigorously analyzed\textsuperscript{228,229}. Despite this, all stem cells are difficult to identify because they comprise a small cell population in the...
organ in which they reside (e.g. HSC represent a rare population of 0.01-0.05% of WBM\textsuperscript{220}). Also, since stem cells can undergo both self-renewal and differentiation into functionally specialized mature cells\textsuperscript{160}, their definitive identification requires their ability to ‘function’ as stem cells be demonstrated. A wide variety of functional and phenotypical assays have been developed to detect HSC and progenitor cells. In functional assays, cells are recognized based on their functional properties of their progeny. In phenotypic assays, combinations of surface receptors are used to enrich for the stem cell compartment.

3.3.1.1 Functional assays

Stem cells are most rigorously defined by their \textit{in vivo} repopulating capability. In HSC, this is usually demonstrated using assays such as the competitive repopulating unit (CRU) assay. This assay provides a relatively reproducible and specific method for determining the frequency of transplantable cells with long-term \textit{in vivo} reconstituting capacity. However, most \textit{in vivo} tests are limited because of the extended period of time required to perform the test as well as high costs. Therefore, in order to identify the functional properties of stem cells, \textit{in vitro} methods have been developed. One system to detect stem cells is by the use of colony forming assays. Since HSC and progenitor cells can be activated to proliferate, a strategy in identifying putative stem cells is to stimulate the cells to proliferate and then focus on their essential ability to generate differentiated cell types. CFC assays can be used to quantify the frequency of hematopoietic progenitors that form discrete colonies in semisolid gels (agar or methyl cellulose) in the presence of colony stimulating factors\textsuperscript{231,232}. Lineage-restricted committed hematopoietic progenitors can be evaluated as CFU-GM, BFU-E, CFU-G/M. On the other hand, multipotent hematopoietic precursors can be distinguished as CFU-GEMM colony\textsuperscript{233}.

3.3.1.2 Phenotypic markers

Isolation of BM derived HSC has been performed by cell surface antigen expression\textsuperscript{229,234}. Numerous cell surface markers have been used to identify and isolate HSC from murine BM, though a definitive set of markers, which clearly identifies a HSC, has so far eluded researchers. Despite this, occasional transplants with single purified stem cells have been successful in mouse\textsuperscript{235}. It has been pointed out that the functionality and the purity of HSC maybe limited in repopulating assays since only 20% of the injected cells home to the BM, the site best suited for hematopoietic engraftment\textsuperscript{236}; thus, the enrichments observed by phenotypical assays may actually be an underestimation of the actual HSC content.
The most commonly used markers for identification of murine HSC are CD34, Sca-1, Lin and c-kit. CD34 is a monomeric transmembrane phosphoglycoprotein that is present in capillary endothelial cells and on some BM stromal progenitors. In hematopoietic lineage, CD34 expression is generally lost as cells mature. In mice, it has been reported that CD34 is expressed on hematopoietic progenitors capable of short-term but not long-term reconstitution of lethally irradiated mice. Recent results suggest that CD34 is not necessarily expressed on HSC; for example, clonal CD34<sup>−</sup> can long-term repopulate mice and low expression of CD34 has been shown on HSC. Current knowledge associates CD34 with HSC cell cycle activation which induces CD34 expression in a reversible manner.

Stem cell antigen-1 (Sca-1 - also known as Ly6A/E) is a cell surface protein that is expressed on multipotent HSC in mice. Sca-1<sup>+</sup> HSC are found in the adult BM, fetal liver, and fetal liver but not in the early embryonic yolk sac or interaembryonic hematopoietic sites. Sca-1<sup>+</sup> HSC can also be mobilized to the peripheral blood and spleen in adults. Other than HSC, Sca-1 has also been found on B and T lymphocytes, myeloid cells, and several non-hematopoietic tissues.

C-Kit (CD117) is a transmembrane tyrosine kinase receptor which reacts with its ligand SCF. C-kit is expressed in adult BM on hematopoietic progenitors, including on the Sca-1<sup>+</sup> Lin<sup>−</sup> HSC. It is also expressed on myeloid/erythroid progenitors and lymphoid precursors.

One of the most successful murine phenotypical enrichment protocols has been developed by Weissman and colleagues who enriched for HSC within c-kit<sup>+</sup> Sca-1<sup>+</sup> Lin<sup>−</sup> (KLS - where lineage markers CD2, CD3, CD4, CD5, CD8, NK1.1, B220, Ter119, GR-1 and Mac-1 are expressed on differentiated hematopoietic cells) cell population and demonstrated their ability to
clonally reconstitute hematopoiesis in lethally irradiated hosts. HSC have also been isolated using a 48-hour homing assay in which quiescent cells are segregated and have been seen to repopulate other mice.

In this work, BM cells were enriched for HSC to varying degrees. In the majority of experiments, Lin- populations were tested and compared with Lin+ and WBM populations. In some experiments that required the use of purified HSC, KLS cells were used.

### 3.3.2 Identification of hepatic cells

#### 3.3.2.1 Functional assays

Albumin is the most abundant serum protein accounting for 50% of serum proteins. It is synthesized exclusively by hepatocytes and it is one of the most commonly used markers to detect hepatocyte function. By adhering to specific substances, albumin aids in the transportation of many substances such as drugs, lipids, hormones, and toxins within the bloodstream. Once the drug reaches the liver, it is detached from the albumin and metabolized to a water-soluble form that can be excreted. Albumin has a half-life of 21 days and can be easily detected in serum; therefore, it is very sensitive in detecting differentiated hepatocyte functions.

A number of other liver secreted proteins can be used to detect hepatocyte function. The most popular of these proteins is CYP450. CYP450 encompasses a highly diverse 'superfamily' of enzymes responsible for the metabolism of toxic carbohydrates. Also, many other secreted proteins and metabolic enzymes have been used to analyze hepatocyte function. However, these markers were not analyzed due to the unavailability of murine specific antibodies or anticipated difficulties with low expression.

#### 3.3.2.2 Phenotypic markers

Hepatocytes and other liver cells can be identified with monoclonal antibodies that react specifically with their specific cytoskeletal proteins. Each cell type has a specific and stable pattern of expression of cytoskeletal subunits such as cytokeratins. Cytokeratins (CKs) are a family of over 20 polypeptides expressed in different epithelial cells during differentiation. Therefore, different endoderm tissues (such as liver) can be distinguished and characterized by their specific cytokeratin expression patterns. CKs are divided into 2 groups (acidic and basic) which are differentially expressed as pairs. Most cells express one acidic and one basic of the 21 different cytokeratin proteins.
CK8 is a basic intermediate filament that is expressed in simple epithelial cells such as trachea, small intestine, bladder, pancreas, colon and mammary gland ducts. Furthermore, CK8 and CK18 are the first cytokeratin expressed in embryos\textsuperscript{273}. Differentiated hepatocytes express the pair CK8 and CK18\textsuperscript{272}, while cholangiocytes express CK7 and CK19 in addition to hepatocyte CKs\textsuperscript{274}. These expression patterns are especially strong in hepatocytes and cultured hepatocytes have been shown to continue the expression of hepatocyte like cytokeratins long after they lose expression of other functional markers\textsuperscript{275}.

Very little is known about the role of CKs in hepatocytes\textsuperscript{271}. It is believed that CKs provide cells with mechanical integrity and may be very important for endoderm differentiation. In CK8 knockouts, 50% of mice die before E12-13\textsuperscript{276} due to growth retardation and liver dysfunction; interestingly, CK18 knockout mice appear normal and functional\textsuperscript{277}. Based on these observations, it appears that CK8/CK18 intermediate filaments are important for liver integrity.

Different reactivities of cytokeratin antibodies with the specific cytokeratins of different tissues have been noted with conventional antiserum\textsuperscript{278-280} and monoclonal antibodies\textsuperscript{281}. Thus, great care was taken in choosing the antibodies that reacted with the infrequently studied murine liver system. In mice, TROMA-1,2,3 stain CK8, CK18 and CK19 respectively\textsuperscript{282,283}. These antibodies have been shown to react with murine liver cytokeratins although they react differently depending on the molecular weight (MW) of the protein\textsuperscript{284}.

E-cadherin was also used to characterize murine hepatocytes. E-cadherin is a regulator of junctions in epithelial cells. In hepatocytes, E-cadherin is expressed on cell surface where it aids in regulating cell-cell contact with other hepatocytes and endothelial cells\textsuperscript{100}.

Flow cytometry was used as the preferred analytical method for the detection of liver specific phenotypical markers due to its capability to quantify population dynamics and detect simultaneous markers. Flow cytometry is a fast and accurate technique in which individual cells are analyzed (as supposed to cell populations). Although flow cytometry has been used for isolation of hepatic progenitor cells\textsuperscript{80,285} and characterization of hepatocyte suspensions\textsuperscript{268}, its widespread use has not been adopted for analyzing hepatocytes. Thus, the development of this technique for measuring cell surface and intracellular markers was one of the goals of the project.
4 MATERIALS AND METHODS

4.1 Bone marrow stem cell extraction and enrichment

4.1.1 Bone marrow isolation

All procedures involving mice were performed according to the University of Toronto approved protocols. BM cells were collected from the femur and tibia of 5 weeks or older male C57Bl/6 mice (Charles River laboratories, Wilmington, MA), as well as YFP+ mice or their YFP parent line (generous gifts from Dr. W. Stanford). The process of BM isolation is illustrated in Figure 4-1. Mice were euthanized using cervical dislocation, and their femurs and tibias were then dissected and placed within pre-cooled Dulbecco's Modified Eagle Medium (DMEM - Gibco BRL, Life Technologies, Grand Island, NY) containing 1000 U/ml penicillin and 1000 μg/ml streptomycin (10X-DMEM - Gibco BRL). The bones and isolated cells were maintained on ice to prevent any deleterious effects on cells.

![Figure 4-1 Schematic of BM isolation process](image)

The bones were then cleaned of any attached muscle tissue. To flush out the BM, scissors were used to remove a small portion of the bone from each end of the femur or tibia. A 22½ gauge needle (Becton Dickinson (BD), Franklin Lakes, NJ) attached to a 3 ml syringe (BD) was then inserted through the cartilage plate at the distal end of the femur and the marrow was flushed out with 10X-DMEM into a 15 ml centrifuge tube (Corning Glass Works, Corning, Ithica, NY). The bones were flushed two more times to ensure maximum recovery of the marrow cells. Typically, the cells from 2-4 mice were flushed with the same medium and thus pooled into a single batch and clumps were broken up by aspiration through the needle.

Any large bone fragments were then removed by running the cells through a 70 μm cell strainer (Falcon, BD, Franklin Lakes, NJ). In some experiments erythrocytes, were lysed with a solution containing 8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer (Red Blood Cell Lysing Buffer, Sigma, St. Louis, MO) for 8 minutes. The nucleated cells were then washed with phosphate buffered solution (PBS - Gibco BRL) and counted prior to further enrichment or analysis using a hemocytometer. Viable cell numbers were determined by trypan blue (Sigma) dye exclusion. If BM
preparation and sorting could not be performed on the same day, the BM was kept in the refrigerator overnight prior to staining and sorting.

4.1.2 Stem cell enrichment

Lineage negative (Lin') BM cells were isolated using the commercially available negative selection system (StemSep®, StemCell Technologies Inc. (STI), Vancouver, BC, Canada). In this system, unwanted cells were labeled with multiple antibodies that were attached to magnetic particles. The cells binding the magnetic particles on their membrane were then separated within a high gradient magnetic column. StemSep depletion cocktail contains antibodies to murine CD5 (T and B cells), CD45RA (B and T cells and lymphoid progenitors but not HSC), CD11b (macrophages and monocytes), GR-1 (granulocytes) and TER119 (erythroid cells and erythroblasts). Cells that do not express lineage markers are not labeled with antibodies and are thus ready for further manipulation.

To isolate Lin' cells, WBM cells were suspended at 5 x 10^6 cells/ml in PBS containing 2% fetal bovine serum (FBS – Gibco BRL) with 5% normal rat serum (STI) and incubated for 15 min. Subsequently, they were incubated with 10 μL/mL of the enrichment cocktail for 15 min at 4 °C. The cells were then washed and resuspended at 5 x 10^7 cells/ml in DMEM containing 2% FBS and incubated with 100 μL/ml of anti-biotin tetrameric antibody complex for 15 min at 4 °C. Afterwards, the cells were mixed with 60 μL/ml of magnetic colloid solution for 15 min and then directly passed through the column.

To isolate c-kit^+ Lin' Sca-1^+ (KLS) populations, column enriched Lin' cells were stained with 2 μg/mL of directly labeled FITC-CD45, PE-Sca-1 and APC-c-kit or appropriate IgG isotype antibodies (BD Pharrningen, Franklin Lakes, NJ) for 1 hour. Desired cells were then isolated using fluorescence activated cell sorter (FACS), performed at Princess Margaret Hospital by a MoFlow FACS (Cytomation, Fort Collins, COL).

4.2 Primary hepatocyte isolation and culture

Hepatocytes were isolated from livers of male C57Bl/6 mice. To enhance the yield of viable cells, all media were pre-warmed to 37 °C. To isolate primary liver cells, the abdominal region was cut and all liver lobes were dissected from the mice and placed on a 60 mm petri dish (Falcon, BD) containing 2 mL of 0.1% (w/v) collagenase (Sigma). The lobes were then minced with scissors into small pieces. The chopped liver sections were then placed into 20 ml of 0.1% (w/v) collagenase in a 50 mL centrifuge tube and were vigorously pipetted. The solution was kept at 37 °C for 25 minutes with frequent mixing. The resulting cell mixture was then washed with PBS and re-suspended in 15 mL of cell dissociation buffer (Gibco BRL) for 20 minutes with frequent mixing. Finally, to remove
dissociated cells from non-digested tissue aggregates, the cells were filtered through a 70 μm coarse filter and then washed with PBS.

Collagen (Type IV, Sigma) coated dishes were used to enhance the viability of primary hepatocytes in culture. To coat dishes, 10 μg/cm² of a solution containing 0.01% (w/v) of collagen in 0.1 M acetic acid (Sigma) was allowed to bind with the dish overnight at 4 °C. Excess fluid was then removed and the coated dishes were sterilized by overnight exposure to UV light in a sterile tissue culture hood (Forma Scientific Inc., OH). Finally the dishes were rinsed with PBS prior to initiation of the cultures.

Isolated hepatocytes from each mouse were cultured in a 25 cm² tissue culture dish (Sarstedt Inc., Newton, NC) with or without collagen coatings and their metabolic functionality and albumin expression was measured at regular intervals. The media used for hepatocyte cultures were either the HepatoZYME - serum free medium (HepatoZYME-SFM - Gibco BRL, which has been specifically designed for maintenance of metabolic activity of hepatocyte culture) or a 1:1 mixture of DMEM and Ham's F12 (Gibco BRL) containing 10% FBS and other supplements (see Section 4.3.1).

4.3 Cell culture

4.3.1 Cell lines

All cell lines were purchased from American Tissue Type Collection (ATCC, Rockville, MD) and maintained in 25 cm² tissue culture flasks in a 95% air/5% CO₂ humidified incubator at 37 °C. To prevent bacterial infection, 100 U/mL penicillin and 100 μg/ml streptomycin (Gibco BRL) were added to all culture media. NIH-3T3 fibroblasts were maintained in DMEM medium containing 10% (v/v) FBS. These cells were subcultured every 5-7 days by the addition of 1 mL of 0.25% Trypsin-1 mM EDTA (Gibco BRL). The flask was then incubated for a few minutes until the cells were detached from the bottom of the dish. Detached cells were then suspended in 10 mL of fresh medium and centrifuged for 5 minutes at 1000 rpm. Following centrifugation, the medium was aspirated and the cells were then resuspended in normal medium and subcultured using a 1:10 subculture ratio.

AML12 cells were maintained in a medium comprised 90% of 1:1 (v/v) mixture of DMEM and Ham's F12 medium (DF medium) with 0.005 mg/ml insulin (Gibco BRL), 0.005 mg/ml transferrin (Sigma), 5 ng/ml selenium (Sigma), and 40 ng/ml dexamethasone (Sigma), and 10% FBS (Gibco BRL). AML12 cells were passaged every week (using a 1:4 subculture ratio) and fed every 3-4 days.

4.3.2 Bone marrow cultures

To initiate BM cultures, 2 x 10⁷ WBM or 5 x 10⁷ Lin⁻ cells were cultured on to each well of a six-well plate (Falcon, BD) pre-coated with Matrigel® (Collaborative Biomedical Products, Bedford, MA), each containing 5 mL of DF medium along with 10% FBS and other supplements, unless otherwise
indicated. The medium was routinely changed every 3-4 days (beginning after week one during which no feeding is done) by removing half of the volume (2.5 ml), without taking out any of the cells, and replacing it with an equivalent volume of fresh medium. The cells of a dish were harvested, counted and analyzed using the CFC assay or flow cytometry. The medium supernatant was frozen at -20 °C for subsequent analysis for albumin content analysis (see Section 4.5.1).

For experiments designed to determine the clonal endoderm differentiation ability of the KLS cells, 1 to 100 KLS or 1000 Lin' CD45' cells were placed in wells (using FACS) that had been pre-coated with Matrigel® and pre-filled with 75 μl of either 1 μg/ml HGF or hematopoietic medium (HM-100 ng/ml SCF, 100 ng/ml FL, 100 ng/ml HIL6 and 20 ng/ml TPO) or the combination of two media in HF without serum. The presence of single cells in each well was confirmed one day after culture initiation. These cultures were maintained for 6 days after which, an additional 75 μl of the original medium supplemented with 10% FBS was added to each well. After 17 days the number of viable cells in each positive well was then recounted for clone size determination and medium supernatant was frozen at -20 °C for subsequent albumin content analysis. To ensure that the secreted albumin was derived from cells, wells treated with various media were used as controls.

### 4.3.3 Bone marrow co-cultures with feeder cells

To initiate co-cultures, 1 x 10⁵ 3T3 or AML12 feeder cells were cultured on to each well of a six-well plate pre-coated with Matrigel® (see Section 4.3.3.1) for 24 hours to obtain an adherent layer. BM cells were then isolated from YFP- mice or stained with CFSE and cultured directly or after enrichment onto the cultures at 2 x 10⁷ WBM, 5 x 10⁶ Lin' or 1 x 10⁷ Lin* cells. For control cultures, dishes without feeder layers were used (similar to BM cultures). Unless otherwise indicated, the medium used for the co-cultures was the DF medium plus supplements (see Section 4.3.2). Half of the medium was changed every 3-4 days without removing non-adherent cells. The cells of each dish were harvested, counted and analyzed using CFC assay or flow cytometry. The medium supernatant was frozen at -20 °C for subsequent analysis for albumin content (see Section 4.5.1).

In co-cultures in which direct cell contact was prevented, 0.2 μm tissue culture inserts (Nunc Products) were used to separate the feeder cells from the BM cells. In these cultures, the feeder cells were cultured as previously described. However, Matrigel® coating was performed on the surface of the tissue culture inserts and BM populations were cultured directly on the membranes. All reagents and analysis procedures were similar to the other cultures.
4.3.3.1 Matrigel®

Matrigel® was used to establish 3 dimensional (3-D) cultures or to coat dishes. Frozen (-20 °C) aliquots were thawed initially at 4°C overnight and then mixed in a 1:1 (v/v) ratio with cold DMEM tissue-culture medium (4 °C).

To coat dishes, wells were pretreated with Matrigel® solution (diluted 1:10 with DMEM) or with 15 μl/cm² of 1:1 diluted Matrigel® and incubated overnight at 37 °C. To establish 3-D cultures, 150 μl/cm² of Matrigel® solution that contained cells was added to each dish and maintained at 37 °C. To recover cells from 3-D Matrigel® cultures, dispase (Gibco BRL) was used. However, in coated dishes, the use of dispase was not necessary as trypsin was sufficient to recover the cells.

4.3.3.2 Cytokines

All cytokines were stored at -80 °C; they were obtained as gifts or purchased from companies as follows: recombinant mouse (rm) TPO (Sigma); recombinant human (rh) FL (Immunex, Seattle, WA); rh HIL-6 (Gift from Dr. Stefan Rose-John286), rh HGF (R & D Systems, Minneapolis, MN) and rm SCF (Calbiochem, La Jolla, CA).

4.3.3.3 CFSE staining

Carboxyfluorescein diacetate succinimidyl ester (CFSE - Sigma) stock solution was prepared at 5 mM in dimethyl sulfoxide (DMSO - Sigma) in small aliquots that were stored at -20 °C. To label, cells were incubated at a concentration of 5 x 10⁶ cells/mL in a solution of 10-50 μM CFSE in Hank’s basal salt solution (HBSS - Gibco BRL) for 10 min at 37 °C. Further uptake of dye was ceased by the addition of 10 fold volume of cold HBSS with 10% FBS. The cells were then washed three times with HBSS containing 5% FBS and incubated overnight in regular medium. To analyze the cells under flow cytometry, 10⁶ cells were suspended in 100 μL of PBS along with 2 μg propidium iodide (PI - Sigma) to stain dead cells.

4.4 Hematopoietic analysis

4.4.1 Colony-forming ability

Cells were assayed to determine their ability to form erythropoietic (BFU-E), granulopoietic / monocytic (CFU-GM) and multi-lineage (CFU-GEMM) colony forming cells (CFC). CFC assays were performed by plating an appropriate number of cells (to give < 100 colonies per 1 mL culture — for WBM this represented about 2 x 10⁶ cells) in FCS-supplemented methylcellulose containing Iscove’s medium (MethoCult™, STI). The following growth factors were included as part of the medium to
induce colony formation: Epo 3 units/mL, 10 ng/mL rh IL-3, 10 ng/mL rh IL-6 and 50 ng/mL rm SCF. Methylcellulose cultures were aliquotted using a 3 mL syringe (BD) and 16g blunt-end needle (STI) in 1.1 mL volumes into 35 mm petri dishes (BD) and then incubated at 37 °C in humidified atmosphere of 5% CO₂ in air. Colonies were then scored in situ using well established criteria; 35 mm dishes were placed within 60 mm grided dishes (Nunc Products) after 7-12 days of incubation. All CFC assays were done in triplicate.

4.4.2 Hematopoietic morphological markers and analysis

The following cell surface antibodies were used to detect various antigens found on hematopoietic cells: FITC or PE conjugated rat anti-murine CD45, FITC conjugated rat anti-murine CD34, PE conjugated rat anti-murine Sca-1 and APC or biotin conjugated rat anti-murine c-kit (BD Pharmingen, Mississauga, ON, Canada). For all directly labeled antibodies, appropriately labeled rat IgG isotype antibodies (BD Pharmingen, Mississauga, ON, Canada) were used as control. For antibodies conjugated with biotin, the appropriate streptavidin-fluorochrome construct was used as control.

To stain, test samples were washed once in PBS containing 2% FBS. The samples were then resuspended in HBSS solution containing 2% FBS at ≤ 1 x 10⁷ cells/mL with 2 μg/mL of specific monoclonal antibodies at 4°C for one hour. For biotin conjugated samples, the cells were washed with HBSS + 2% FBS and resuspended at ≤ 1 x 10⁷ cells/mL with 10 μg/mL of streptavidin-fluorochrome construct for 45 minutes. 7-aminoactinomycin D (7-AAD – Molecular Probes, Eugene, OR) or PI was added at 2 μg/mL to stain dead cells.

Stained cells were analyzed using the EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA). The 488 nm argon ion laser was used for excitation with emission being measured at appropriate band pass filters. Data was collected and analyzed using the EXPO32 software (Beckman Coulter Inc.).

4.5 Hepatic analysis

4.5.1 Serum albumin detection

Albumin enzyme linked immunoassay (ELISA – Bethyl Laboratories, Montgomery, TX) was performed at room temperature using double antibody sandwich method. The lower limit of sensitivity of the mouse albumin ELISA was about 100 pg/mL. The wells of a microtiter plate were pretreated with 100 μl of a coating buffer comprised of purified goat anti-mouse albumin antibody at 10 μg/mL in a diluent containing 0.05 M sodium bicarbonate (Sigma) adjusted to pH 9.6 and incubated for 60 minutes. The plate was washed twice with PBS containing 0.05% Tween 20 (Sigma) and then blocked with 200 μl of a 1% (w/v) bovine serum albumin (BSA – Sigma) solution in PBS for 30 minutes. After washing, 100 μl of purified murine albumin standards and samples (diluted in 1% BSA dissolved in PBS with
0.05% Tween 20) were added and incubated for 60 minutes. After 2 washes, 100 μl of diluted (1/5000) detection antibody/enzyme conjugate solution was added to each well which was then incubated for 60 minutes. The wells were then washed 3 more times and 100 μl of 3,3',5,5'-tetramethyl benzidine (TMB – Bio-Rad Laboratories, Hercules, CA) substrate solution (prepared by 1:9 (v/v) mixing of the two substrate reagents) was added to each well. The plate was incubated for 5-30 minutes based on the intensity of the colour change.

After the incubation, the TMB reaction was then stopped by adding 100 μl of 2 M H₂SO₄ (Sigma) to each well. A microplate spectrophotometer (VERSA-max, Molecular Devices, Sunnyvale, CA) was then used to analyze the absorbance intensity of each plate at 450 nm.

4.5.2 MTT

The metabolic activity of the isolated hepatocytes was measured by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – Sigma) assay. This assay is based on the ability of the cells to convert MTT, a yellow tetrazolium salt, to purple formazan crystals via the mitochondrial activity of the cells. These salt crystals are insoluble in aqueous solution but may be dissolved by adding a solubilization solution and it is then read with a spectrophotometer.

Cells were incubated with 1.1 μM MTT in normal medium. After 4 hours, the supernatant was carefully aspirated and DMSO was added to dissolve the MTT crystals. The intensity of the color of the solution was determined using a microplate spectrophotometer (VERSA-max, Molecular Devices, Sunnyvale, CA) at an excitation of 519 nm and analyzed using Softmax Pro V3.1 (Molecular Devices).

4.5.3 Hepatic morphological markers and analysis

Two different approaches were used to analyze the cell surface as well as intracellular markers of hepatic cells. The cell surface markers used were c-Met (tyrosine kinase cell surface protein287 activated by HGF288 that is expressed in many tissues120) and E-cadherin. The following antibodies were used: c-Met antibody (sc-162, Santa Cruz Biotechnology Inc.) and secondary PE conjugated rabbit anti-goat IgG (Biomedia, Foster City, CA), rat anti-mouse E-cadherin (Sigma) and FITC-anti-rat IgG (Sigma). To stain the cells already established protocols were used289,290. Briefly, cells were resuspended in HBSS solution containing 2% FBS at ≤ 1 x 10⁷ cells/mL for 1 hour with 2 μg/mL of specific monoclonal antibodies. For secondary antibodies the cells were washed and resuspended with appropriate dilution of the secondary antibody for 1 hour.

Rat anti murine CK8, 18 and 19 antibodies (TROMA 1.2.3 - markers for mouse endoderm283) were obtained from Developmental Hybridoma Bank (TROMA-1) and as generous gifts from Dr. R. Kemler (Max-Planck Institute, Germany for TROMA 1.2.3). These antibodies were used to stain cells.
for intracellular antibodies, using standard protocols (IntraPrep permeabilization kit, Immunotech-Beckman Coulter Co., Marseille, France). Briefly, the cells were suspended at ≤ 1x10^7 cells/ml and aliquotted in 100 ul samples into 5 mL test tubes (Falcon BD). Aliquots were then diluted at 1:1 ratio with aqueous fixation medium containing 5.5% (v/v) formaldehyde and incubated for 15 min at room temperature. Cells were then washed with PBS, centrifuged and the supernatant was discarded. To permeabilize, cells were incubated for 5 minutes in 100 μl of saponin rich solution, which was added to the cells without mixing. The antibodies was added directly to the saponin solution at a 1:5 dilution and incubated for 3 hours at room temperature. The samples were then washed once in PBS, and resuspended in 100 μl of PBS containing 5% rat serum and 10 μg/mL of the PE-conjugated secondary antibody (Immunotech-Beckman Coulter Co.) or biotinylated goat anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and incubated for 1 hour. For biotinylated antibodies, the cells were subsequently washed in PBS and resuspended in 100 μl of 100 μg/mL streptavidin-fluorochrome substrate. Stained cells were analyzed using EPICS XL flow cytometer as previously described (see Section 4.4.2). For negative controls, cells were stained with isotype-matched controls for irrelevant specificity.

4.6 Image analysis

All samples were viewed using an Olympus CK40 RFL epifluorescent light microscope (Carsen Group Inc. (CGI), Markham, ON, Canada) with an attached digital camera (CGI). The microscope was equipped with a laser and filters for FITC and PE (CGI).

4.7 Data analysis

Data from experiments that were performed in duplicate or triplicates were analyzed as the mean ± SD. Student t-test was used to determine the statistical significance between paired data. Group means were compared using single factor analysis of variance (ANOVA) to determine statistical significance. P < 0.05 was used to detect statistical difference between samples.
5 RESULTS

5.1 Bone marrow Purification and characterization

Whole bone marrow (WBM) and enriched cell populations were analyzed using phenotypic markers and CFC assays. The percentages of various hematopoietic markers on viable PI WBM cells are shown in Table 2. The majority of the BM derived cells were CD45+, indicating that they were hematopoietic. The expression of hematopoietic progenitor markers CD34, c-kit and Sca-1 were also analyzed. The results obtained were similar to previously reported data \(^{100,292}\). For example, the typical expression of CD34+, c-kit+, Lin+ and Sca-1+ in BM has been previously reported at 1.7% \(^{292}\), 7.7%, 6.6% and 4.2% respectively \(^{100}\).

Table 2: Percentage of various hematopoietic markers in mononucleated WBM cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>% Purity ± SD</th>
<th>Marker</th>
<th>(% Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CFC</td>
<td>0.4 ± 0.1</td>
<td>CD34+</td>
<td>1.6</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>0.3 ± 0.05</td>
<td>CD45+</td>
<td>99.0</td>
</tr>
<tr>
<td>BFU-E</td>
<td>0.06 ± 0.01</td>
<td>c-kit+</td>
<td>9.3</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>0.035 ± 0.005</td>
<td>Sca-1+</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c-kit+ Sca-1+</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Different types of CFC progenitors were measured based on the phenotypic properties of the derived colonies. Example figures of various colony types are presented in Figure 5-1. Typically, CFU-GM colonies contained small, uniformly round cells with distinct cytoplasmic membranes that often formed grape-like clusters. CFU-GM colonies were formed in a wide range of sizes. BFU-E colonies contained predominantly erythroid cells with a minimum of three clusters (usually three to eight clusters), each containing 8 to 32 erythroblasts. The cells were small and refractile, and the clusters had irregular shapes. Unlike their human counterparts, some murine BFU-E colonies were not reddish-brown. CFU-GEMM colonies represent the most primitive of the clonogenic progenitor types. CFU-GEMM colonies were recognized by their ability to form large colonies that contained erythroid clusters, granulocytes, monocyte / macrophages as well as megakaryocytes. Most CFU-GEMM colonies contained dense cores with erythroid clusters visible usually at the edges of the colony at higher magnifications.

In general, the rate of colony formation in unpurified BM cells was very low. For example, less than 1 in 200 WBM cells had the ability to form colonies in the CFC assay. The majority of these
CFCs were comprised of CFU-GM colonies (~80%), followed by BFU-E (~16%) and CFU-GEMM (~4%).

As indicated, HSC and progenitor cells comprised a small fraction of the overall BM content. To obtain more purified cell populations, cells were enriched either as Lin− or c-kit+ Lin− Sca-1+ (KLS) populations. To remove cells that expressed hematopoietic lineage markers (Lin), cells were stained with CD5, CD45RA, CD11b, GR-1 and TER119, which stain differentiated hematopoietic cells. Table 3 shows the recovery and enrichment of various hematopoietic progenitors after Lin− isolation.

Table 3: Percentage of various hematopoietic markers in mononucleated Lin− cells, after column separation

<table>
<thead>
<tr>
<th>Markers</th>
<th>% Positive ± SD</th>
<th>% Recovery ± SD</th>
<th>Fold Enrichment ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sca-1−</td>
<td>22.0 ± 1</td>
<td>44.1 ± 1</td>
<td>3.7 ± 1</td>
</tr>
<tr>
<td>c-kit+</td>
<td>44.8 ± 1</td>
<td>33.1 ± 1</td>
<td>5.5 ± 1</td>
</tr>
<tr>
<td>CD34+</td>
<td>39.3 ± 1</td>
<td>84.7 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>CD45+</td>
<td>99.8 ± 1</td>
<td>11.8 ± 1</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers</th>
<th>% Purity ± SD</th>
<th>% Recovery ± SD</th>
<th>Fold Enrichment ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFC</td>
<td>3.8 ± 1</td>
<td>36 ± 7</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>3 ± 0.9</td>
<td>44 ± 8</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>BFU-E</td>
<td>0.5 ± 0.9</td>
<td>28 ± 5</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>0.3 ± 0.2</td>
<td>33 ±9</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

As expected, column enrichment enhanced the proportion of cells that expressed various hematopoietic progenitor markers. The enrichment of these markers varied greatly depending on their expression on differentiated hematopoietic cells. For example, since CD45 is ubiquitously expressed on hematopoietic cells (including HSC), the Lin− separation process did not lead to its enrichment. For hematopoietic progenitor markers, the resulting enrichment varied from 3.7 fold for Sca-1 (which is also expressed on T-cells) to 17 fold for CD34. Column enrichment also enhanced the frequency of various types of CFCs from 6 to 9 fold.
Figure 5-2 Phenotypic analysis for isolation of KLS cells and the restricted gates used to sort HSC.

In some experiments, phenotypically defined KLS cells were isolated from Lin- enriched populations. These cells expressed CD45, and were therefore hematopoietic in origin (see Figure 5-2(d)). Furthermore, KLS cells are highly enriched in HSC activity, and contain ~20% CFCs and ~5% CRU cells

Figure 5-2 illustrates the approach used to isolate these cells. Column enriched Lin- cells were gated on flow cytometry based on their cellular morphology, their ability to exclude PI dye and express c-kit and Sca-1. The c-kit- Sca-1- cells constituted about 1.5% of the Lin- population, translating to about 1 in 1000-5000 of mononucleated BM cells.

To examine HGF receptor expression on hematopoietic progenitor cells, c-Met (HGF receptor) expression on WBM and Lin- cells was analyzed. As shown in Figure 5-3, a small fraction of WBM cells express c-Met (~3%), while this population was enriched in the Lin- cells (~ 6 fold to 18%). These results correlate with previously published reports regarding c-Met expression on hematopoietic progenitors, which showed that about 30-50% of the human CD34+ cells as well as a high percentage of CFCs simultaneously expressed c-Met. Furthermore, as expected, freshly isolated WBM cells did not express CK8 or CK18, markers that are associated with simple epithelial cells (see Figure 5-3).

In contrast, the staining pattern of AML12 hepatocytes revealed that the majority of CK8+ cells simultaneously co-express c-Met. This may indicate that cells which express the highest levels of CK8 expression also express the c-Met receptor and thus have the capability to respond to HGF protein.
5.2 Development of the co-culture systems

To develop *in vitro* liver microenvironments, significant effort was dedicated to the design of co-culture systems. To establish such cultures, the use of primary hepatocytes as well as, hepatocyte and fibroblast cell lines was investigated. Also, the effectiveness of various cell tracking techniques for distinguishing BM derived cells from feeder cells was examined.

5.2.1 Primary hepatocytes isolation and culture

To evaluate the feasibility of using primary hepatocytes in co-culture with BM cells, hepatocyte cultures were established. Isolated liver cells were cultured either on treated or non-treated collagen coated petri dishes, and monitored for the expression of hepatocyte markers and overall metabolic activity.
Figure 5-4 Micrographs of primary hepatocyte cultures cultured on collagen after 0 (a), 7 (b) and 15 (b) days.

As shown in Figure 5-4, freshly isolated hepatocytes were typically found in cell aggregates that contained approximately 85-98% viable cells. Despite numerous attempts with various enzymatic digestions ranging from dispase to collagenase, a complete single cell suspension of hepatocytes was not obtained. Isolated hepatocytes did not proliferate in culture but instead after 7 and 15 days, fibroblastic cells overgrew the primary hepatocyte cultures. This culture overgrowth occurred regardless of the properties of the coated dishes. The majority of hepatocyte aggregates disappeared during the two-week culture period. However, based on MTT and albumin secretion results (see Figure 5-5 and Figure 5-6), it was clear that the loss of hepatocyte viability and function occurred within the first few days of culture.

Figure 5-5 Cell specific MTT activity for isolated primary hepatocytes in various culture conditions.
(SFM = HepatoZYME-SFM and F12 represents DF medium)

At different times after liver culture initiation, cell specific metabolic activity was analyzed using MTT assay. As indicated in Figure 5-5, the rate of formazan absorbance per cell decreased with time from ~2 x 10^-5 abs units/cell for freshly isolated hepatocytes to ~4 x 10^-6 abs units/cell after a week of culture. This represents a significant decrease in cell specific metabolic activity. This loss can be
attributed to a decrease in hepatocyte metabolic function as well as changes in population dynamics due to overgrowth of fibroblastic cells.

Figure 5-6 Albumin secretion rate in various primary hepatocyte cultures.
(SFM = HepatoZYME-SFM and F12 represents DF medium)

To test for hepatocyte function within these cultures, the extent of albumin secretion from hepatocyte cultures in various conditions were measured. As demonstrated in Figure 5-6, despite the use of collagen coated flasks or HepatoZYME-SFM, the rate of albumin secretion in all cultures decreased to nearly undetectable levels within one week after culture initiation. These results are strong evidence that the use of primary hepatocytes, at least under the conditions tested, was not feasible in order to facilitate the long-term co-culture of BM derived cells with hepatocytes.

5.2.2 Hepatocyte and fibroblast characterization

To overcome the difficulties faced in maintaining primary hepatocytes in vitro, AML12 hepatocytes and NIH-3T3 fibroblasts were used. These cell lines were also used for the development of analytical methods to characterize morphological and functional properties of primary hepatocytes and hepatic cell lines.
In culture, NIH-3T3 and AML12 cell lines demonstrated phenotypical features that were characteristic of their primary fibroblastic and hepatic counterparts. As shown in Figure 5-7, NIH-3T3 cells were adherent and spread to form fibroblastic cells which resembled the fibroblasts from primary BM or liver cultures. On the other hand, AML12 cells had polygonal morphology with distinct intracellular features such as nucleus and endoplasmic reticulum.

The rate of albumin secretion from NIH-3T3 and AML12 cells was also measured. Albumin was not detected from NIH-3T3 cells (< 2 x 10^8 ng/100 cell/day); however, AML12 cells secreted albumin at approximately 0.04±0.02 ng/100 cells/day. This rate is lower than the secretion rates obtained from primary hepatocyte spheroid cultures or in reactors, which has been reported to be on the order of 1 to 10 ng/100 cells/day respectively. Furthermore, no significant difference in the rate of albumin secretion from AML12 cells was observed with time (data not shown). Despite a documented decrease in hepatic function with time, the strict use of AML12 cells with passage numbers between 37 to 47 facilitated consistent hepatic properties.

The ability of TROMA-1 antibody to stain CK8 in AML12, NIH-3T3, primary hepatocytes and isolated BM cells was tested using flow cytometry. As it can be seen in Figure 5-9, both primary hepatocytes and AML12 cells expressed significantly higher levels of CK8 compared to their respective isotype controls. Interestingly, human anti-cytokeratin antibodies that were tested did not stain liver cytokeratins, though they have been shown to react with non-liver murine endoderm cells. As expected, NIH-3T3 or freshly isolated WBM cells did not express CK8. The expression of CK18 as measured using the TROMA-2 antibody was also examined. The staining pattern of CK18 was similar to that of CK8. AML12 cells expressed CK18, while CK18 expression was not detected in WBM and NIH-3T3 cells (see Figure 5-10).
Figure 5-9 Typical CK8 expression of AML12 (a), NIH/3T3 (b), primary hepatocytes (c) and WBM cells (d). Red and black represent isotype and stained samples respectively.

Figure 5-10 Typical CK18 expression of AML12 (a), NIH/3T3 (b) and freshly isolated WBM cells (c). Red and black represent isotype and stained samples respectively.
Figure 5-11 Expression of E-cadherin (a) and c-Met (b) on AML12 cells. Red and black represent isotype and stained samples respectively.

To further characterize AML12 and NIH-3T3 cells, the expression of other morphological markers such as E-cadherin and c-Met was also studied. As illustrated in Figure 5-11, AML12 cells expressed E-cadherin and c-Met. Table 4 summarizes the expression of various phenotypic markers on primary hepatocytes, AML12 and NIH-3T3 cells. As indicated, AML12 and NIH-3T3 cells did not express the hematopoietic markers CD45 and c-kit. In addition, AML12 cells expressed all hepatic markers that were examined, while NIH-3T3 stained negatively for all these markers.

Table 4: Expression of various hematopoietic and hepatic markers for primary hepatocytes, AML12, NIH-3T3 cells

<table>
<thead>
<tr>
<th></th>
<th>NIH-3T3</th>
<th>AML12</th>
<th>Primary Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>(-)ve</td>
<td>(-)ve</td>
<td>ND</td>
</tr>
<tr>
<td>c-kit</td>
<td>(-)ve</td>
<td>(-)ve</td>
<td>ND</td>
</tr>
<tr>
<td>CK8</td>
<td>(-)ve</td>
<td>(+)ve</td>
<td>(+)ve</td>
</tr>
<tr>
<td>CK18</td>
<td>(-)ve</td>
<td>(+)ve</td>
<td>ND</td>
</tr>
<tr>
<td>E-Cad</td>
<td>(-)ve</td>
<td>(+)ve</td>
<td>ND</td>
</tr>
<tr>
<td>c-Met</td>
<td>(-)ve</td>
<td>(+)ve</td>
<td>ND</td>
</tr>
</tbody>
</table>

(ND – not determined)

The use of surface markers was particularly intriguing in the study of hepatocytes since it allows for the analysis of cells without the need for fixation or permeabilization. Interestingly, although CK8 or CK8-like protein expression has been reported on the surface of hepatocytes 295,296, neither CK8 nor CK18 could be detected on AML12 cells by surface staining. Attempts were also made to analyze cell specific albumin expression from hepatocytes. However, results from these experiments were
inconsistent due to low albumin secretion or heterogeneity of albumin secretion\(^{297}\) from hepatocytes (data not shown).

5.2.3 Tracking of bone marrow derived cells in vitro

In order to distinguish BM derived cells from feeder layers, CFSE staining and YFP expression were used to track cells in co-cultures.

5.2.3.1 CFSE

The feasibility of CFSE dye to track BM derived cells was analyzed by staining freshly isolated BM derived cells prior to co-culture with unstained feeder cells. Despite published accounts on the ability to track cell division dynamics, in our cultures CFSE stained BM cells did not maintain high level of expression after one week. Figure 5-12 shows the flow cytometric results from co-culture experiments between CFSE stained WBM and AML12 cells. As it can be seen, despite clear distinction between initial cell populations, the CFSE expression from BM cells was not sufficient to allow for their tracking after 7 days.

![Figure 5-12 CFSE stained WBM (center) and unstained AML12 (left) cells on day 0 were distinguishable based on CFSE expression; however, after 7 days in co-culture, the two populations were indistinguishable (right).](image)

This was surprising considering that co-cultures with labeled and unlabeled NIH-3T3 cells seemed to maintain their CFSE expression for longer periods than BM cells (data not shown). This can be attributed to the significant cell death in BM cultures which may result in the release of CFSE content in the form of lysed cell membranes. These lysed membranes or soluble CFSE may in turn stain the feeder cells. Furthermore, primitive BM progenitors quickly proliferate in culture, resulting in the rapid decrease of dye expression in their progeny.
5.2.3.2 Yellow fluorescent protein (YFP)

To overcome the difficulties encountered in tracking CFSE labeled BM cells in co-cultures, BM cells derived from YFP+ mice were utilized. These mice had normal hematopoietic systems and their BM cells were capable of giving rise to various types of CFCs (see Figure 5-13). Furthermore, YFP expression was strong enough to be detected from individual cells that formed CFC colonies. This property was used to distinguish BM derived CFC colonies from other colonies during analysis.

Figure 5-13 YFP expression of CFU-GM from YFP+ BM cells in CFC assay.
(Bar indicates 50 μm – cultures were harvested 7-12 days after initiation)

Flow cytometric analysis confirmed that nucleated BM cells expressed significantly higher levels of YFP in comparison with background feeder cells (such as AML12 cells – Figure 5-14 (a-c)). However, since the YFP gene shuts down at later stages of erythroid differentiation, the analysis from WBM (which included erythrocytes), indicated the presence of a peak with low YFP expression which was eliminated after erythrocytes were lysed. Lin- subpopulation expressed high levels of YFP, and maintained its expression after 7 days in culture with or without the presence of background feeder cells (Figure 5-14 (d-f)).
Figure 5-14 BM derived cells from YFP+ mice had distinguishably higher YFP fluorescence than AML12 cells. AML12 expression is shown in (a). WBM cells had two distinct peaks (b), the peak with the lower expression represents the erythrocyte population which disappears after lysing of RBCs (c). Lin- cells also express YFP (d) and maintain this expression in culture after 7 days alone (e) or in co-culture with AML12 cells (f).

Based on the ability to distinguish YFP+ BM derived cells from YFP- feeder cells, all subsequent co-culture experiments utilized YFP+ cells as the desired tracking tool.
5.3 Effects of in vitro cultures on hematopoietic progenitor cell fate

Although the primary focus of this project was to study the endoderm differentiation of BM derived cells, the creation of these cultures revealed important information about the behavior of hematopoietic cells in liver-like environments.

5.3.1 Effects of AML12 and NIH-3T3 fibroblasts on hematopoietic differentiation of BM derived cells

As an initial approach to understand the effects of the liver microenvironment on BM derived progenitor cell fate, Lin- cells were co-cultured with AML12 hepatocytes and NIH-3T3 fibroblasts. The hematopoietic differentiation of BM derived progenitors in various conditions was examined based on YFP+ cell counts as well as CFC assays. As demonstrated in Figure 5-15, a significantly higher number of cells were derived from co-cultures with fibroblasts in comparison with AML12 or without feeder layers. NIH-3T3 co-cultures led to >3 fold expansion of the total number of BM cells within a week after culture initiation, and they maintained their YFP+ cell numbers after two weeks of culture. Surprisingly, co-cultures with hepatocytes also allowed for moderate expansion of BM derived cells after a week of culture. However, the total number of YFP+ cells in these cultures greatly diminished after two weeks.

With respect to hematopoietic progenitor maintenance, co-cultures with fibroblast and hepatocyte feeders maintained similar frequencies of CFCs to total BM derived cells throughout the experiment (see Figure 5-16). However, cultures without addition of exogenous cytokines or feeder layers lost their ability to give rise to CFCs within the first week of culture. Despite the similarity in the proportion of CFC in both hepatic and fibroblastic co-cultures, higher cell counts yielded significantly higher overall progenitor numbers in fibroblastic cultures.
Figure 5-15 BM derived cell numbers in various co-cultures that were initiated with \(5 \times 10^5\) Lin- cells.  
Control represents cultures without feeder cells.

Figure 5-16 Effects of co-culture on the frequency of CFCs to total cells in cultures that were initiated with \(5 \times 10^5\) Lin- cells.  
Control represents cultures without feeder cells.

Figure 5-17 Effects of co-culture condition on CFC types, 7 days after culture initiation.

Cultures with exogenous HGF had significantly higher proportion of BFU-E progenitors in comparison to the input population while the hepatic co-cultures had significantly reduced ratio of BFU-E progenitors \((p < 0.05)\). No CFCs were detected in control cultures without feeder layers.

Co-culture conditions affected the types of hematopoietic progenitors produced, as measured by CFC assay. Co-cultures with fibroblast cells gave rise to a significantly higher proportion of BFU-E colonies compared to co-cultures with AML12. The values for cultures without feeder cells could not be determined due to the inability to recover sufficient number of cells.

### 5.3.2 TPO and HGF alter bone marrow progenitor cell fate

To elucidate the effects of liver growth factors on hematopoietic differentiation of BM progenitors, two specific cytokines, TPO and HGF (10ng/ml) were investigated. These growth factors were
particularly interesting since they were secreted by hepatic and fibroblastic cells (hepatocyte cell lines secrete TPO\textsuperscript{152} and NIH-3T3 cells secrete HGF\textsuperscript{201}). Cultures supplemented with HGF or TPO yielded higher overall cell and CFC counts in comparison with cultures without exogenous cytokines (see Figure 5-18 and Figure 5-19). In general, cultures supplemented with exogenous TPO generated more cells. Cultures supplemented only with HGF generated lower cell numbers when compared to TPO supplemented cultures; however, they generated significantly higher total cell numbers than cultures without feeder layers. The frequency of CFCs compared to total number of BM derived cells present in a co-culture at any point in time decreased for all co-culture conditions in a logarithmic fashion. However, TPO supplemented cultures maintained higher proportions of CFCs during the two-week trial in comparison with HGF supplemented cultures. CFC colonies disappeared within the first week in BM cultures without exogenous addition of TPO or HGF. Also, HGF supplemented cultures had a higher proportion of BFU-E progenitors than non-HGF supplemented cultures (see Figure 5-20).

![Figure 5-18](image1.png)  ![Figure 5-19](image2.png)

**Figure 5-18** Effects of TPO or HGF supplementation on BM cell counts in cultures initiated with 5 x 10\(^2\) Lin\(^-\) cells.  **Figure 5-19** Effects of TPO and HGF on the frequency of CFCs compared to total cells in BM cultures initiated with 5 x 10\(^5\) Lin\(^-\) cells.
**5.3.3 Effects of HGF supplementation on hematopoietic development**

The emergence of a higher proportion of BFU-E colonies in co-cultures with NIH-3T3 led us to investigate the effects of exogenous HGF on co-cultures. To examine the effects of HGF on hematopoietic differentiation of BM derived cells, HGF was added to AML12 and NIH-3T3 co-cultures. As shown in Figure 5-21, HGF (10 ng/ml) supplementation to co-cultures resulted in an increase in the total cells numbers (~2 fold increase) versus untreated cultures.

![Figure 5-20](image-url) Effects of HGF and TPO supplementation on BM derived CFC Types in cultures initiated with 5 x 10^5 Lin^- cells after 7 days.

HGF supplemented cultures had a significantly higher proportion of BFU-E progenitors in comparison with other culture conditions (p<0.05).
Figure 5-21 Effects of HGF supplementation on YFP⁺ cell counts in cultures initiated with $5 \times 10^5$ Lin⁻ cells.

Figure 5-22 Effects of HGF supplementation on the frequency of CFC colonies to total cells at any point in time for cultures initiated with $5 \times 10^5$ Lin⁻ cells.

Figure 5-23 Effects of HGF supplementation on CFC types in cultures initiated with $5 \times 10^5$ cells at day 7.

As illustrated the ratio of BFU-E colonies was much lower in co-cultures with AML12 cells in comparison with HGF cells. Furthermore, exogenous addition of HGF enhanced the proportion of BFU-E colonies. The exogenous addition of HGF to fibroblastic cultures did not significantly enhance the proportion of BFU-E colonies while its addition to hepatic cultures increased the proportion of BFU-E cultures ($p < 0.05$).
Also, co-cultures supplemented with HGF enhanced the total number of CFCs and expressed higher BM derived cell counts (see Figure 5-23 and Figure 5-22). Interestingly, the addition of HGF to hepatocyte feeder layers also enhanced the proportion as well as the total number of BFU-E progenitors compared to cultures with no HGF supplementation.

5.4 Bone marrow derived endoderm differentiation

5.4.1 Co-culture of Lin' cells with AML12 or 3T3 cells

As a first approach to dissect the liver microenvironment in studying the factors that influence BM derived hepatic differentiation, YFP⁺ BM cells were co-cultured with AML12 and 3T3 cells. In initial experiments, Lin' cells were directly co-cultured with feeder cells and monitored for CK8 and CK18 expression as well as albumin secretion. Unfortunately, due to the secretion of albumin by hepatocytes and AML12 cells, detection of serum albumin was not used in co-cultures with AML12 cells.

![Figure 5-24 Expression of CK8/CK18 and YFP on NIH-3T3 (a), AML12 (b), and Lin' cells (d)](image)

Figure 5-24 illustrates the expression of CK8 and YFP on cells that initiated the co-cultures. As shown previously, NIH-3T3 and AML12 cells were distinguishable from YFP⁺ BM derived cells. Also, AML12 hepatocytes expressed CK8 and CK18 which was not expressed on fibroblast or BM derived cells. In addition, YFP⁺ CK8⁺ cells were not observed in any of the cultures, thus indicating that the cells that were used to initiate such co-cultures did not express both YFP and CK8 simultaneously.
It utilized FS-SS gates (a) followed by discrimination of events in which PI-PI peak distribution did not increase linearly (b).

Figure 5-26 CK8 and YFP expression of Lin⁺ cells after two days in co-culture with AML12 cells.

The graphs which did not use doublet discriminator (a) showed significantly higher number of CK8⁺YFP⁺ cells than plots in which doublet discriminator was used (b).

In co-cultures containing Lin⁺ and AML12 cells, there was no detectable amount of endoderm differentiation based on microscopic analysis (see Table 5). However, initial flow cytometric analysis yielded results contradicting those of the microscopic analysis (see Figure 5-26). These results indicated that Lin⁺ BM cells (i.e. mature BM populations, which do not have the capability to give rise to non-hematopoietic cells) gained CK8 expression while in co-culture with AML12 cells. As confirmed by fluorescent microscopy, the presence of CK8⁺YFP⁺ cells were due to cell aggregates that contained CK8⁺ AML12 and YFP⁺ BM cells. These aggregates were formed because cells were not completely dissociated prior to flow cytometric analysis. Thus, despite FS-SC discrimination, the presence of BM derived cells that were attached to 'sticky' hepatocytes yielded an aggregate co-expressing YFP and CK8.
By utilizing the doublet discriminator (see Figure 5-25), the percentage YFP⁺ events that expressed CK8 due to clumping significantly decreased. However, even the use of doublet discriminator did not eliminate this phenomenon (see Figure 5-26).

Figure 5-27 CK8 and YFP expression of Lin⁻ cell in co-culture with AML12 cells, 2 days (a), 7 days (b) and 15 days (c) after culture initiation.

Figure 5-27 illustrates the flow cytometric results from co-cultures with AML12 cells, in which, despite the use of the doublet discriminator, a small population of cell aggregates remained. Evidence that showed no individual cells co-expressed YFP and CK8 was observed using fluorescent microscopy (see Table 5). Further evidence of the inability of AML12 cells to give rise to CK8⁺ YFP⁺ BM cells was demonstrated by co-culturing Lin⁻ YFP⁺ cells with AML12 cells separated by a permeable barrier. In these cultures, a membrane prevented direct cell contact between the feeder and BM derived cells. As shown in Figure 5-28, the flow cytometric analysis of the BM derived cells cultured in this manner did not show the emergence of a YFP⁺ CK8⁺ cell population.

Figure 5-28 CK8 and YFP expression of Lin⁻ cells cultured without direct cell contact with AML12 feeder layers, 2 days (a), 7 days (b) and 15 days (c) after culture initiation.

These experiments demonstrated the importance of single cell suspensions in the analysis of co-cultures that contain hepatocytes. Also, the inability to detect endoderm markers on BM derived cells
indicated that co-culture with hepatocyte-like cells was not sufficient in inducing BM derived hepatic differentiation.

To investigate the nonparenchymal (i.e. stellate cell) component of the liver, BM derived progenitors were co-cultured with NIH-3T3 cells. These co-cultures were much easier to analyze due to the absence of hepatic markers (such as albumin, CK8 and CK18) in freshly isolated WBM and fibroblastic cells. Figure 5-29 indicates the CK8 and YFP expression of Lin⁻ cells that were co-cultured with NIH-3T3 cells through a two-week period. After 7 days, a population of Lin⁻ cells expressed CK8 (and CK18) markers. The expression of CK8/CK18 in the BM cultures peaked on day 7, and then it became undetectable by day 15. In Lin⁺ cells, no CK8⁺ YFP⁺ cells emerged throughout the culture period.

Albumin was also detected in these co-cultures over the first week of culture (see Figure 5-32). However, its expression quickly diminished over the culture period. This coincided with the presence of YFP⁺ CK8⁺ cells within the culture. These results indicated the emergence of a transiently appearing CK8⁺ YFP⁺ population in co-cultures with NIH-3T3 cells, which is only present in the Lin⁻ subpopulation.
Figure 5-29 Flow cytometric expression of CK8 and YFP on BM derived Lin−(a-b) and Lin+ (c-d) cells in co-culture with NIH-3T3 cells.

In Lin− co-cultures with NIH-3T3 cells, YFP+ CK8+ cells did not appear after 7 (a) or 15 days (b). However, in Lin+ cultures YFP+ CK8+ cells appeared after 7 days (c) and lost their CK8 expression within two weeks of culture (d).

5.4.2 Effects of hepatocyte growth factor (HGF) on BM progenitors

5.4.2.1 Exogenous HGF supplementation to co-cultures

Previous reports, in which HGF has been shown to induce transition of mesenchymal cells to epithelial and endoderm tissues, along with our findings from co-cultures with NIH-3T3 fibroblasts (which have been shown to secrete HGF), led us to question the effects of HGF in inducing endoderm differentiation of murine BM derived cells. Aside from being a hepatic mitogen that is secreted by mesenchymal cells (such as fibroblasts), HGF has been shown to play a regulatory role in murine BM progenitor cell fate decisions. In fact, it was demonstrated that c-Met (HGF receptor) is expressed on a large population of hematopoietic progenitors (see Section 5.1). Since the primary structure of HGF is >90% homologous in humans and rodents, rhHGF cross-reacts with
murine c-Met. Thus, rhHGF was used in subsequent experiments in order to test the effects of HGF on BM derived endoderm differentiation.

To examine the exogenous effects of HGF, co-cultures were set up as in previous experiments with the addition of medium supplemented with HGF. In these cultures, the presence of individual BM derived cells (i.e. YFP*) expressing endoderm marker (i.e. CK8) was observed (see Figure 5-30). In all co-cultures, it was observed that HGF enhanced the proportion of BM derived cells that express endoderm markers CK8 and CK18. Table 5 represents the microscopic analysis of the co-culture systems in which exogenous HGF was added.

Table 5: Percentage of CK8* YFP* cells detected in various co-cultures using fluorescent microscopy after 7 and 14 days.

<table>
<thead>
<tr>
<th>Day 7</th>
<th>AML12</th>
<th>3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF medium</td>
<td>(Not detected -- 0 / 457)</td>
<td>(0.6 % -- 4 / 687)</td>
</tr>
<tr>
<td>HF medium + 10 ng/ml HGF</td>
<td>(1.5 % -- 8 / 544)</td>
<td>(1.2 % -- 10 / 789)</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>(Not detected -- 0 / 362)</td>
<td>(Not detected -- 0 / 523)</td>
</tr>
<tr>
<td>HF + 10 ng/ml HGF</td>
<td>(0.9 % -- 5 / 507)</td>
<td>(0.2 % -- 1 / 423)</td>
</tr>
</tbody>
</table>

Figure 5-30 The presence of individual cells expressing CK8, after 7 days of co-culture with NIH-3T3 and AML12 cultures supplemented with HGF (10ng/ml) was confirmed using fluorescent micrographs.

(Bar indicates 50 μm)

In normal NIH-3T3 co-cultures (i.e. not initiated with HGF supplemented medium), rare BM derived cells that expressed CK8 (~0.6%) were observed after 7 days of culture. However, the CK8* population was no longer detectable after 14 days. In these co-cultures, the addition of exogenous HGF increased the overall number and frequency of CK8* YFP* cells generated after 7 days of co-cultures (see Figure 5-31) and nearly doubled the rate of albumin secretion (see Figure 5-32).
In co-cultures with AML12 cells, the addition of HGF led to the generation of rare CK8* YFP* cells that remained present after two weeks of culture.

Figure 5-31 CK 8 expression of Lin- cells cultured with 3T3 fibroblasts in absence (a) or presence (b) of 10 ng/ml HGF seven days after initiation of the cultures.

Figure 5-32 Albumin secretion from Lin- cells co-cultured with NIH-3T3 feeder cells. (No albumin was detected in HF medium and NIH-3T3 cultures or Lin- cultures without feeder layers (no HGF)).

5.4.2.2 HGF induces hepatocyte differentiation in a dose dependent manner

The dose response of BM derived progenitors to HGF was analyzed by culturing 5 x 10^5 Lin- cells on dishes pre-coated with Matrigel® and then measuring the rate of albumin secretion and cellular proliferation. BM derived Lin- cells were cultured in HF medium without serum for 6 days in a range
of HGF concentrations. The cells were then cultured in HF medium containing 10% FBS in the presence of 10 ng/ml HGF (see Figure 5-33).

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 6</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Free</td>
<td>Serum added</td>
<td></td>
</tr>
<tr>
<td>HGF (various concentrations)</td>
<td>HGF (10 ng/ml)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5-33 Culture conditions used in experiments to determine effects of HGF concentration.**

The percentage of viable cells in these cultures dropped significantly within the first week. Due to insufficient number of cells retrieved from each well, cell counts could not be performed. However, despite this difficulty, no qualitative difference in the number of viable cells in different HGF concentrations was detected. Based on the limited number of cells retrieved from these cultures it is estimated that only about 5 - 10% of the initial Lin- populations remained viable past one week. Also, due to the inability to recover a significant number of cells, only a limited number of cells were used in flow cytometric analysis. These results further indicate that c-Met+ CK8+ cells may be responsible for the albumin detected in cultures, since their emergence correlated with the expression of albumin (see Figure 5-34). Typically, the flow cytometric analysis in these cultures was limited to <500 cells, thus large scale experiments may be required to demonstrate this phenomenon in a statistically significant manner. As shown in Figure 5-34, the emergence of CK8+ cells was detectable on day 6 in HGF cultures and was maintained at least to day 16. Interestingly, the majority of CK8+ cells co-express c-Met, which further strengthens the hypothesis that HGF induces their hepatic differentiation behavior.
Figure 5-34 Flow cytometric analysis of CK8 and c-met expression on Lin⁻ BM cultures supplemented with exogenous HGF after 6 and 16 days.

Results indicate the emergence of CK8⁺ cells in cultures initiated with 1000 ng/ml HGF (a-b) while no CK8⁺ cells were detected in cultures that were not supplemented with HGF (c-d).

Figure 5-35 illustrates the rate of albumin secretion from various conditions over the 21-day experiment. As indicated, albumin secretion rate was regulated in a dose dependant manner, with sustained expression (after 21 days) only observed in cultures that were initiated with 1 µg/ml HGF. Interestingly, all cultures containing HGF seemed to secrete detectable levels of albumin above controls within the first week of culture. However, soon after most cultures initiated with low HGF concentration quickly lost their ability to secrete albumin. This behavior was similar to NIH-3T3 co-cultures in which albumin-secreting cells were detected in culture at early time points.
Figure 5-35 Effects of HGF concentration on albumin expression in BM cultures initiated with 5 x 10^5 Lin^- cells.

(* indicates p<0.05 in comparison with non-HGF supplemented control)

Interestingly, in cultures that continuously secreted albumin, spheroids containing ~5-20 cells were observed. These spheroids were morphologically similar to the aggregates formed when AML12 cells were cultured on Matrigel® (see Figure 5-36). The presence of non-viable cells was detected in these cultures (dark and granular cells visible in Figure 5-36) indicates that only a small fraction of Lin^- cells survived.

Figure 5-36 Morphological features of AML12 hepatocytes cultured on Matrigel® in comparison with (a)HGF (1000 ng/ml) supplemented BM cultures (b).

(Bar indicates 50 μm).
5.4.2.3 Frequency of differentiation and effects of hematopoietic cytokines on HGF mediated generation of endoderm-like cells

To further investigate the mechanisms that regulate HGF on inducing BM derived hepatic differentiation, the addition of cytokines that have shown to be important in expansion of HSC in culture was analyzed. Also, to define whether BM derived hepatic progenitors were hematopoietic, HSC phenotypically defined by c-kit' Sca-1' Lin' CD45' (KLS) markers were tested against Lin' CD45' cell populations. These populations were plated at 1 to 100 KLS cells or 1000 Lin' CD45' cells in medium that was supplemented with either 1 µg/ml HGF, or HM or combination of HGF and HM.

After 17 days, cultures containing HM or 1 µg/ml HGF plus HM induced the proliferation of Lin' cells while no significant proliferation was detected in cultures that contained HGF alone. In these cultures, only a fraction of the wells that were initiated with 1 KLS cell contained live cells regardless of the type of medium used. However, majority of wells contained cellular debris and dead cells, which indicated that viable cells had been present previously. Despite this, it was clear from wells initiated with ≥ 10 KLS cells that cell proliferation was greater in cultures that contained hematopoietic cytokines. Cultures that contained HM plus HGF formed the largest colonies, providing further evidence that HGF works synergistically with hematopoietic cytokines to enhance hematopoietic cell proliferation.

Albumin secretion was measured to determine the frequency of albumin secreting cells as well as the rate of secretion. Figure 5-37 illustrates the amount of albumin detected after 17 days from wells initiated by 1000 CD45 Lin' cells. No statistical difference between the cultures was observed (p < 0.05). A background level of albumin was detected in all wells due to the Matrigel<sup>®</sup> coating. The fact that the various media did not significantly modify albumin secretion rate indicates that the different cytokines did not cross-react with ELISA antibodies. Furthermore, as Figure 5-37 indicates, CD45 Lin' cells did not give rise to progeny which secreted albumin regardless of the microenvironmental cues provided.
To determine the frequency of KLS cells that gave rise to albumin secreting cells, albumin measurements per each well that was initiated from one KLS cell were compared with background medium (HM medium alone was chosen since it had the highest background level). The values were then compared using stringent criteria (p < 0.01) to eliminate false positives. Table 6 indicates the obtained frequencies of wells which secrete albumin and thus contain BM derived hepatic cells. As indicated, about 10.1% of wells containing 1 KLS cells in HGF, and only 4.2% of the cells that combined HGF with HM gave rise to detectable levels of albumin. No significant albumin was detected from any of the wells containing medium rich only in hematopoietic cytokines.

Table 6: Albumin secretion of KLS cells plated individually in 96 well plates after 17 days in various mediums.

<table>
<thead>
<tr>
<th></th>
<th>% of (+)ve wells --- (# of (+)ve wells / # of total wells)</th>
<th>Average albumin measured from (+)ve wells (ng/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>10.1% — (7 / 69)</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>Hematopoietic medium</td>
<td>0% — (0 / 58)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>HGF + Hematopoietic medium</td>
<td>4.2% — (3 / 72)</td>
<td>9 ± 2</td>
</tr>
</tbody>
</table>

Positive wells are determined by stringent criteria (p < 0.01) in comparison to highest background levels.

Furthermore, the average levels of detectable albumin in various positive wells were highest in cultures containing only exogenous HGF compared to conditions in which HGF and HM were added. This could be interpreted either as a proliferative or functional effect of HGF on the resulting cells. Thus either the endoderm-like cells generated in HGF cultures secreted albumin at higher rates than
HGF+HM conditions or that they generated more endoderm-like cells. Based on typical albumin expression of cultured hepatocytes, the values obtained in these results suggest similar values as would be obtained by the secretion of 1 to 10 hepatocytes (depending on albumin secretion levels) in cultures for the duration of the experiment. Therefore the observed proliferation data in each well correlated with the albumin secretion results.

![Figure 5-38 Albumin secretion after 17 days in 1000 ng/ml HGF (HGF) with addition of 1000 Lin'CD45' (HGFCD45), or 1 (HGF1), 10 (HGF10) or 100 (HGF100) KLS cells to each well. (* indicates p<0.01 in comparison to 1000 ng/ml HGF alone without cells).](image)

Figure 5-38 represents the average albumin secretion from all the plates comprised of 1, 10 and 100 KLS cells, as well as 1000 Lin' CD45' cells in HGF medium. As expected, all conditions in which KLS cells were added to the medium resulted in significantly higher measured albumin values. Surprisingly, the albumin secretion per well does not directly increase based on the number of KLS cells present in each well. This could be due to cellular interactions at early stages of cell fate determination of KLS cells. The presence of a large number of KLS cells or their hematopoietic progeny may in fact prevent endoderm differentiation. These results are also demonstrated in cultures with medium containing HGF and hematopoietic cytokines (see Figure 5-39). In these cultures, the addition of 10 or 100 KLS cells did not dramatically increase the albumin secreted. However, more experiments are required to determine the exact reasons for these observations.
Figure 5.39 Albumin secretion after 17 days in cultures initiated with HM and 1000 ng/ml HGF (HGFHM) with addition of 1 (HGFHM1), 10 (HGFHM10) or 100 (HGFHM100) KLS cells to each well.

(* indicates p<0.01 in comparison with HGFHM medium)
6 DISCUSSION

6.1 Development of the co-culture systems

A co-culture system was developed to analyze the fate of BM derived cells in hepatic microenvironments. This was accomplished by finding techniques to distinguish BM derived cells from feeder layers, to characterize murine hepatocytes and to select proper feeder cells that would mimic various aspects of the liver microenvironment.

Despite preliminary attempts to maintain primary hepatocytes using various extracellular matrices and media, these cells did not sustain normal metabolic activity or hepatocyte-specific markers such as albumin. In addition, the overgrowth of fibroblastic cells in primary hepatic cultures was undesired as it skewed the cell population dynamics. This overgrowth also prevented the separation of parenchymal from nonparenchymal cells in the attempt to distinguish the cell type that was responsible for endoderm differentiation of BM derived cells. As expected, the yield of isolated hepatocyte was much lower than the published values for the two-step collagenase method\(^\text{301}\); however, the main reason for abandoning the use of primary hepatocytes as the desired feeder layer was their poorly maintained phenotype in culture.

As discussed previously, the test experimental system utilizes AML12 and NIH-3T3 cell lines. These cell lines were ideal for these studies because they were representative of hepatocytes and stellate cells. The AML12 cell line expressed all functional, phenotypical, and morphological characteristics of hepatocytes that were tested. Furthermore, these cells secreted the hepatic mitogen TGF-\(\alpha\) in an autocrine loop. Therefore, signals provided by AML12 cells provide a suitable microenvironment for the maintenance of hepatic functional and morphological features.

To mimic the mesenchymal component of the liver microenvironment, the NIH-3T3 cell line was used. It is believed that the factors produced by liver mesenchymal cells control the differentiation and growth of hepatocytes. For example in liver development, the cardiac mesoderm is required to induce endoderm cells to proliferate and form hepatic primordium\(^\text{302}\). Also, a second induction by the mesenchymal cells is needed to enable cells to express hepatic markers\(^\text{302}\). The mesenchymal cells found in close proximity with hepatocytes are the stellate cells. These cells are believed to be the main source of ECM (collagen, fibronectin, laminin, heparin, heparan sulphate) and growth factors. Furthermore, these cells are believed to be important in controlling the developmental fate of oval cells\(^\text{303}\). Based on previous research\(^\text{200}\), NIH-3T3 cells have been shown to be a good representative of liver mesenchymal cells. In fact NIH-3T3 cells induce differentiation of oval cells into hepatocytes at higher frequencies than stellate cell lines\(^\text{200}\). Although this is surprising, since stellate cells reside in
the liver, this behavior may be explained by the inactivation of stellate cells in culture, as they may no longer produce the growth factors and matrix components necessary to elicit differentiation.

6.2 Effects of in vitro culture conditions on hematopoietic progenitor cell fate

Although the focus of this work was to study the factors which induce hepatic differentiation of BM derived stem cells, it was shown that co-cultures with fibroblasts increase the number of CFCs and total cells in comparison to co-cultures with AML12 cells. This is not surprising since fibroblast feeder cells have been used as stromal cells for in vitro BM cultures in order to maintain CFCs and LTC-ICs. NIH-3T3 fibroblasts secrete numerous cytokines (such as SCF and FL) which are shown to be important in HSC maintenance. Furthermore, NIH-3T3 cells secrete biologically active HGF that has been shown to be an important hematopoietic regulator. Interestingly, NIH-3T3 co-cultures greatly enhanced the frequency of BFU-E progenitors. This trend was similar to cultures in which HGF was added exogenously, which may suggest that BFU-E enhancing effects of fibroblasts are attributed to HGF. Also, since HGF acts synergistically with SCF, GM-CSF and IL-3, it may combine with the other NIH-3T3 secreted cytokines in enhancing the total hematopoietic cell numbers from these co-cultures.

Surprisingly, AML12 cells supported moderate proliferation of hematopoietic cells and maintenance of CFCs. This was the first demonstration that AML12 cells were capable of supporting hematopoiesis. Traditionally, hepatocytes have not been associated with adult hematopoietic maintenance, mainly due to the difficulties in maintaining primary hepatocytes cultures. However, embryo derived hepatocyte cell lines (such as MMH) have been shown to support in vitro hematopoiesis. These cells express cytokines involved in the survival and self-renewal of early progenitor cells (SCF and FL), as well as those acting at various stages of progenitor differentiation (LIF, IL-6, GM-CSF, M-CSF, G-CSF and TPO). Although the expression of some of these cytokines is shut off during development, the expression of TPO and GM-CSF, G-CSF, and FL continues in adult hepatocytes. This cytokine profile may explain the reason why co-cultures with AML12 cells gave rise to considerably higher proportion of CFU-GM colonies in comparison to erythroid progenitors. As stated previously, TPO induces megakaryocyte differentiation, whereas GM-CSF and G-CSF induce formation of granulocytes and monocytes (which can be detected by CFU-GM colonies). Furthermore, TGF-α has been shown to induce granulocytic and monocytic differentiation of human leukemia cells. Therefore, its presence may further enhance the formation of CFU-GM colonies in AML12 co-cultures.
Although membrane bound TGF-α has been shown to be present on erythroblasts and other erythroid cells, AML12 co-cultures did not enhance erythropoiesis. This could be due to the fact that TGF-α may require the presence of TGF-β to enhance the self-renewal of erythrocytic progenitors. Since TGF-β is not secreted by hepatocytes, the presence of TGF-α alone may not be sufficient to enhance BFU-E generation.

Co-cultures with supplemented HGF, contained significantly higher overall cell numbers, probably due to synergistic effects of HGF with other hematopoietic cytokines (SCF or IL-3, IL-12, IL-13, IL-14). Furthermore, this effect is shown to be concentration dependent, as indicated by the increase in overall cell numbers in co-cultures with NIH-3T3 cells (since HGF is already present in these cultures – see Figure 5-21). Based on our analysis, the ratio of the progenitor cells to the total number of cells within these cultures did not change significantly in the presence of exogenous HGF; however, the resulting change in the overall number of CFC was due to total population expansion. Also, HGF enhanced the total number of BFU-E cells in both co-cultures, further demonstrating its importance in erythroid differentiation. The BFU-E stimulating activity of HGF is still under investigation. However, recent data suggests that HGF induces the activation of signal transduction pathways linked to Epo receptor and therefore plays a crucial role in the commitment of multipotent myeloid progenitors into erythroid lineage.

6.3 Bone marrow derived endoderm differentiation

There is strong evidence to support the notion that adult BM contains stem cells that are capable of differentiating into all hematopoietic and hepatic lineages. The goal of this project was to design in vitro systems to differentiate BM derived cells into hepatocytes, and by doing so, study the factors which regulate BM derived hepatic differentiation.

BM derived cells were induced to differentiate into CK8+ or CK18+ endoderm-like cells that secreted albumin in certain culture conditions. The co-cultures of Lin− cells with AML12 hepatocytes without HGF did not induce hepatic differentiation from BM derived cells, as detected by the expression of CK8 or CK18. This suggests that soluble or bound signals by the AML12 cell line are not sufficient to induce differentiation of BM cells into hepatocytes-like cells. Furthermore, these results suggest that TGF-α secreted in these cultures does not induce BM derived endoderm differentiation. However, the addition of HGF to these co-cultures resulted in the formation of CK8+ YFP+ cells. Therefore, even though TGF-α and other signals from AML12 cells did not induce BM derived endoderm differentiation, they may be helpful in maintaining differentiated phenotype of BM derived hepatocyte-like cells.
The co-cultures with NIH-3T3 cells gave rise to a rare cell population (<1%) which transiently expressed CK8 / CK18 as well as secreted albumin. However, even though these cultures secreted albumin, the resulting albumin expression was much less than if all the CK8− cells were secreting albumin at typical hepatocyte levels. For example, it was estimated that the albumin secretion rate in cultured hepatocytes ranged from 1 to 10 ng albumin / day /100 hepatocyte294. Based on these secretion rates, the number of functionally active BM derived hepatocytes in NIH-3T3 co-cultures was very low. This could be interpreted in two ways, either a small fraction of CK8− YFP+ cells expressed albumin or a larger portion of the cells secreted albumin at lower levels. The ability to obtain cell specific albumin measurements, as determined by flow cytometry268 or immunohistochemistry, could be used to answer this question. If only a small fraction of these cells secreted albumin, then the other cells may be dedifferentiated hepatocytes or other types of BM derived epithelial tissues such as cells of lung, liver (cholangiocytes), skin, stomach and gastrointestinal tract45.

The addition of exogenous HGF to NIH-3T3 co-cultures increased the rate of albumin secretion as well as the proportion of YFP− CK8− cells. Therefore, the increased albumin secretion rates may be due to the increased number of CK8− cells.

In experiments in which exogenous HGF was added to Lin− BM cultures (without feeder cells), hepatic differentiation was induced in a dose dependent manner. In these cultures, hepatic markers were maintained only in cultures that were initiated at high HGF concentrations. The need for high HGF concentrations could be attributed to several factors. For example, the quick internalization and degradation of surface bound HGF/c-Met complex on hepatocytes313, rapidly depletes soluble HGF at low concentrations. Therefore, the use of heparin (and/or heparan sulphate proteoglycans)314,315 or immobilization to (bioreactors or tissue engineering scaffolds) surfaces may increase the ‘effective’ concentration of HGF. Another reason for high HGF concentrations could be due to high physiological concentrations required to induce BM derived endoderm differentiation. For example, it has been shown that extremely high HGF concentrations were present in blood after liver injury in vivo141. Also, perhaps high HGF concentrations are required in vitro, due to de-differentiation of hepatocyte-like cells in culture at low HGF concentrations. This is further supported since albumin was detected after 6 days in all cultures which were initiated with exogenous HGF. Thus, the use signals that enhance hepatocyte maintenance in culture may lower the minimum required HGF concentration.

The morphology of the ‘candidate’ hepatic cells in these cultures greatly resembled those of AML12 cells on Matrigel®. In both cases, the cells grew in a clonal manner and gave rise to spheroids. Hepatocyte spheroids have been shown to enhance the maintenance of hepatic genes in culture, while reducing the proliferative capacity of the cells316. Based on the albumin secretion rate of hepatocytes in primary spheroid cultures, the rate of albumin secreted from 5 x 10^5 BM derived Lin− cells in 1 μg/ml
of HGF, correlated to about ~3000 functional hepatocytes. Since KLS cells constituted about 1% of Lin- cells, this translates to approximately 5 x 10^3 KLS cells present within these cultures. Based on the KLS hepatic efficiency obtained in these results (~10%), it can be deduced that each KLS cell differentiated to hepatic lineage gave rise to approximately 6-8 functionally active hepatocytes (these values also correspond to the albumin secretion rates obtained from clonal analysis). However, some spheroid colonies had more than 6-8 cells which may indicate the presence of other cells (such as bile duct cells) or hepatocytes with secretion levels below those of primary hepatic cultures. In any case, it appears that KLS cells in culture do not, under the conditions tested, have a high proliferative capacity. Therefore, further research may be required to expand the hepatic yield from BM derived cells, as well as to identify other cell types that are generated from their differentiation in vitro.

It was demonstrated that rare KLS cells have the capability to give rise to albumin producing cells in a clonal manner. These results support recent observations by a number of groups45,100, who suggest that KLS are the population responsible for liver engraftment in BM transplants. Furthermore, it was demonstrated that these cells are CD45- (or hematopoietic in origin), which further supports that HSC (and not other BM derived cells) are capable of giving rise to both blood and liver lineages. The frequency of KLS cells that gave rise to albumin secreting cells was ~10% in medium containing HGF. This indicates that the KLS cells that are highly enriched for HSC may also be highly enriched for cells capable of giving rise to hepatic cells. The inability to detect albumin in medium that contained only hematopoietic cytokines also points to the importance of HGF.
Fibroblasts: Stellate cells

Hepatocytes

TPO
TGF-alpha

Multipotent progenitors

ûîbr

diierated hematopoietic cells

CKB*/CKl
û+
albumin

Hematopoietic differentiation

Endoderm differentiation

Figure 6-1 Schematic of soluble factors that may influence HSC cell fate in liver-like microenvironments.

Phenotypically defined c-kit⁺ Lin⁻ Sca-1⁺ CD45⁺ (which may also co-express c-Met) BM stem cells are influenced in the liver microenvironment by signals from hepatocytes and stellate-like cells. HGF and hematopoietic cytokines (SCF, TPO, FL and HIL-6) may act in competing pathways to determine early cell fate decisions into hematopoietic or endoderm lineage. HGF gives rise to CK8⁺/CK18⁺ albumin secreting cells while hematopoietic cytokines give rise to hematopoietic cells. Furthermore, HGF enhances the proportion of BFU-E progenitors and may act synergistically with hematopoietic cytokines to enhance the total number of cells.

The frequency of KLS cells that gave rise to albumin secreting cells in medium comprised of HGF plus hematopoietic cytokines (SCF, TPO, FL, HIL6) was lower than cultures with HGF supplemented medium. Furthermore, the rate of albumin secretion in positive wells was also significantly lower. These results may suggest that hematopoietic and hepatic differentiations are controlled by cytokine networks that regulate their behavior in competing pathways (see Figure 6-1). Therefore, hematopoietic cytokines may induce the hematopoietic differentiation of BM derived cells, while liver regenerating cytokines may induce hepatic differentiation of BM derived cells. Although the absence of hematopoietic cytokines results in apoptosis or necrosis of BM derived cells, it is possible that HGF induces HSC to differentiate into endoderm cells independent of the action of hematopoietic cytokines; therefore, the presence of hematopoietic cytokines may compete in directing the differentiation of BM derived HSC into endodermal or mesenchymal fates. These explanations are further supported in NIH3T3 co-cultures (that secrete hematopoietic cytokines), in which lower amounts of albumin is detected.
in comparison to cultures that are only supplemented with HGF. Furthermore, the experiments in which more than one KLS cell was plated per well may also be explained by this 'competing pathway' hypothesis. In these cultures, some cells may have generated progeny that secreted hematopoietic cytokines that may further inhibit the hepatic differentiation of BM derived cells. In addition, this hypothesis may be used to explain the reason that hepatic differentiation of BM derived cells has not been demonstrated in any other tissues.

The explanation above assumes a deterministic (or instructive) effect of cytokines on HS cell fate decisions. In this scheme, cytokines and growth factors induce the differentiation of stem cells. For example, HGF induces BM derived cells to differentiate into hepatic lineage. An alternative theory suggests a stochastic mechanism in which HSC fate is determined intrinsically. Therefore, a stem cell will have a pre-determined probability of giving rise to a progenitor cell of a specific lineage whose survival and proliferation is determined by microenvironmental factors. Based on this scheme, HGF allows for the survival and proliferation of independently committed hepatic progenitors. The fact that no BM derived hepatocytes-like cells were detected in liver regenerating conditions (i.e. co-culture with TGF-α secreting AML12 cells) may suggest that the effect of HGF is deterministic. However, because of the uncertainties with the other signals that AML12 cells secrete this alone cannot exclude the possibility that the endoderm differentiation from HSC is stochastic. The evidence regarding embryonic lethality of HGF/c-Met knockout mice (due to retarded liver development) further supports that HGF is essential in liver development and may induce hepatic differentiation in a deterministic manner. However, this could also be explained in that only HGF (and no other liver regenerating cytokine) allows for the survival of the BM derived liver progenitor. Clearly, more research is required to determine the stochastic or deterministic nature of HGF on HSC differentiation.
7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The behavior of BM derived cells in liver-like environments was studied. YFP+ BM derived cells were easily tracked in co-cultures with fibroblastic and hepatic cells which did not express the fluorescent gene. The ability to track BM derived population revealed significant information regarding the frequency and the type of progeny generated by these cells in co-cultures (see Figure 6-1). It was observed that hepatocyte cell lines enhanced hematopoiesis in vitro compared to BM cultures without feeder layers. However, this maintenance was not as profound as the degree of maintenance provided in co-cultures with fibroblasts. Furthermore, fibroblastic cultures seemed to enhance the generation of erythroid progenitors, while hepatocytes did not support the formation of BFU-E colonies in a manner that may suggest the importance of HGF.

The ability of BM derived cells to generate endoderm-like cells in vitro was analyzed. It was observed that YFP+ CK8+ endoderm-like cells were not generated in co-cultures with hepatocytes. However, in fibroblast cultures, transiently appearing YFP+ CK8+ cells emerged within the first week of culture. To further study these mechanisms, the role of HGF on BM derived cells was analyzed. It was observed that c-Met (HGF receptor) expression is enriched in BM progenitors. Furthermore, HGF supplementation enhanced the frequency of BM derived endoderm cells. The effects of HGF concentration on BM derived cell indicated that hepatocyte-like cells appeared in these cultures in a dose dependent manner with high concentrations (1 μg/ml HGF) inducing long-term differentiation of BM derived cells into hepatocytes. The clonal ability of purified HSC to give rise to albumin secreting cells was also analyzed. It was observed that ~10% of KLS cells gave rise to albumin secreting cells when exposed to HGF rich medium, while no albumin was detected in cultures without exogenous HGF. Also, the addition of hematopoietic cytokines (SCF, TPO, HIL-6 and FL) reduced the frequency as well as the amount of albumin secreted in these cells. Together, these results indicate that HGF secreted by mesenchymal cells may regulate the differentiation of BM cells to hepatocytes in culture. This understanding may lead to the development of in vitro cultures in order to expand hepatocytes or pre-treat cells prior to transplantation.

7.2 Recommendations and future work

Future work should be aimed at further development and optimization of the culture conditions developed in this thesis. To further demonstrate the hepatic nature of the cells derived in these cultures, the ability of individual cells to co-express cytokeratin 1fs along with albumin and other early (such as AFP and GGT) and late liver specific markers (such as CYP450 and urea) should be examined. This is important in determining the functionality of the generated hepatocytes, since it has been shown that
primary hepatocyte cultures which express albumin and cytokeratin markers, quickly lose their ability to express other functional hepatic markers such as CYP450294.

Also, albumin production reported in this thesis is calculated based on time-averaged albumin production. These results do not necessarily reflect the profile of secretion within the specified period. For example, the albumin secretion over a week of culture can be achieved in a day within the specified time as supposed to being evenly secreted over the entire length of the test. Due to these factors, an aliquot of the serum should be taken each day rather than at medium exchanges.

The need for extremely high concentrations of HGF must be eliminated in order to enhance the commercial viability of this approach in clinical applications. A number of approaches can be taken to enhance the functional effectiveness of HGF. For example, if one of the features of high HGF concentration is to allow for in vitro maintenance of hepatocytes, then the addition of other factors that enhance hepatocyte maintenance may decrease the need for HGF (such as the presence of floating Matrigel317 which has been shown to stabilize the differentiated phenotype on cultured hepatocytes). Also, as previously mentioned, HGF can be covalently bound to culture dishes or tissue engineering scaffold surfaces in order to decrease the internalization of HGF/c-Met complex. Another approach is to add factors that may enhance the stability of HGF. For example, HGF binds not only to c-Met but also to heparan sulphate proteoglycans on cell surface and soluble heparin in serum314,315,318,319. Heparin in low concentrations has been shown to enhance HGF potency in the activation of c-Met receptor, most likely through the stabilization of the molecules and enhanced receptor dimerization314,315. In addition, the basal HGF production by fibroblasts may be upregulated by the addition of heparin or phorbol ester125, IL-1, TNF-α115,116,320,321, EGF, PDGF, aFGF, bFGF115 and agents such as forskolin, cholera toxin and PGE2322-324. Therefore, the addition of fibroblasts to cultures that are supplemented with appropriate stimulatory factors may upregulate HGF levels to sufficiently high values without exogenous addition of HGF.

It was demonstrated that the addition of cytokines found to be important in ex vivo expansion of HSC was not effective in increasing the production of albumin or the frequency of cells which gave rise to albumin secreting cells. Therefore the use of other cytokines which are deemed to be important in liver regeneration, such as EGF, FGF, IL-6, TNF-α, insulin, TGF-α and OSM325, should be evaluated in determining the optimum ‘soup’ required for differentiating and expanding BM derived hepatocytes in vitro.

To utilize this culture system to enhance liver therapy, future studies could utilize in vivo models (such as the FAH+ or partial hepatectomy or CCl4 treated mice), to determine the feasibility of ‘priming’ HSC in culture prior to implantation. Various studies can be performed that involve pre-culturing cells with HGF, EGF or other hepatic regenerating cytokines prior to transplantation.
However, since these cytokines may induce downstream endoderm differentiation in culture (which may decrease their capability to engraft the host and expand), the use of cytokine cocktails known to expand LT-HSC may be the preferred approach in pre-treating BM cells prior to transplantation. It would be interesting to study the relationship between the BM and hepatic engraftment under similar liver regenerating conditions. If long-term hematopoietic engraftment correlates with liver engraftment then primitive cells along with regenerative liver microenvironment are the key components in restoring liver function. Thus future research into co-transplanting genetically engineered cells or other factors that modify the existing liver microenvironment may be ultimately critical to successful liver therapies. It is also important to verify that hematopoietic reconstitution and liver engraftment occur in the c-Met” population of HSC.
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