The role of Lhx2 in hematopoietic stem cell function, liver development and disease

Ewa Wandzioch

Department of Molecular Biology
Umeå University
Umeå, Sweden, 2004
Front cover: albumin/HE staining of $Lhx2^{-/-}$
liver section at E14.5
To my family

If you are looking for Home
and find instead a sand-pit,
try looking for a sand-pit.
Then you’d be sure not to find it,
which would be a Good Thing,
because you might find something
that you weren’t looking for,
which might be just
what you were looking for.

A.A Milne
# TABLE OF CONTENTS

1. **ABSTRACT**  
2. **PAPERS IN THIS THESIS**  
3. **ABBREVIATIONS**  
4. **INTRODUCTION**  
   4.1. **THE LIVER**  
      4.1.1. Hepatic fibrosis  
      4.1.2. Liver regeneration  
      4.1.3. Overview of the fetal liver development  
      4.1.4. Tissue interactions in liver development  
      4.1.4.1. Endoderm-specific genes required for early stages of liver development  
      4.1.4.2. Role of mesenchymal signals in liver development  
   4.2. **THE HEMATOPOIETIC SYSTEM**  
      4.2.1. Hematopoietic stem cells  
      4.2.1.1. Hematopoietic stem cell phenotype  
      4.2.1.2. Functional assays for hematopoietic stem cells  
      4.2.2. Regulatory mechanisms in hematopoiesis  
      4.2.2.1. Regulation of self-renewal  
      4.2.2.2. Regulation of proliferation, differentiation and survival  
      4.2.3. Role of c-Kit/Steel factor signaling in the hematopoietic system  
      4.2.3.1. Signal transduction from the c-Kit receptor  
      4.2.3.2. Ras-Raf-Mek-Erk pathway  
      4.2.3.3. PI-3K pathway  
   4.3. **LIM-HOMEODOMAIN PROTEINS**  
      4.3.1. Function of Lhx2 during embryonic development  
      4.3.2. Role of Lhx2 in regulation of self-renewal of HSCs  
   4.4. **RELATIONSHIP BETWEEN THE DEVELOPING HEMATOPOIETIC AND HEPATIC SYSTEMS**  
   
   7  
   9  
   10  
   11  
   11  
   13  
   15  
   16  
   16  
   17  
   17  
   20  
   21  
   22  
   22  
   24  
   25  
   26  
   28  
   29  
   30  
   31  
   33  
   34  
   35  
   36
5. AIMS OF THIS THESIS

6. RESULTS AND DISCUSSION

6.1. During fetal liver development Lhx2 is expressed in the septum transversum mesenchyme and hepatic stellate cells

6.2. Functional Lhx2 expression in the hepatic mesenchyme is required for proper liver expansion

6.3. Loss of Lhx2 from developing stellate cells causes their activation and leads to hepatic fibrosis and distorted liver architecture

6.4. Lack of Lhx2 expression in the developing stellate cells induces regenerative response of the hepatoblasts

6.5. Models for the possible role of Lhx2 in fetal liver development

6.6. Role of Lhx2 in fetal liver hematopoiesis

6.7. In vitro self-renewal of multipotent HPC line is dependent on Lhx2 expression and occurs via secreted factor(s)

6.8. The HPC lines represent a relevant in vitro model system to study basic properties of HSCs

6.9. A cross-talk between the PI-3K and Erk pathways occurs in HPCs

6.10. Inhibition of PI-3K signaling in HPCs induces apoptosis

7. CONCLUSIONS

8. ACKNOWLEDGEMENTS

9. REFERENCES

10. PAPERS I-IV
1. ABSTRACT

During embryonic development, generation of functional organs is dependent on proper interactions between different cell types. Elucidation of the mechanisms operating during organ formation might provide insights into the origin of many pathological disorders in the adult. Gene inactivation studies in mice have provided invaluable tool to study the function of genes critical for morphogenesis of distinct organs. A LIM-homeodomain transcription factor Lhx2 has previously been reported to play a role in fetal liver development and hematopoiesis, as its inactivation leads to lethal anemia due to underdeveloped liver. This thesis focuses on the function of Lhx2 in the development of these two organ systems.

Reciprocal signaling between ventral foregut endoderm and mesenchyme of the septum transversum regulates the liver formation, expansion and differentiation. A fully formed liver is composed of endoderm-derived hepatocytes and cholangiocytes and a variety of mesenchyme-derived cell types, such as endothelial cells and hepatic stellate cells. In early stages of liver development Lhx2 is expressed in the liver-associated septum transversum mesenchyme, a part of which becomes integrated into the liver organ and develops into hepatic stellate cells. Functional Lhx2 expression in the hepatic mesenchyme is necessary for normal liver outgrowth and differentiation. Loss of Lhx2 from developing hepatic stellate cells leads to their activation and excessive deposition of collagen fibres, resulting in hepatic fibrosis and severely distorted liver architecture. Transfection of Lhx2 to human stellate cell line downregulates genes associated with stellate cell activation and fibrogenesis. Thus, Lhx2 is the first gene identified to negatively regulate events leading to hepatic fibrosis. Elucidation of the molecular mechanisms involved in this process might therefore be instrumental for the development of novel therapies useful in treatment of this disorder.

Fetal liver is also a major site of hematopoiesis in the embryo and provides physiological conditions necessary for the efficient expansion of hematopoietic stem cells (HSCs). The hematopoietic defect observed in Lhx2-deficient embryos is cell-nonautonomous, indicating that Lhx2 might control secreted factors involved in the
self-renewal of HSCs. This putative second role of Lhx2 has been investigated by analyzing the mechanism whereby Lhx2 expression generates in vitro self-renewing HSC-like cell lines. Interestingly, in agreement with the cell nonautonomous phenotype of the lethal anemia in Lhx2\/- embryos, the mechanism of self-renewal is dependent on Lhx2 expression and occurs via secreted factor(s). Identification of these factor(s) might potentially allow ex vivo expansion of HSCs for therapeutic purposes.

The Lhx2-immortalized HSC-like cell lines share many basic features with HSCs and self-renew in vitro in presence of Steel factor (SF). SF/c-Kit signaling mediates a wide variety of biological activities in cells at many different levels in the hematopoietic hierarchy. We used the HSC-like cell lines as an in vitro model system to compare signal transduction pathways from c-Kit receptor in stem cells versus differentiated hematopoietic cells. HSCs require PI-3K dependent activation of Raf1-Mek-Erk cascade for their survival and self-renewal in response to SF, whereas activation of Erk is PI-3K independent in committed myeloid and mast cells. Thus, the mode of SF/c-Kit signaling is dependent on the differentiation status of the cells.
2. PAPERS IN THIS THESIS

This thesis is based on the following papers that will be referred to in the text by their corresponding Roman numerals (I-IV).


* The first two authors contributed equally to this work

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### 3. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
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<tr>
<td>AGM</td>
<td>aorta, gonad and mesonephros</td>
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<td>ASMA</td>
<td>α-smooth muscle actin</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<td>BMP</td>
<td>bone morphogenic protein</td>
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<td>BM-HPC</td>
<td>bone marrow-derived hematopoietic progenitor cell</td>
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<tr>
<td>CAFC</td>
<td>cobblestone area – forming cell</td>
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<tr>
<td>CFC</td>
<td>colony-forming cell</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>Epo</td>
<td>Erythropoietin</td>
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<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>Flt3L</td>
<td>flt3 ligand</td>
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<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
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<td>GTP</td>
<td>guanine triphosphate</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HPC</td>
<td>hematopoietic progenitor cell</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IFN</td>
<td>interferon</td>
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<td>LIM-HD</td>
<td>LIM-homeodomain</td>
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<tr>
<td>LTR</td>
<td>long term repopulating</td>
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<td>LTC-IC</td>
<td>long-term culture initiating cell</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/Erk kinase</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PI-3K</td>
<td>phosphoinositide 3-kinase</td>
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<td>PDK</td>
<td>3-phosphoinositide-dependent-kinase</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>PKB</td>
<td>protein kinase B</td>
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<td>Shc</td>
<td>src homology containing</td>
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<td>SF</td>
<td>Steel factor</td>
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<tr>
<td>SoS</td>
<td>Son-of-Sevenless</td>
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<td>STR</td>
<td>short term repopulating</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
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4. INTRODUCTION

4.1. THE LIVER

The liver is the largest gland in the body and functions as an interface between the digestive tract and the blood. Major functions of the liver are inactivation of toxic substances, production of bile, storage of fat, glycogen and vitamins, control of serum proteins and regulation of blood glucose level. The primary functional cells of the liver are hepatocytes, normally arranged as one or two-cell thick interconnecting plates. In the liver, the two input circulations (hepatic artery and hepatic portal vein) discharge their blood into liver sinusoids located in between hepatocyte plates and carrying the blood from the portal regions of the liver lobule towards central vein (Figure 1).

Figure 1. The structure of the liver. Plates of hepatocytes are separated from the bloodstream by a single sheet of endothelial cells lining the surface of the sinusoid. The space in between endothelial cells and hepatocytes is termed space of Disse. Oxygenated blood from hepatic artery and venous blood from portal vein mix within the sinusoids and feed into central vein (white arrows indicate the direction of blood flow). Bile from the bile canaliculi located between adjacent hepatocytes flows in a direction opposite to that of blood (indicated by thick black arrows), and is collected in bile ducts, located in the portal regions and lined by cholangiocytes. Adapted from www.mc.vanderbilt.edu/histology/images/histology/liver/display/liver0016.jpg
INTRODUCTION

The liver exhibits an exocrine function through the generation of bile, critical for lipid digestion. The bile is secreted across the apical surface of the hepatocytes into the canaliculi formed by junctional complexes connecting the plasma membranes of adjacent hepatocytes (Figure 1 and 2). The bile flow progresses in a direction opposite to that of blood, i.e., from the center of the lobule to its periphery (Figure 1). In the portal regions, the bile enters bile ducts, which are lined by cholangiocytes and transport the bile through the hepatic duct to the gall bladder and duodenum.

The basal surface of the hepatocytes is separated from adjacent sinusoidal endothelial cells by the subendothelial space of Disse, where the exchange of substances between the blood and hepatocytes takes place (Figure 1 and 2). The hepatic sinusoidal endothelium differs from that of other capillaries in that it has multiple pores, commonly referred to as fenestrae, to maximize exchange between hepatocytes and sinusoidal circulation (Figure 2). Moreover, the basement membrane-like ECM underlying sinusoidal endothelial cells has a low-density structure that minimally impedes sinusoid-hepatocyte exchange (Hahn et al., 1980; Kmiec, 2001). This arrangement facilitates the endocrine-like function of the liver, which is to condition the venous blood, received from the digestive tract, through secretion of plasma proteins and removal of toxic products.

Figure 2. Cellular elements of the hepatic sinusoid. Hepatic stellate cells reside in a space of Disse and extend their cytoplasmic processes along the endothelial cells. Fenestrations in the endothelial sheet allow exchange of molecules between the hepatocytes and the bloodstream. Kupffer cells are located in the lumen of the sinusoid. Courtesy of Don Rockey and Scott Friedman.
A number of specialized cells reside in the space of Disse, including hepatic stellate cells and Kupffer cells. Kupffer cells are macrophages which ingest bacteria, bacterial toxins and damaged erythrocytes. Hepatic stellate cells (also known as Ito cells, fat storing cells or lipocytes) store vitamin A and synthesize and deposit ECM proteins in the space of Disse (Ramadori, 1991; Enzan et al., 1997) (Figure 2). Thus, the liver has developed a highly specialized architecture, which is critical for its function and is usually disrupted in common liver diseases such as hepatic fibrosis and cirrhosis.

### 4.1.1. Hepatic fibrosis

Liver fibrosis and its end stage cirrhosis can be viewed as a sustained wound healing response to chronic liver injury induced by a variety of insults including hepatitis B and C, alcohol abuse, chronic biliary obstruction, autoimmune disorders imposing damage on hepatocytes or biliary epithelium and various congenital abnormalities (Desmet, 1992; Friedman, 2000; Hui and Friedman, 2003). Hepatic fibrosis is characterized by excessive deposition of ECM proteins of which collagen type I predominates. If the injurious stimulus is present for a prolonged period of time, an abundant fibrous scar tissue will accumulate in the liver (Rojkind et al., 1979; Hahn et al., 1980; Friedman, 2000).

Activation of hepatic stellate cells is the key event in liver fibrosis. Stellate cells are normally maintained in a quiescent state by adhesions with endothelial cells, hepatocytes and perisinusoidal low-density ECM comprised mainly of type IV collagen, laminin and proteoglycans (Friedman et al., 1989; Wake, 1999). Following liver injury, hepatic stellate cells undergo transition from quiescent, retinoid-storing cells into proliferative, myofibroblasts-like cells. Cellular changes accompanying stellate cell activation include the appearance of abundant α-smooth muscle actin (ASMA) filaments, in addition to desmin, an intermediate filament normally expressed by stellate cells (Yokoi et al., 1984; Ballardini et al., 1988; Ramadori et al., 1990). Changes in ECM composition, in concert with various mediators released by the damaged neighboring cells (i.e. hepatocytes, Kupffer cells, endothelial cells, platelets etc.), are believed to contribute to stellate cell activation (Hui and Friedman, 2003). Activated stellate cells proliferate, increase in number and synthesize excessive
amounts of collagen I and other ECM proteins, which become deposited in the space of Disse (Friedman et al., 1985; Bissell, 1992; Gressner and Bachem, 1995; Bedossa and Paradis, 2003) (Figure 3). Furthermore, activated stellate cells express several matrix-degrading proteases termed matrix metalloproteinases (MMPs), as well as specific tissue inhibitors of the MMP family (TIMPs), all of which contribute to aberrant ECM remodeling and increased fibrogenesis (Milani et al., 1994; Arthur, 2000). Consequently, an ECM rich in interstitial collagens replaces the normal perisinusoidal ECM, which perturbs the cellular organization of the liver and severely compromises its function (Kmiec, 2001; Hui and Friedman, 2003).

![Figure 3. Sinusoidal changes during liver injury](image)

The main characteristics of hepatic fibrosis in humans have been described based on patient material (e.g. liver biopsies). The process of stellate cell activation has been investigated using experimental animal models with inducible liver injury (e.g. partial hepatectomy, bile duct ligation and CCl4-induced liver damage). Although the external stimuli triggering stellate cell activation have been studied extensively, the genes controlling stellate cell responses are largely unknown.
4.1.2. Liver regeneration

The liver is unique in that it responds to physical or chemical injury by the process of regeneration. In most cases of liver injury, the regeneration of the lost hepatic tissue involves replication of the mature hepatocytes (Michalopoulos and DeFrances, 1997). Proliferation of hepatocytes leads to the formation of small, avascular hepatocyte clusters that subsequently become invaded by endothelial and stellate cells and after several days the typical liver structure becomes restored, given that the injurious stimulus is no longer present (Court et al., 2002).

Under conditions when hepatocyte proliferation is inhibited, liver regeneration involves hepatic stem cells, so called oval cells (Fausto et al., 1993; Thorgeirsson, 1996; Lowes et al., 2003; Fausto, 2004). Subsequent to severe liver damage, oval cells proliferate and detach from the terminal biliary ductules in the portal regions of the liver lobule. Proliferating oval cells form ductular structures, often referred to as ductular reaction (Fausto et al., 1993). The cells within these ductular structures express both cholangiocyte and hepatocyte markers (Vandersteenhoven et al., 1990; Rubin et al., 1995), and are capable of differentiation into mature hepatocytes after liver regeneration is complete (Forbes et al., 2002).

Although the precise role of stellate cell activation in the regenerating liver is currently unknown, activated stellate cells have been reported to remain in close contact with the ductular structures (Paku et al., 2001), and are likely to be involved in the regenerative response (Mabuchi et al., 2004). Transgenic mouse models have recently shed some light on how aberrant stellate cell activation influences liver regeneration. Foxf1, a transcription factor of the Forkhead Box family, has been found to be expressed in stellate cells in the developing and adult liver. Haploinsufficiency in Foxf1-/- mice revealed defective stellate cell activation and reduced synthesis of collagen I and ASMA proteins after liver injury (Kalinichenko et al., 2003). The inhibition of stellate cell activation was associated with impaired liver regeneration and hepatic apoptosis, confirming that stellate cell activation plays an important role in the regenerative process in adult liver.
4.1.3. Overview of the fetal liver development

The liver emerges from the ventral foregut endoderm as a thickening of the foregut epithelium that subsequently forms a liver bud. The pre-hepatic cells, also called hepatoblasts, proliferate, delaminate from the foregut epithelium and migrate as cords into the surrounding loose connective tissue of septum transversum mesenchyme (LeDouarin, 1975; Medlock and Haar, 1983a). As the pre-hepatic cells migrate, they closely associate with primitive sinusoid endothelial cells that form capillary-like structures between the hepatic cords (Medlock and Haar, 1983a; Enzan et al., 1997). The sinusoidal endothelium becomes well established between E12-14 in the mouse, although the fenestration typical for the adult sinusoidal endothelium is absent (Enzan et al., 1997). It is believed that the septum transversum-derived mesenchymal cells trapped in the subendothelial spaces give rise to stromal cells of the liver, such as stellate cells (Enzan et al., 1997; Kalinichenko et al., 2003), although their origin is still a matter of controversy.

Around E10.5, the developing liver is colonized by numerous hematopoietic cells and becomes the primary site of hematopoiesis in the embryo until birth (Russell, 1979; Medlock and Haar, 1983b). Along with the expansion and differentiation of hematopoietic cells, the liver organ increases in size (Vassy et al., 1988), immature bipotential hepatic cells initiate differentiation to hepatocytes and cholangiocytes (Luzzatto, 1981; Rogler, 1997; Shiojiri, 1997; Spagnoli et al., 1998), and the general liver architecture is laid down. Hence, from this time point, the hepatic and the hematopoietic system develop simultaneously and establishment of a functional three-dimensional liver structure seems to be a pre-requisite for sufficient expansion of the hematopoietic system, as anemia is a common phenotype in embryos displaying defective liver morphogenesis (Bladt et al., 1995; Schmidt et al., 1995; Hentsch et al., 1996; Keng et al., 2000).

4.1.4. Tissue interactions in liver development

Adult liver is composed of an endodermal component, comprising hepatocytes and cholangiocytes and a mesenchymal component, which includes endothelial cells, stellate cells and other types of connective tissue cells. During embryonic
development, reciprocal signaling between mesenchyme and endoderm has been shown to be crucial at many stages of liver morphogenesis, including induction, outgrowth and differentiation (LeDouarin, 1975; Houssaint, 1980). Gene inactivation studies in mice have identified several endoderm- or mesenchyme-specific genes that are required at distinct stages of liver morphogenesis (reviewed in (Zaret, 2002; Duncan, 2003)).

4.1.4.1. Endoderm-specific genes required for early stages of liver development

A divergent homeobox transcription factor Hex is expressed in a region of the foregut endoderm that corresponds to the site of the future liver (Thomas et al., 1998; Keng et al., 2000). In the absence of functional Hex, the endoderm is specified to hepatic fate but the liver bud fails to form, indicating that Hex is required for the earliest stages of liver bud formation (Keng et al., 2000; Martinez Barbera et al., 2000) (Figure 4b).

The liver bud is separated from the surrounding septum transversum mesenchyme by a basement membrane (Medlock and Haar, 1983a). As the liver bud grows, this basement membrane is progressively disrupted; the hepatoblasts delaminate from the foregut and invade the septum transversum mesenchyme (LeDouarin, 1975; Medlock and Haar, 1983a). This process is disrupted in the embryos deficient for a homeobox transcription factor Prox1 (Oliver et al., 1993). Prox1\textsuperscript{-/-} hepatoblasts generate the liver bud, but by E9.5 they fail to migrate into septum transversum mesenchyme because of insufficient ECM remodeling capacity and increased E-cadherin-based adhesions between the hepatoblasts (Figure 4c). Interestingly, the lack of hepatoblasts does not seem to impair the overall formation of the liver-like organ composed mainly of mesenchymal and hematopoietic cells (Sosa-Pineda et al., 2000).

4.1.4.2. Role of mesenchymal signals in liver development

In classic experiments utilizing grafts of quail tissue into donor chick embryos, Nicole LeDouarin demonstrated that induction of hepatogenesis depended on interaction between ventral foregut endoderm and precardiac mesoderm (LeDouarin, 1975; Fukuda, 1979). Further grafting experiments have shown that another stimulus
emanating from the septum transversum mesenchyme was required for proliferation and complete differentiation of the hepatoblasts (LeDouarin, 1975).

Some of these inductive signals have recently been identified in the mouse system. The current model suggests that fibroblast growth factors (FGFs) secreted from the pre-cardiac mesoderm (Jung et al., 1999), in concert with bone morphogenic proteins (BMPs) emanating from the septum transversum mesenchyme (Rossi et al., 2001), initiate the liver program in the ventral foregut endoderm at E8.5, as evidenced by the expression of hepatic genes such as albumin and α-fetoprotein (AFP) in this region (Cascio and Zaret, 1991; Gualdi et al., 1996) (Figure 4a). Proliferation of endodermal cells committed to the hepatic fate generates the liver bud at E9. The proliferative stimuli include BMPs (Rossi et al., 2001) and as yet unidentified factors secreted by endothelial cells lying between the liver primordium and septum transversum (Matsumoto et al., 2001) (Figure 4b). Several mutations specific to septum transversum mesenchyme cause a failure of the hepatic endoderm to proliferate, suggesting that septum transversum mesenchyme is an important source of

![Figure 4. Stages of early liver development. (A) Ventral foregut endoderm is specified to the hepatic fate by FGF signals from the cardiac mesenchyme and BMPs from the septum transversum mesenchyme. (B) BMPs emanating from septum transversum and unknown signals from endothelial cells induce hepatoblast proliferation and formation of the liver bud. Expression of Hex in the endoderm is required for the bud formation. (C) Digestion of the basement membrane surrounding the liver bud and migration of hepatoblasts into the septum transversum mesenchyme requires endodermal expression of Prox1. (D) Liver expansion is dependent on mesenchymal expression of Hlx, HGF and BMP. Differentiation of hepatoblasts is influenced by mesenchyme-derived ECM. The figure is based on reviews by (Duncan, 2003, Zaret 2002)
paracrine factors promoting hepatic expansion. Hepatocyte growth factor (HGF) is one of the factors secreted by septum transversum mesenchymal cells and its receptor c-met is expressed in hepatic endoderm (Sonnenberg et al., 1993a; Sonnenberg et al., 1993b). Inactivation of either of these genes results in liver hypoplasia and apoptosis of hepatocytes (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Similarly, inactivation of a divergent transcription factor Hlx in the septum transversum mesenchyme results in severe liver expansion defect (Hentsch et al., 1996; Lints et al., 1996). Although Hlx downstream target genes have not been identified, the data imply that it might control expression of novel secreted factors that control proliferation of hepatoblasts (Figure 4d).

Prior to E12 in the mouse, the hepatoblasts remain in a morphologically undifferentiated state and express early hepatic markers AFP and albumin (Shiojiri and Mizuno, 1993; Gualdi et al., 1996). As they are originally derived from the endodermal epithelium of the foregut, they also express E-cadherin, a marker of epithelial cells (Nitou et al., 2000). From midgestation until early neonatal stage, hepatoblasts undergo gradual maturation during which they acquire various specific functions necessary for their metabolic activities. Along with this process, hepatoblasts differentiate into either hepatocytes or bile duct cells (Rogler, 1997; Spagnoli et al., 1998). Formation of bile ducts is induced around E13.5 in the mouse (Shiojiri, 1997; Lemaigre, 2003). Developing immature cholangiocytes express bile duct specific cytokeratin (CK) together with early hepatic markers as they begin to acquire phenotypic traits of bile duct epithelium (Shiojiri and Mizuno, 1993; Shiojiri, 1997; Terada et al., 1998), while the cells of mature bile ducts are positive for CK19 and do not express hepatic markers (Shiojiri and Mizuno, 1993; Shiojiri, 1997).

Previous tissue grafting experiments indicated that the presence of septum transversum is required for complete differentiation of hepatocytes (LeDouarin, 1975; Houssaint, 1980). While the molecular nature of this observation is not yet elucidated, interactions between hepatoblasts and mesenchyme-derived ECM are likely to be responsible for the delivery, location and timing of the differentiation stimuli. Consistent with this hypothesis, early in vivo transplantation studies of fetal liver fragments have indicated that hepatoblasts differentiate to hepatocytes when
transplanted into the testis, where sinusoids are well developed, whereas they form bile ducts when placed under the newborn skin, where the connective tissue is abundant (Shiojiri, 1984). In agreement with these observations, the presence and composition of ECM as well as cell-ECM adhesions have been shown to modulate the differentiation of hepatoblasts (Ben-Ze'ev et al., 1988; DiPersio et al., 1991; Hynes, 1992; Stamatoglou and Hughes, 1994; Fassler and Meyer, 1995; Shiojiri, 1997) and promote ductal plate formation in the ECM-rich regions of the developing liver (reviewed in (Shiojiri, 1997; Lemaigre, 2003)).

Collectively, the evidence supports a central role for the septum transversum mesenchyme and its derivatives in the control of liver development, but the specific molecular mechanisms and the extrinsic mesenchymal signals involved in different aspects of liver morphogenesis are yet to be discovered.

4.2. THE HEMATOPOIETIC SYSTEM

The mammalian hematopoietic system comprises blood and the blood-forming organs, such as bone marrow and thymus. Blood is a highly specialized tissue composed of a variety of functionally diverse hematopoietic cells, suspended in an aqueous plasma solution. The plasma functions principally as a vehicle for a transport of nutrients, metabolites, antibodies and hormones throughout the body. The hematopoietic cells, comprising the cellular component of the blood, supply oxygen (red blood cells or erythrocytes), protect against foreign organisms (white blood cells or leukocytes) and control the process of bleeding through the activation of the blood clotting cascade (platelets produced by megakaryocytes).

The generation of blood cells, i.e. hematopoiesis, occurs in the specialized hematopoietic organs. In adult mice and humans the bone marrow (BM) is the predominating site of hematopoiesis. Since all mature blood cells are relatively short-lived, they have to be continuously replaced by the newly generated cells. Billions of new blood cells are produced daily from a small pool of hematopoietic stem cells (HSCs). The mammalian hematopoietic system is organized as a hierarchy in which HSC is the founder of all blood cell lineages. The HSCs are thus immature and multipotent cells that have the capacity of multilineage differentiation as well as the
ability to generate more stem cells through the process of self-renewal (Till and McCulloch, 1961; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990). The term self-renewal will be used herein to describe the ability of HSC to replicate itself in its multipotent and immature state, while the term proliferation will refer to dividing progenitor cells that have initiated the differentiation process. The HSCs have to make a choice of either self-renewing or committing to differentiation. The daughter cells that entered the differentiation pathway will generate a large number of highly proliferating intermediate multilineage progenitors, which in turn will give rise to precursors that are more restricted in their proliferation and differentiation potential, and finally to functionally mature cells (Figure 5). Mature hematopoietic cells are released into the circulation where they perform their specific functions, critical for the survival of the individual.

4.2.1. Hematopoietic stem cells

In early sixties Till, McCulloch and Siminovitch, discovered the presence of immature and multipotent clonogenic hematopoietic progenitor cells within the blood forming organs (Till and McCulloch, 1961; Siminovitch et al., 1963). Later on, when the methods allowing isolation and purification of HSCs have been developed, transplantation experiments in mice have shown that injection of one or very few cells could reconstitute all hematopoietic lineages in the lethally irradiated host, revealing an enormous regenerative capacity of a single HSC (Smith et al., 1991; Osawa et al., 1996). The marrow from transplanted recipients could in turn repopulate secondary and tertiary immuno-compromised hosts, thus demonstrating that self-renewal of HSCs occurs in vivo (Jordan and Lemischka, 1990; Keller and Snodgrass, 1990). Depending on the extent of HSC self-renewal abilities, two subsets of multipotent HSCs have been identified: short term (ST-HSCs) and long term (LT-HSCs) reconstituting cells (Morrison and Weissman, 1994). The ST-HSCs have very limited self-renewal capacity and therefore can only provide radioprotection and short-term hematopoietic reconstitution (STR). The LT-HSCs, on the contrary, are capable of “indefinite” self-renewal. Owing to this unique property, LT-HSCs are indispensable for the maintenance of a life long hematopoiesis and thus constitute the true HSC population.
INTRODUCTION

4.2.1.1. Hematopoietic stem cell phenotype
Purification of homogenous HSC populations has been difficult due to their low frequency in the BM (<1 in $10^4$ nucleated marrow cells) (Boggs et al., 1982; Moore and Shapiro, 1994; Morrison et al., 1995b), the fact that they divide infrequently (Bradford et al., 1997; Cheshier et al., 1999), and the lack of specific cell markers related to HSC function. Currently, most methods for isolation of HSCs rely on a combination of positive and negative selection using monoclonal antibodies specific for different cell surface markers, followed by fluorescence-activated cell-sorting (FACS) (reviewed in (Wognum et al., 2003)). HSCs do not express lineage markers (lin) associated with terminal maturation of specific blood cell types. Murine HSCs have been shown to express cell-surface antigen Sca-1 and tyrosine kinase receptor c-Kit (Spangrude et al., 1988; Uchida and Weissman, 1992; Osawa et al., 1996). Negative selection of lin' cells combined with positive selection for Sca-1' and c-Kit' cells results in cell population highly enriched for BM-derived stem cells with LT-HSC activity. HSCs isolated from fetal liver express c-Kit, Mac-1, AA4.1 and Sca-1(Morrison et al., 1995a; Sanchez et al., 1996), and yolk sac-derived HSCs express CD34, c-Kit, Mac-1 and AA4.1, but not Sca-1 (Yoder et al., 1997a), indicating that expression of HSC-associated cell surface antigens vary dependent on their ontogenic origin.

4.2.1.2. Functional assays for hematopoietic stem cells
Evaluation of biological function of HSCs and hematopoietic progenitor cells is based on various functional in vivo and in vitro assays. It is now widely accepted that the only valid test that defines a HSC is one that demonstrates its capacity for sustained (for at least 6 month) reconstitution of the hematopoietic system following transplantation into immuno-compromised hosts (reviewed in (Szilvassy, 2003)). Additionally, a number of in vitro assays have been established for qualitative and quantitative analysis of different hematopoietic progenitor and HSC populations (Dexter et al., 1977; Ploemacher et al., 1991), although none of them has proven to specifically detect HSCs.
INTRODUCTION

Figure 5. A schematic overview of the murine hematopoiesis. All types of the blood cells originate from hematopoietic stem cell (HSC). The HSC generates progenitor cells which undergo maturation through a number of intermediate stages with progressively restricted potential for proliferation and differentiation. The arrow above the HSC indicates that it can self-renew. Indicated in the figure are hematopoietic cell populations which the different functional assays detect: LT-HSC-long-term reconstituting HSCs; ST-HSC-short-term reconstituting HSCs; LTC-IC-long-term culture initiating cell; CAFC-cobblestone area forming cell; CFC-colony forming cell. Modified from (Keller, 1992)
The colony forming cell (CFC) assay in semisolid media measures the clonogenic responses of tested cells to various combinations of hematopoietic cytokines. Since colonies formed in response to cytokine stimulation are of single cell origin (colony forming cell, CFC), the initial differentiation stage of the founder CFC can also be inferred, by analyzing the cellular content of the colony. Thus, in response to broad cytokine cocktail, multipotent progenitors including HSCs will form multilineage colonies, whereas more committed precursors will give rise to bi- or single lineage colonies.

In the cobblestone-area-forming cell (CAFC) assay the test population is plated onto a preformed stromal cell monolayer. HSCs and progenitor cells will grow in a specific fashion, forming cobblestone areas and generating cells that are delivered into the culture medium. The temporal pattern of cobblestone area formation defines the differentiation status of the tested cells. The more immature the input cells are, the longer they will maintain growth as cobblestone areas. Cells still forming cobblestone areas 35 days after plating are considered to be candidate HSCs (Ploemacher et al., 1991).

The long-term culture-initiating cell (LTC-IC) assay employs the same conditions as CAFC assay, but the cultures are followed for the long-term maintenance (5-8 weeks) of clonogenic hematopoietic progenitors released into the culture media. The clonogenic potential of these cells is evaluated using CFC assays. The LTC-IC assay thus enables identification of the most immature cells, comparable to LT-HSCs (Cho and Muller-Sieburg, 2000). The schematic overview of the murine hematopoietic system and hematopoietic cell populations detected in various functional in vitro and in vivo assays are depicted in Figure 5.

4.2.2. Regulatory mechanisms in hematopoiesis
The steady-state hematopoiesis relies on the ability of HSCs to maintain balance between self-renewal and commitment to differentiation. The emerging concept is that this ability is controlled by a combination of multiple cell-intrinsic and external regulatory mechanisms (discussed in (Krause, 2002; Zhu and Emerson, 2002)). This complexity is due to the fact that HSCs constitute an integral part of the environment.
where they reside. Hematopoiesis is restricted to the extravascular compartment of the adult BM, where HSCs and their differentiating progeny remain in close proximity with a heterogeneous population of stromal cells of mesenchymal origin that comprise a hematopoietic microenvironment (Mayani et al., 1992). The development of hematopoietic cells is regulated by multiple cell-cell interactions, cell-ECM interactions and exposure to variable concentrations and combinations of soluble growth factors and cytokines (Rafii et al., 1997; Torok-Storb et al., 1999; Prosper and Verfaillie, 2001). Stromal cells secrete a variety of soluble proteins and ECM-components that affect the growth of hematopoietic cells (Dexter, 1982; Dorshkind, 1990; Deans and Moseley, 2000). ECM proteins in the BM include collagens, fibronectin, laminin and proteoglycans which provide a scaffold for developing hematopoietic cells, transduce intracellular signals via specific ECM-receptors (i.e. integrins, selectins, cadherins and mucins), and bind many exocrine factors, thus modulating their concentrations and availability to the cells (Simmons et al., 1997; Verfaillie et al., 1999; Chan and Watt, 2001). Hence, ECM acts in concert with cell-cell interactions and soluble factors to regulate hematopoiesis. The type of the hematopoietic response to the overall external stimulation is likely to be dependent on the intracellular mechanisms including differential gene expression and the cell cycle status.

4.2.2.1. Regulation of self-renewal

Dividing HSCs are subjected to three different choices: self-renewal, differentiation and apoptosis/survival. The putative signals promoting self-renewing divisions should not only stimulate self-replication, but also prevent differentiation and apoptosis. Identification of extrinsic factors promoting HSC self-renewal would allow ex vivo expansion and manipulation of HSCs for numerous clinical applications. Although factors directly supporting a long-term in vitro culture of self-renewing HSCs have not yet been identified, recent studies have implied that some extracellular signaling molecules can induce ex vivo expansion of HSCs.

Different combinatorial cocktails of cytokines, including SF, interleukins (IL-1,-3 and -6), Flt3 ligand (FLt3L), thrombopoietin (TPO) and granulocyte-colony...
stimulating factor (G-CSF) have been shown to promote a limited *ex vivo* expansion of HSCs (summarized in (Heike and Nakahata, 2002)). Moreover, a long-term maintenance has been achieved by co-culture of HSCs on stromal cells isolated from BM (Fraser et al., 1992) or fetal liver (Moore et al., 1997), although the *ex vivo* expansion was relatively inefficient. An *ex vivo* expansion of HSCs with transplantable LT-HSC activity has been attained in cultures containing both hematopoietic and stromal cells from total BM, supplemented with either TPO (Yagi et al., 1999) or FGF1 (de Haan et al., 2003). Furthermore, signaling pathways involved in cell-cell interactions classically studied in context of embryonic development, such as Notch (Karanu et al., 2000; Varnum-Finney et al., 2000a; Varnum-Finney et al., 2000b; Stier et al., 2002), Sonic hedgehog (Bhardwaj et al., 2001) and Wnt (Murdoch et al., 2003; Reya et al., 2003; Willert et al., 2003), have recently emerged as candidates for regulating self-renewal, although *ex vivo* expansion of LT-HSCs by those pathways has not yet been definitively demonstrated.

Finally, several transcription factors have been shown to play important roles in self-renewal of HSCs. Inactivation of polycomb group of proteins with gene-suppressing activity such as Bmi-1 and rae28 causes failure of HSC to self-renew (Ohta et al., 2002; Park et al., 2003) and overexpression of the transcription factor HOXB4, a member of Hox homeobox gene family, causes selective expansion of HSCs (Sauvageau et al., 1995; Antonchuk et al., 2002). Hence, multiple signaling pathways and transcription factors appear to be involved in regulation of self-renewal, but the molecular mechanisms integrating these signals to control HSC development remain unresolved.

**4.2.2.2. Regulation of proliferation, differentiation and survival**

Apart from regulation by specific transcription factors (Orkin, 2000; Zhu and Emerson, 2002), the process of hematopoiesis is governed by a number of growth factors and cytokines that regulate survival, proliferation and differentiation of hematopoietic cells (Moore, 1991; Ogawa, 1993). Cytokines are a large group of soluble or membrane bound glycoprotein mediators that include interleukins (ILs), colony-stimulating factors (CFS), tumor necrosis factors (TNFs), transforming growth factors (TGFs) and
interferons (IFNs). Different cytokines can have overlapping, synergistic or antagonistic actions, the cellular outcome being dependent on the cytokine combination, concentration and the differentiation stage of the responding cell.

The development of CFC-assays has allowed detailed analysis of cytokine action on different aspects of hemapoiesis. Optimal clonogenic growth and multilineage differentiation of HSCs and early progenitor cells has been shown to require the synergistic action of Steel factor (SF) and various so called early acting cytokines (Metcalf, 1993a; Ogawa, 1993). Early-acting activity has been ascribed to several cytokines (e.g, SF, TPO, IL-3, Flt3L, IL-6, LIF, IL-11) (Ogawa and Matsunaga, 1999), the most potent being combinations of SF, Flt3L, IL-6, IL-3 and TPO (de Vries et al., 1991; Heimfeld et al., 1991; Hannum et al., 1994; Sui et al., 1995; Sitnicka et al., 1996). The same cytokines, when acting individually, do not induce proliferation but can promote survival of immature hematopoietic cells (Katayama et al., 1993; Li and Johnson, 1994; Keller et al., 1995; Matsunaga et al., 1998). On the other hand, the so called late-acting cytokines (e.g., Epo, M-CSF, G-CSF, IL-5 or IL-7) have no effect on early progenitors and HSCs (Ogawa, 1993), but efficiently induce proliferation and differentiation of lineage-committed hematopoietic precursor cells (Metcalf, 1993a; Socolovsky et al., 1998). Certain cytokines, such as TGF\(\beta\), have been shown to inhibit proliferation of early hematopoietic progenitor cells (Keller et al., 1988).

Hematopoietic cytokines are normally produced by stromal cells located within hematopoietic tissues and exert their biological functions through cytokine receptors expressed on the hematopoietic cells. The receptors have been grouped into two families: cytokine receptor family, lacking intrinsic tyrosine kinase activity (Kishimoto et al., 1994) and the tyrosine kinase receptor family, with flt3, M-CSFR and c-Kit receptors being members of the latter (Linnekin, 1999; Taylor and Metcalfe, 2000). The signal transduction pathways from these receptors have been extensively investigated, but how a relatively limited number of signaling pathways interact to control pleiotrophic biological responses of different cell types is not yet clear.
INTRODUCTION

4.2.3. Role of c-Kit/Steel factor signaling in the hematopoietic system

Steel factor (SF, also known as Kit ligand, Stem Cell Factor or Mast cell growth factor) is a growth factor existing in both membrane-bound and soluble form, and triggers its biological effects through activation of the tyrosine kinase receptor c-Kit. Insights into the in vivo role of SF and c-Kit have been provided by molecular characterization of the set of naturally occurring mutations at the Steel (Sl) and White spotting (W) loci, encoding SF and c-Kit respectively (Yarden et al., 1987; Chabot et al., 1988; Geissler et al., 1988; Brannan et al., 1991; Broxmeyer et al., 1991; Galli et al., 1994). These mutations are associated with hematopoietic deficiencies of varying severity, such as lack of tissue mast cells, immature progenitor defects and macrocytic anemia (McCulloch et al., 1964; Russell, 1979; Barker, 1994). Severe W and Sl mutations lead to impaired fetal liver hematopoiesis and embryonic or perinatal death due to anemia (Russell, 1979). Whereas failure to establish hematopoiesis in c-Kit mutants is due to intrinsic defect in HSCs (cell-autonomous defect) and can be rescued by transplantation of wild type HSCs, the SF mutants contain defective stromal cells incapable of producing SF and thus unable to support the development of hematopoietic cells (cell-nonautonomous defect) (reviewed in (Broudy, 1997)). Consistent with these data, the c-Kit receptor has been found to be expressed on HSCs, the majority of hematopoietic progenitor cells and mast cells (Ogawa et al., 1991), while SF is produced by fibroblasts (Nocka et al., 1990), endothelial cells (Weiss et al., 1995) and stromal cells within the hematopoietic tissues (Heinrich et al., 1993).

Blocking c-Kit/SF interaction abrogates hematopoietic development in vitro and in vivo (Ogawa et al., 1991; Okada et al., 1991), indicating that c-Kit/SF signaling is required for development of the hematopoietic system and the maintenance of steady state hematopoiesis by HSCs. Studies using a recombinant form of SF alone and in combination with other hematopoietic growth factors/cytokines showed that it promotes survival and self-renewal of HSCs (de Vries et al., 1991; Metcalf and Nicola, 1991; Bodine et al., 1992), as well as proliferation and development of erythroid, myeloid and lymphoid cells (Ogawa et al., 1991; Melemed et al., 1997; Waskow et al., 2002). Importantly, the SF responsiveness decreases along with cell maturation, as c-
Kit receptor is progressively downregulated during differentiation of all hematopoietic lineages except mast cells, which retain a high level of expression (Fukuda et al., 1995). In mast cells, SF/c-Kit interaction stimulates diverse cellular responses including proliferation (Nocka et al., 1990), survival (Yee et al., 1994), differentiation (Tsai et al., 1991a; Tsai et al., 1991b), chemotaxis (Meininger et al., 1992) adhesion to fibronectin matrix (Dastych and Metcalf, 1994) and release of anti-inflammatory mediators (Columbo et al., 1992). Thus, SF/c-Kit signaling appears to mediate a wide range of biological activities in cells at many different levels in the hematopoietic hierarchy. Whether similar or distinct signaling pathways are employed upon c-Kit activation in different blood cell types remains to be elucidated.

4.2.3.1. Signal transduction from the c-Kit receptor

The c-Kit gene encodes a 145-kDa growth factor receptor with ligand-dependent tyrosine kinase activity, which is structurally related to the platelet-derived growth factor receptor (PDGF-R), the macrophage colony-stimulating factor receptor (c-fms), and the fms-like tyrosine kinase-3 (Flt-3) (Ullrich and Schlessinger, 1990). Structurally, c-Kit contains five immunoglobulin-like domains extracellularly, a juxtamembrane domain, and an intracellular catalytic kinase domain divided into two regions by an insert of variable length (Figure 6). Two isoforms of c-Kit exist and differ in Gly-Asn-Asn-Lys (GNNK) insertion within the extracellular domain (Reith et al., 1991; Voytyuk et al., 2003). The activation of the c-Kit receptor is mediated by SF induced dimerization and autophosphorylation of the receptor on tyrosine residues, thereby creating docking sites for cytoplasmic signaling molecules containing Src homology 2 (SH2) domains (reviewed in (Pawson, 1995; Linnekin, 1999; Taylor and Metcalf, 2000). Downstream c-Kit, multiple signal transduction components are activated, including the phosphoinositide-3 kinase-PKB pathway and the Ras-Raf-Mek-Erk cascade (Rottapel et al., 1991; Ronnstrand, 2004).
4.2.3.2. Ras-Raf-Mek-Erk pathway

Ras belongs to a large family of GTP-regulated molecular switches for many signaling pathways that modulate different aspects of cell behavior. The Ras-Raf-Mek-Erk (the traditional MAP kinase pathway) comprises a set of ubiquitous and highly conserved proteins that orchestrate the delivery of signals from the cell membrane to the nucleus (reviewed in (Karnitz and Abraham, 1995; Chang et al., 2003)). Receptor autophosphorylation appears to regulate Ras via the tyrosine phosphorylation of the adaptor protein Shc, which associates with the SH2 domain of another adaptor protein Grb2 (Cutler et al., 1993). Since Grb2 is constitutively bound to the Ras guanine-nucleotide exchange protein Son of Sevenless (SoS), recruitment of Grb2 to the activated receptor results in co-localization of SoS and Ras at the membrane. SoS catalyzes exchange of GDP to GTP and activates Ras that in turn activates Raf-1 by interacting with its Ras binding domain (RBD) (Marais et al., 1995). Ras-mediated membrane translocation of Raf-1 results in its activation and subsequent initiation of a protein kinase cascade leading to phosphorylation of Mek and Erk (Marshall, 1996a; Marshall, 1996b; Linnekin, 1999).

Figure 6. The c-Kit receptor. Extracellular part of the c-Kit receptor contains a ligand binding domain located N-terminally and a dimerization domain. Two c-Kit isoforms differ by the presence of Gly-Asn-Asn-Lys (GNNK) insertion. An intracellular catalytic domain is divided into two regions. Courtesy of Charlotte Edling.
Activated Erk translocates to the nucleus where it regulates expression of many transcription factors, such as NF-κB, Ets-1, AP-1 and c-Myc (Chang et al., 2003). A schematic outline of the signalling events initiated by activated c-Kit receptor and leading to activation of Erk is shown in Figure 7. The Ras-Raf-Mek-Erk cascade is considered to be an important signaling pathway for mitogenic responses to many growth factors (Marshall, 1995).

4.2.3.3. PI-3K pathway

Class Ia phosphoinositide 3-kinases (PI-3Ks) is a family of enzymes capable of phosphorylating the third carbon in the inositol ring of membrane inositol phospholipids (Toker and Cantley, 1997), and play a central role in growth factor signaling. PI-3K is comprised of a p110 catalytic subunit and a regulatory (or adapter) subunit, the most common family of which is derived from the p85α gene Pik3r1 (Vanhaesebroeck et al., 1997; Okkenhaug and Vanhaesebroeck, 2001). The p85 subunit contains several motifs involved in protein-protein interactions, including src
homology domains SH2 and SH3, which bind specific phosphotyrosines in the cytoplasmic domain of tyrosine kinase receptor proteins, thus recruiting the p110 catalytic subunit to the membrane (Vanhaesebroeck et al., 1997; Okkenhaug and Vanhaesebroeck, 2001).

Stimulation with SF leads to autophosphorylation of c-Kit receptor and subsequent binding of p85α through the SH2 domain to the phosphorylated Y719 in the kinase insert domain (Serve et al., 1994). This localizes the p110 catalytic subunit with potential substrates including phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2). The phosphorylation products, phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) and phosphatidylinositol -3,4,5-trisphosphate (PI-3,4,5-P3) function as second messengers and serve to activate proteins containing lipid-binding domains, so called pleckstrin-homology (PH) domains (Salim et al., 1996; Lemmon and Ferguson, 1998). Signaling molecules binding to PI-3,4-P2 and PI-3,4,5-P3 include 3’phosphoinositide-dependent-kinase-1 (PDK-1) and protein kinase B (PKB, also known as Akt). Activation of PKB occurs in

![Figure 8. Signaling from c-Kit receptor via PI-3K pathway.](image)
two steps: targeting to the cell membrane by the N-terminal PH domain and subsequent phosphorylation on serine/threonine residues by PDK1 (Downward, 1998; Stephens et al., 1998; Brazil et al., 2002). Fully activated PKB can detach from the membrane and phosphorylate many downstream targets within the cell, including Bad and forkhead transcription factor (Foxy-3), which results in prevention of apoptosis (Datta et al., 1997; Toker and Cantley, 1997; Blume-Jensen et al., 1998; Biggs et al., 1999; Brunet et al., 1999; Engstrom et al., 2003). A simplified outline of PI-3K pathway is presented in Figure 8.

A large body of evidence gained from both in vitro and in vivo experiments, indicates that PI-3K has multiple roles in SF-mediated cellular responses and that these roles vary with cell lineage (Linnekin, 1999). Abrogation of PI-3K signaling in murine mast cells results in inhibition of SF-mediated proliferation and survival as well as impaired adhesion, membrane ruffling and actin assembly (Serve et al., 1995; Vosseller et al., 1997). In vivo loss of adapter p85α function leads to defective development of erythroid progenitors and B cells (Fruman et al., 1999; Fruman et al., 2000; Huddleston et al., 2003) due to reduced capacity to activate PI-3K-dependent signaling pathways. Thus, PI-3K is clearly necessary for a wide variety of biological responses mediated by SF, but the exact signaling pathways and their functional significance in different cell types is not completely understood.

4.3. LIM-HOMEODOMAIN PROTEINS

LIM-homeobox genes encode proteins containing a DNA-binding homeodomain and two tandem LIM domains (Hobert and Westphal, 2000), named by the initials of the three homedomain proteins Lin11, Is11 and Mec3 in which it was first discovered (Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990) (Figure 9). LIM-domain is a cysteine- and histidine-rich zinc finger-like motif involved in protein-protein interactions. By means of their LIM domains, LIM-HD proteins have a potential to combinatorially interact with other transcriptional regulators, which allows them to regulate a wide range of developmental processes (Bach et al., 1999; Hobert and Westphal, 2000; Becker et al., 2002). Based on sequence homology within the homeodomain, LIM-HD proteins can be divided into subgroups. The members of the
INTRODUCTION

Apterous group include the Drosophila *apterous* gene (Bourgouin et al., 1992; Cohen et al., 1992), the C.elegans gene *ttx-3* (Hobert et al., 1997), and two vertebrate genes, *Lhx2* (also called LH-2 or LH2A) and *Lhx9* (LH2B) (Xu et al., 1993; Nohno et al., 1997).

4.3.1. Function of Lhx2 during embryonic development

The LIM-HD transcription factor Lhx2 controls cell-fate decisions as well as proliferation of different cell populations during embryonic development. Lhx2 expression is detected in the developing forebrain, hindbrain and midbrain, in the neural retina and optic stalk of the eye, fetal liver, spinal cord, olfactory epithelium, fetal thymus and thymocytes and in mesenchymal cells within the progress zone of the developing limb bud (Xu et al., 1993; Nohno et al., 1997; Porter et al., 1997; Lee et al., 1998; Rodriguez-Esteban et al., 1998; Woodside et al., 2004). Studies in chick have shown that during limb development Lhx2 contributes to the formation of the apical ectodermal ridge (AER), a region regulating limb bud outgrowth. Upon expression of a dominant negative variant of Lhx in the chick limb primordia, the AER did not form and the limb bud outgrowth was arrested (Rodriguez-Esteban et al., 1998). Although high expression of Lhx2 was detected in the mouse limb bud, Lhx2-knockout embryos display no apparent defects in limb development, presumably due to functional overlap with two other LIM-HD genes, Lhx9 and Lmx-1 (Porter et al., 1997; Rodriguez-Esteban et al., 1998; Bertuzzi et al., 1999).

Analyses of the Lhx2-deficient mice revealed that during development of the nervous system Lhx2 expression is required cell-autonomously for proliferation and specification of a variety of neuronal progenitor cells. Lhx2−/− embryos exhibited a severe proliferation defect of the cortical ventricular zone progenitors which resulted in hypoplasia of the cerebral cortex (Porter et al., 1997). In addition to regulating
proliferation, Lhx2 was also shown to play a role in specification of the cortical ventricular zone progenitors (Monuki et al., 2001).

Expression of Lhx2 is also necessary for the formation and maturation of sensory organs. \( Lhx2^{-/-} \) embryos were anophtalmic because the eye development was arrested prior to the formation of the optic cup (Porter et al., 1997). Lhx2 expression in olfactory neuron progenitors appeared to be required for their differentiation into a heterogeneous population of individually and regionally specified mature olfactory sensory neurons (Hirota and Mombaerts, 2004; Kolterud et al., 2004).

The phenotype of the \( Lhx2 \)-deficient mice also indicates that Lhx2 plays a role in fetal liver development and hematopoiesis. The \( Lhx2 \)-null embryos displayed a 7-fold reduction in fetal liver cellularity due to defective liver expansion and died around E16 because of severe anemia. The hematopoietic defect observed in \( Lhx2^{-/-} \) embryos was cell-nonautonomous, since HSCs isolated from \( Lhx2^{-/-} \) livers were able to reconstitute lethally irradiated recipients, and \( Lhx2^{-/-} \) ES cells contributed to hematopoietic cells in chimeric animals (Porter et al., 1997). This indicates that the function of Lhx2 during development of the hematopoietic system is confined to fetal liver environment.

### 4.3.2. Role of Lhx2 in regulation of self-renewal of HSCs

A putative role of Lhx2 in the control of cell fate decision and/or self-renewal of HSCs is revealed by the generation of Lhx2-immortalized multipotent hematopoietic progenitor cell lines. Ectopic expression of Lhx2 in immature hematopoietic progenitors obtained by in vitro differentiation of embryonic stem (ES) cells allowed the generation of multipotent hematopoietic progenitor cell (HPC) lines. The HPC lines are dependent on SF for their self-renewal \textit{in vitro} and can be efficiently induced to differentiate into erythrocytes, megakaryocytes, macrophages, neutrophils and mast cell when exposed to a broad spectrum of hematopoietic growth factors. The cell surface markers and transcription factors expressed by HPCs are consistent with fetal-derived multipotent hematopoietic progenitor/stem cells (Pinto do et al., 1998).

Expression of Lhx2 in hematopoietic progenitor/stem cells derived from BM led to the generation of stem cell-like lines termed BM-HPCs. These cell lines express
cell surface markers characteristic for BM-derived HSCs and similarly to HPC lines are dependent on SF for self-renewal in vitro. Furthermore, BM-HPCs are able to generate erythroid, myeloid and lymphoid cells upon transplantation into lethally irradiated mice, as well as long-term repopulate stem cell deficient recipients (Pinto do et al., 2002). Although the mechanism of Lhx2-induced immortalization of HPC and BM-HPC lines has not yet been identified, the specificity by which Lhx2 immortalizes multipotent, SF-responsive hematopoietic progenitor cells is striking and indicates that Lhx2 may operate in a specific cellular and molecular context to regulate stem cell function.

4.4. RELATIONSHIP BETWEEN THE DEVELOPING HEMATOPOIETIC AND HEPATIC SYSTEMS
The mammalian hematopoietic system is originally derived from the mesodermal layer formed during gastrulation and the hematopoietic activity is subsequently detected in distinct organs during embryonic development. The first signs of murine hematopoiesis appear at E7.5 in the extra-embryonic mesoderm of the yolk sac (Moore and Metcalf, 1970; Metcalf, 1971; Russell, 1979; Keller et al., 1999) and at E8.5-9 in the intraembryonic splanchnopleuric mesoderm surrounding the dorsal aorta, the region that later develops into the aorta, gonad and mesonephros (AGM) region (Muller et al., 1994; Medvinsky and Dzierzak, 1996; Sanchez et al., 1996). The extraembryonic hematopoiesis is transient and produces a wave of large, nucleated erythrocytes known as primitive erythrocytes (Moore and Metcalf, 1970; Keller et al., 1999). By E10.5, the major hematopoietic activity localizes to the fetal liver, which becomes colonized by hematopoietic progenitors and HSCs migrating presumably from both the AGM region and the yolk sac (Johnson and Moore, 1975; Yoder et al., 1997b; Palis et al., 2001; Kumaravelu et al., 2002). At this time point, the definitive type of enucleated erythrocytes emerge (Barker, 1968). The two waves of embryonic hematopoiesis are controlled by different transcription factors, which is exemplified by the absence of both primitive and definitive hematopoiesis in mice lacking the basic helix-loop-helix transcription factor SCL/Tal1, whereas inactivation of runt family
transcription factor AML-1 abrogates definitive hematopoiesis, but generation of primitive erythrocytes is normal (Shivdasani et al., 1995; Okuda et al., 1996).

Fetal liver remains the main hematopoietic organ until shortly after birth, when the hematopoiesis shifts to the BM (Russell, 1979). The fact that a 38-fold increase in the number of HSCs occurs in fetal liver during midgestation indicates that fetal liver environment provides the physiological conditions promoting efficient expansion of HSCs (Ema and Nakauchi, 2000). The molecules putatively involved in this process have not yet been characterized.

The mutual in vivo relationship between the developing hematopoietic and hepatic systems has not been thoroughly investigated. The data obtained from knockout studies indicate that early liver development can proceed normally when the number of hematopoietic cells in the liver is significantly reduced, as it was evidenced in the embryos lacking the runt family transcription factor AML-1 (Okuda et al., 1996). On the contrary, mutations specific to the hepatic mesenchyme often lead to hepatic failure and anemia, because an underdeveloped liver cannot sufficiently support fetal liver hematopoiesis (Schmidt et al., 1995; Hentsch et al., 1996). $Hlx^{null}$ mutant mice belong to this category. Although $Hlx$ deficiency leads to severe anemia and embryonic death, the $Hlx^{-/-}$ hematopoietic cells are able to reconstitute hematopoiesis in lethally irradiated wild type recipients. Thus, the hematopoietic defect observed in $Hlx$ deficient embryos might be either directly related to the drastically reduced liver size or to the lack of mesenchyme-derived factor promoting hematopoietic expansion, the latter being supported by disproportionally reduced numbers of multipotent progenitors in mutant livers (Hentsch et al., 1996). Lack of the endoderm-specific gene $Prox1$ causes failure of hepatoblasts to migrate into the septum transversum mesenchyme. Interestingly, the absence of hepatocytes from most of the liver lobes of $Prox1^{-/-}$ embryos does not seem to impair the overall formation of the liver-like organ, which suggests that mesenchymal component provides the sufficient morphogenetic information required for this process (Sosa-Pineda et al., 2000). Since no hematopoietic defect has been reported in $Prox1^{-/-}$ embryos, it is possible that the same hypothetical mesenchyme-derived stimuli control expansion of both hepatoblasts and HSCs in the developing liver.
5. AIMS OF THIS THESIS

The aim of this thesis was to analyze molecular mechanisms operating during the development of the hepatic and the hematopoietic systems, by studying the function of \( Lhx2 \) gene in these processes. The specific aims were:

- To characterize spatial and temporal expression of Lhx2 during murine liver development.

- To elucidate the role of Lhx2 in liver morphogenesis by comparing fetal liver development in \( Lhx2^{-/-} \) and wild type embryos.

- To further characterize and determine the mechanism whereby Lhx2 expression generates immortalized HPC lines, in order to gain some insights into the putative role of Lhx2 in the self-renewal of HSCs \textit{in vivo}.

- To use the HPC line as a model system for biochemical characterization of signal transduction pathways from c-Kit receptor in immature and differentiated hematopoietic cells.
6. RESULTS AND DISCUSSION

6.1. During liver development Lhx2 is expressed in the septum transversum mesenchyme and hepatic stellate cells (I, II)

Although the LIM homeobox gene Lhx2 was originally cloned from the pre-B cell line cDNA library and was reported to be expressed in immature B-cells in the developing rat liver (Xu et al., 1993), the identity of these cells has never been confirmed by co-staining with B-cell markers. The cell nonautonomous hematopoietic defect observed in Lhx2 null mutant livers suggests that Lhx2-expressing cells are more likely to be of non-hematopoietic origin. To clarify this issue and to get some insights into the role of Lhx2 in liver morphogenesis, we performed a detailed spatial and temporal analysis of Lhx2 expression by in situ hybridization. Expression of Lhx2 was first detected at E9, after the liver bud had formed and prior to the formation of hepatic cords, and was confined to the loose connective tissue of liver-associated septum transversum mesenchyme surrounding the liver bud (Figure 10A) (paper I). In the course of liver bud outgrowth, hepatoblasts intermingled with Lhx2 positive septum transversum-derived mesenchymal cells, a part of which became integrated into the liver and developed into sinusoid-associated hepatic stellate cells (Figure 10B), as confirmed by their co-expression of desmin, a marker specific for hepatic stellate cells (Yokoi et al., 1984; Tsutsumi et al., 1987) (paper II). These desmin+ stellate cells continued to express Lhx2 in the adult liver (paper I), which provides further evidence that at least a subpopulation of hepatic stellate cells originates from the septum transversum mesenchyme. Lhx2 expression was also observed in a discontinuous layer of cells located just beneath or at the outermost connective tissue layer surrounding all liver lobes (Figure 10B) (paper I). Lhx2 expression in this location decreased throughout the embryonic development and was not detected in postnatal life (paper I). The identity of these cells is not yet clear, but they might represent a stellate cell-related population, since they also express desmin (Figure 10, lower panel). This cell population might have a specific, Lhx2-dependent function during liver morphogenesis that is not required in adult liver.
RESULTS AND DISCUSSION

6.2. Functional Lhx2 expression in the hepatic mesenchyme is required for proper liver expansion (I).

Gene inactivation studies in mice have identified several genes necessary at distinct morphogenetic stages during liver development, and established the importance of reciprocal mesenchymal-epithelial interactions in this process. Furthermore, mesenchyme-derived signals have been shown to play crucial role in liver specification, expansion and differentiation. The liver hypoplasia observed in Lhx2\textsuperscript{+/−} embryos (Porter et al., 1997), together with the early onset of Lhx2 expression in the septum transversum mesenchyme surrounding the liver bud suggested that Lhx2 might control mesenchymal-epithelial interactions promoting proliferation of hepatoblasts at the stage of liver bud expansion. To investigate this hypothesis, we analyzed early liver
RESULTS AND DISCUSSION

development in $Lhx2^{-/-}$ embryos. We found that the cells normally expressing Lhx2 were present in the mutant livers, indicating that Lhx2 was not required for their generation. However, the mesenchymal-epithelial signaling appeared to be defective in the $Lhx2^{-/-}$ embryos, since the mutant livers were consistently 10-20% smaller than the wild type livers already at E 10.5. To exclude that the reduced liver expansion in the mutant embryos was due to altered expression of genes reported to be involved in the regulation of early steps in liver morphogenesis, we analyzed expression of Hlx, HGF, Hex and Prox1 (Schmidt et al., 1995; Hentsch et al., 1996; Keng et al., 2000; Sosa-Pineda et al., 2000). The lack of obvious differences in expression of these genes in wild type and $Lhx2^{-/-}$ livers suggests that a novel pathway might be used by Lhx2 to control liver outgrowth.

6.3. Loss of Lhx2 from developing stellate cells causes their activation and leads to hepatic fibrosis and distorted liver architecture (II)

To gain further insights into the role of Lhx2 in liver development, we compared the cellular organization of the wild type and mutant livers at E14.5, just prior to the death of $Lhx2^{-/-}$ embryos. These analyses revealed that Lhx2 deficient embryos displayed a severely distorted hepatic architecture. Hepatoblasts in the mutant livers were organized into ductular structures surrounded by abundant fibrous tissue (Figure 11A). In accordance with the basic characteristics of adult hepatic fibrosis, excessive deposition and expression of interstitial collagens type I and III (Figure 11B), the glycoprotein fibronectin, and the basement membrane components collagen type IV and laminin (Rojkind et al., 1979; Hahn et al., 1980; Herbst et al., 1997), were detected in $Lhx2^{-/-}$ livers by qRT-PCR, immunohisto- and histochemistry. Hence, $Lhx2^{-/-}$ embryos appeared to exhibit the primary features of adult liver fibrosis and would thus represent a novel example of hepatic fibrosis arising during embryonic development due to a gene deficiency. As the first signs of increased ECM deposition were detected at E11.5 and became more prominent in later developmental stages, the hepatic fibrosis in the mutant embryos seemed to be progressive and preceded the structural changes observed at E14.5 (Figure 11C).
RESULTS AND DISCUSSION

Interstitial collagens and other ECM proteins in fibrotic livers are synthesized by activated stellate cells (Friedman, 2000). Immunohistochemical analysis of liver sections from wild type and mutant liver at E14.5 revealed that \( Lhx2^{-/-} \) livers contained 4 fold increased proportion of mesenchymal cells expressing desmin, many of which also co-expressing ASMA, and thus resembling activated and proliferative myofibroblastic cells implicated in adult hepatic fibrosis. It thus appears that loss of \( Lhx2 \) expression in the developing stellate cells induces their activation and consequent fibrogenesis, meaning that normal \( Lhx2 \) function would be to prevent both these processes.

To verify whether overexpression of \( Lhx2 \) could suppress genes associated with activated stellate cell phenotype in human cells, we took advantage of recently generated human stellate cell line LX-2 retaining key features of activated hepatic stellate cells (Xu et al., 2004). As expression of collagen1\( \alpha \) (I) and ASMA in this cell line was significantly downregulated after transfection of \( Lhx2 \) cDNA, it is likely that \( Lhx2 \) inhibits stellate cell activation and fibrogenesis also in humans. Hence, elucidation of the molecular mechanisms used by \( Lhx2 \) to inhibit these processes could provide insights into the treatment of hepatic fibrosis. Up to date, the molecular mechanisms involved in the development and progression of hepatic fibrosis have been studied in experimental animal models for induced liver injury, but gene deficiencies

Figure 11. \( Lhx2^{-/-} \) mice develop hepatic fibrosis. A, albumin positive hepatoblasts form ductular structures (black arrowheads), surrounded by the fibrous tissue (arrow). B, excessive amounts of collagen fibrils detected as reticulin fibers (arrows) are deposited in the perisinusoidal spaces in the mutant liver. C, the architecture of the mutant liver is severely distorted. D, wild type liver exhibiting normal cellular organization. The photographs of the liver sections in C (mutant) and D (wt) were taken under the same magnification at E14.5. All photographs are included in paper II.

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leading to increased fibrogenesis have never been reported. Our findings therefore identify Lhx2 as the first stellate cell-specific gene negatively regulating the events leading to hepatic fibrosis and provide a genetic mouse model to study these processes.

6.4. Lack of Lhx2 expression in the developing stellate cells induces regenerative response of the hepatoblasts (II).

Stellate cell activation is also a part of normal regenerative response of the adult liver. Regeneration is initiated after injury inflicted on the hepatic tissue and is manifested in either mature hepatocyte or oval cell proliferation. The oval cell response occurs when proliferation of mature hepatocytes is inhibited, and involves formation of ductular structures expressing both hepatic and cholangiocyctic markers (Vandersteeenhoven et al., 1990; Rubin et al., 1995; Lowes et al., 2003). Similarly to oval cells, hepatoblasts in fetal liver are bipotent and can differentiate to both hepatocytes and cholangiocytes (Rogler, 1997). The ductular structures observed in the Lhx2<sup>−/−</sup> livers (Fig. 11A) contained cells expressing bile-duct-specific cytokeratin and high levels of E-cadherin in addition to hepatic markers albumin and AFP, thus resembling the atypical ductular reaction occurring during the regeneration process in a damaged adult liver. How the lack of Lhx2 expression in the developing stellate cells triggers the regenerative response of the hepatoblasts remains to be clarified. However, it is possible that the increased amount of ECM components in Lhx2<sup>−/−</sup> livers forces differentiation of hepatoblasts into bile ductules, thus imitating the ductal plate formation process occurring in the ECM-rich portal regions of the developing liver (Shiojiri, 1997; Lemaigre, 2003).
6.5. Models for the possible role of Lhx2 in fetal liver development

a. During early stages of liver formation Lhx2 is expressed in the septum transversum mesenchyme surrounding the liver bud. Since the developing Lhx2-/- liver is 10-20% smaller already at E10.5, it is likely that Lhx2-expressing mesenchymal cells control production of extrinsic signal(s) promoting proliferation of the adjacent hepatoblasts. As the expression of other mesenchyme-specific genes such as Hlx and HGF is unchanged in the mutant livers, it is possible that Lhx2 acts via a distinct and putatively novel signaling pathway. Alternatively, assuming that early Lhx2-expressing septum transversum mesenchymal cells have the same function as stellate cells later in development, Lhx2 could affect proper cell-cell adhesions by negatively regulating ECM production and in this way influence normal liver outgrowth.

b. At later stages of liver development the functional Lhx2 expression in stellate cells is required to suppress fibrogenesis-associated genes and thus ensure normal hepatic ECM content, proper cell-cell interactions and normal liver differentiation. As the function of stellate cells in liver development has thus far been relatively unknown, our results identify a possible role for hepatic stellate cells as a putative liver organizer.
From around E11 until just before birth up to 60% of the liver mass consists of blood cells and the main function of the liver at this time is to support hematopoiesis (Medlock and Haar, 1983b; Medlock and Haar, 1983a). How the process of liver morphogenesis is coordinated with the development of the hematopoietic system is not known, but liver hypoplasia often correlates to anemia and embryonic death around E13-16. Inactivation of \( Lhx2 \) leads to lethal anemia because the defective fetal liver environment cannot support hematopoiesis (Porter et al., 1997). The data presented in this thesis indicate that loss of functional \( Lhx2 \) expression in developing stellate cells leads to dramatic qualitative and quantitative changes in the hepatic ECM, progressive fibrosis and severe distortion of liver architecture. It thus seems reasonable that the reduced blood cell production and consequent anemia in \( Lhx2^{--} \) livers might be secondary to the hepatic failure. The hypoplastic liver lacking proper structure is expected to be unable to support hematopoiesis. Furthermore, the increased amount of collagen-rich ECM in the \( Lhx2 \) deficient livers might directly inhibit proliferation of the hematopoietic cells, induce their pre-mature differentiation or hinder them from proper interactions with other cells.

On the other hand, the phenotype of \( Prox1^{--} \) embryos suggests that hepatic mesenchyme alone might be sufficient for hematopoietic development and defective liver structure might not have any major impact on this process (Sosa-Pineda et al., 2000). It is therefore possible that Lhx2 expressing mesenchymal cells have yet another role in the developing liver, namely to produce paracrine factor(s) promoting self-renewal of hematopoietic cells. Consequently, lack of these signals in \( Lhx2^{--} \) liver environment would impair expansion of the hematopoietic system. The generation of self-renewing hematopoietic stem cell-like lines by immortalization with Lhx2 (Pinto do et al., 1998; Pinto do et al., 2002) supports this hypothesis.
RESULTS AND DISCUSSION

6.7. In vitro self-renewal of multipotent HPC line is dependent on Lhx2 expression and occurs via secreted factor(s) (III)

In an attempt to elucidate the mechanism whereby Lhx2 has immortalized HPC line, we analyzed the growth requirements of the HPCs in detail. Interestingly, optimal self-renewal of HPCs in the presence of SF occurred only at high cell densities (> $10^5$ cells/ml). We employed a transwell system, where low cell density culture ($10^3$ cells/ml) was separated from the high cell density culture (> $10^5$ cells/ml) by a membrane insert with 0.4µm pore size, to assess whether the cell-density dependent self-renewal of HPCs required direct cell-cell contacts or generation of soluble substances (Figure 12). The low cell density culture was self-renewing in the presence of high density culture (Figure 12A), whereas none or very limited self-renewal of low cell density culture was observed in the presence of medium only, or medium supplemented with 50% conditioned medium from the high cell density culture (Figure 12B). The mechanism responsible for the cell-density dependent self-renewal of HPCs appears thus to be mediated by secreted and diffusible factor(s) produced by cells grown at high cell densities (Figure 12C). This implies that the putative secreted factor has to be present at high concentration to affect the cells. In addition, as conditioned medium from high cell density cultures was rather inefficient in stimulating self-renewal of low density cultures, the soluble mediator is most likely also unstable and requires continuous synthesis for optimal function.

Interfering with functional Lhx2 expression either by introduction of antisense Lhx2 cDNA into HPCs or by turning off Lhx2 in the HPC lines with inducible Lhx2 expression (DoxHPC lines; Richter et al., manuscript in preparation) hampered self-renewal and induced cell death and/or terminal differentiation. Since functional expression of Lhx2 appears to be required for self-renewal of the HPC lines, the putative secreted factor is most likely directly or indirectly regulated by Lhx2. As HPCs are unable to self-renew at low cell densities, self-renewal cannot be induced via a strict autocrine loop. Therefore, we decided to use the term “cell nonautonomous” as a description for self-renewal of HPCs occurring in a cell density-dependent manner and induced by Lhx2-dependent mechanism.
The mechanism of Lhx2-induced immortalization of HPCs is in agreement with the cell nonautonomous phenotype of the lethal anemia observed in \textit{Lhx2}\textsuperscript{-/-} mice (Porter et al., 1997) and supports the hypothesis that Lhx2 expression in fetal liver environment is required for production of paracrine factor(s) promoting self-renewal of HSCs. Considering the liver expansion defect in \textit{Lhx2}\textsuperscript{+/-} embryos, it is tempting to speculate

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure12.png}
\caption{Cell-density dependent self-renewal of HPCs occurs via secreted factor(s). \textbf{A}, Low cell density culture is self-renewing in the presence of factor(s) secreted by high cell density culture. \textbf{B}, Low cell density culture cannot self-renew on its own or when supplemented with CM from high cell density culture. \textbf{C}, High concentration of the soluble mediator is required to induce self-renewal of HPCs.}
\end{figure}
that the putative paracrine factor(s) regulated by Lhx2 could influence expansion of both hepatic and hematopoietic cells in the developing liver. This signal(s) might be produced by all types of Lhx2-expressing cells present in the developing liver or either type of them (Figure 13). Since Lhx2 expression in the septum transversum-derived mesenchymal cells surrounding the liver lobes decreases during later gestational stages and is no longer detected in adult liver, these may be the candidate cells for the production of the putative paracrine factor(s) promoting expansion of hepatoblasts and HSCs during liver development.

Figure 13. A model for the putative role of Lhx2 in the expansion of hepatic and hematopoietic cells
Lhx2-expressing septum transversum-derived mesenchymal cells, alone or together with Lhx2-expressing stellate cells, produce secreted factor(s) promoting proliferation/self-renewal of hepatoblasts and HSCs, in addition to inhibiting ECM synthesis.

The identity of this putative factor(s) is still unknown. A wide range of growth factors and growth factors combinations could not substitute for the cell-density dependent self-renewal of HPCs, indicating that a novel factor might be responsible for Lhx2-induced immortalization. Since a soluble factor with self-renewal-promoting activity might be relevant for ex vivo expansion of HSCs, its identification would be critical for a wide variety of clinical applications.

6.8. The HPC lines represent a relevant in vitro model system to study basic properties of HSCs (III).
Analysis of the molecular mechanisms underlying cell fate decisions of HSCs is important for our understanding of the maintenance of the hematopoietic system. Unfortunately, normal HSCs are difficult to study due to their low frequency in
hematopoietic organs and their inability to be maintained *in vitro*. An alternative approach to study the mechanisms controlling self-renewal and differentiation within the hematopoietic system is to analyze immortalized cell lines. However, for this approach to be informative and give insights into the molecular mechanisms comparable to those operating *in vivo*, the cells under study should be characterized with respect to their functional properties. Although HPC lines have been shown to share several characteristics with normal early hematopoietic progenitor/stem cells (Pinto do et al., 1998), their relevance as a model system for studying HSCs requires further functional characterization. In order to validate the stem cell characteristics of the HPC line, the cells were subjected to a wide variety of progenitor/stem cell assays *in vitro*.

HPCs exposed to combinations of different early-acting cytokines responded by clonogenic growth and formation of multilineage colonies in a manner similar to normal HSCs. In agreement with previous reports, the most efficient combination was SF/IL-3/TPO/IL-6/Flt3-L. None of the late acting factors tested had any proliferative effect on HPCs and their proliferation and differentiation was also effectively inhibited by TGFβ1, further confirming their stem cell characteristics. Collectively, the response of HPCs to hematopoietic cytokines is comparable to that of normal hematopoietic progenitor cells and HSCs (Keller et al., 1988; Broxmeyer et al., 1991; Metcalf, 1993b; Sitnicka et al., 1996), indicating that the cytokine receptor expression pattern and their function in HPCs is similar to HSCs.

The ability to functionally interact with stromal cells and sustain hematopoiesis *in vitro* is characteristic of immature hematopoietic progenitor cells and HSCs (Dexter et al., 1977; Breems et al., 1994). HPCs seeded onto the stromal cell line S17 efficiently generated CAs that could be maintained for 4-5 months and can therefore be defined as LTC-IC, a feature commonly associated with normal HSCs isolated from both mice and humans (Ploemacher et al., 1991; Cho and Muller-Sieburg, 2000).

The HSC potential is most commonly tested by their ability to long-term engraft immuno-compromised recipients. Despite striking similarities between HPC line and normal HSCs observed in *in vitro* assays, the HPCs could not long-term
RESULTS AND DISCUSSION

repopulate immuno-compromised recipients. However, HSCs of early embryonic origin, including those derived from ES cells, have previously been shown to be unable to engraft to BM of adult mice, suggesting an intrinsic difference between early fetal and adult HSCs (Muller and Dzierzak, 1993; Sanchez et al., 1996; Potocnik et al., 1997; Yoder et al., 1997b). Consistently, BM-HPC lines obtained by expression of Lhx2 in adult BM-derived hematopoietic progenitors are able to generate all blood cell types after transplantation into lethally irradiated recipients (Pinto do et al., 2002). Thus, based on the expression profile of transcription factors, cell surface markers (Pinto do et al., 1998), the pattern of HPCs response to various cytokines/growth factors and their ability to functionally interact with stromal cells, we concluded that the HPC lines share phenotypic and functional characteristics of normal HSCs. The HPC lines therefore represent a relevant *in vitro* model system and a valuable tool to study basic properties of HSCs at the cellular, molecular and biochemical level.

6.9. A cross-talk between the PI-3K and Erk pathways occurs in HPCs (IV)

Activation of the c-Kit receptor upon binding to its ligand SF triggers many pleiotrophic biological responses including proliferation, survival, differentiation, migration, adhesion and secretion (Columbo et al., 1992; Dastych and Metcalfe, 1994; Galli et al., 1994; Broudy, 1997; Ashman, 1999). The c-Kit receptor is broadly distributed on a wide range of different hematopoietic cells including HSCs, suggesting that c-Kit signaling is important in cells at many different levels in the hematopoietic hierarchy (Broudy, 1997; Ashman, 1999; Linnekin, 1999). Clarification of the signaling events downstream of the c-Kit receptor in different hematopoietic cell types would contribute to our understanding of the molecular regulation of the hematopoietic system. We took advantage of the HPC and BM-HPC lines that exhibit the basic characteristics of HSCs (paper III) (Pinto do et al., 1998; Pinto do et al., 2002) to compare the signal transduction pathways from c-Kit receptor in immature and differentiated hematopoietic cells.

Initially, we compared c-Kit downstream signaling events in the SF-dependent HPC and BM-HPC lines to those of committed myeloid and mast cells either isolated
from BM or differentiated in vitro from HPCs or ES cells. In agreement with previously published data (Rottapel et al., 1991; Duronio et al., 1998), we observed transient tyrosine phosphorylation of c-Kit receptor upon ligation with SF, as well as phosphorylation of PKB and Erk, indicating that both PI-3K-PKB and Ras-Raf-Mek-Erk pathways were activated (Figure 14A).

**Figure 14.** Signaling from c-Kit receptor in HPCs and mast cells. **A,** SF/c-Kit ligation leads to PKB and Erk phosphorylation in both HPCs and mast cells. **B,** Mek inhibitor abrogates Erk phosphorylation in HPCs and mast cells. **C,** PI-3K inhibitor prevents phosphorylation of PKB as well as Raf-1, Mek and Erk in HPCs. **D,** PI-3K inhibitor abrogates phosphorylation of PKB, but not Erk in mast cells. **E,** PI-3K directly or indirectly modulates Raf-1 activity in HPC lines.
RESULTS AND DISCUSSION

To examine the contribution of PI-3K and Mek to these events we employed specific pharmacological inhibitors. As expected, Mek inhibitor PD98059 blocked SF-induced phosphorylation of Erk in all cell types tested (Figure 14B). On the other hand, two different PI-3K inhibitors, LY294002 and wortmannin, which are structurally and mechanistically distinct, unexpectedly abrogated phosphorylation of both PKB and Raf-1, Mek and Erk in HPC lines (Figure 14C), while only PKB phosphorylation was affected in differentiated mast and myeloid cells obtained from different sources (Figure 14D). Furthermore, we were able to show that the PI-3K inhibitors blocked neither Ras nor signaling molecules upstream Ras in HPCs, but clearly inhibited phosphorylation of serine 259 on Raf-1 as well as Raf-1 activation, thus identifying Raf as the point of pathway convergence. Collectively, these data indicate that PI-3K activity is required for Raf-Mek-Erk activation in immature multipotent HPCs (Figure 14E), while activation of Erk in differentiated myeloid and mast cells is PI-3K-independent. This suggests that during differentiation of HSCs, a signaling switch from PI-3K-dependent to PI-3K independent Erk activation occurs. Moreover, this molecular switch appears to be cell type and differentiation stage specific, since it is neither dependent on the amount of c-Kit receptors expressed by the cells, nor on ligand concentration, as it has been reported in other systems (Duckworth and Cantley, 1997). The PI-3K-dependent activation of Erk in the HSC-like cell lines is independent of ontogenic origin of the HSCs and Lhx2 expression. Thus, a cross-talk between the PI-3K and Erk pathways at the level of Raf-1 occurs in HPCs, which is blocked upon differentiation. This clearly shows that the mode of activation of different signaling pathways from c-Kit receptor in hematopoietic cells is dependent on their differentiation status and implies that the cross-talk between different pathways may be a way to confer the specificity of biological responses, as it has been shown in other cell types (Hu et al., 1995; Rommel et al., 1999). The PI-3K-dependent mechanism modulating Raf-1 activity in HPCs remains to be elucidated.
6.10. Inhibition of PI-3K signaling in HPCs induces apoptosis (IV)

SF has been shown to be a survival factor for HSCs and it does not promote their self-renewal unless used in combination with other factors (Li and Johnson, 1994; Broudy, 1997; Luens et al., 1998). In mast cells, SF alone is sufficient to mediate proliferation, enhance survival and induce differentiation of mast cell precursors isolated from BM and peripheral blood (Valent et al., 1992; Yee et al., 1994; Timokhina et al., 1998).

To clarify the biological significance of PI-3K and Erk signaling in HPCs, we monitored viability and proliferation of the cells in the presence of SF and with, or without inhibitors. Treatment with PD98059 inhibited growth of HPCs, indicating that Erk pathway alone promotes self-renewal of HPCs. As SF on its own does not induce self-renewal of normal HSCs (Li and Johnson, 1994), it is possible that Lhx2-dependent mechanism in concert with SF stimulation promotes self-renewal of HPCs. A similar mechanism could operate in vivo, where the factors produced by stromal cells would exert actions equivalent to those induced by Lhx2 (Figure 15). Treatment with PI-3K inhibitor induced prominent apoptosis of HPCs, indicating that SF-dependent PI-3K activity is absolutely necessary for the survival of HPCs. This result is in agreement with the anti-apoptotic effect of PI-3K-PKB pathway downstream c-Kit reported in hematopoietic progenitors analyzed in vitro (Engstrom et al., 2003).

The in vivo relevance of this finding remains to be shown. Targeted disruption of the pik3r1 gene resulting in loss of adapter p85α function did not affect survival of c-Kit+ cells in vivo (Fruman et al., 1999; Lu-Kuo et al., 2000; Huddleston et al., 2003). Moreover, mutation of the tyrosine 719, the PI-3K binding site in the c-Kit receptor did not influence steady-state hematopoiesis (Blume-Jensen et al., 2000). These apparent discrepancies between the role of SF-induced PI-3K signaling in vivo and in vitro might reflect the in vitro usage of pharmacologic inhibitors, which inhibit all PI-3K isoforms. The inhibition of p85α-mediated PI-3K signaling in vivo might have been compensated by the activities of other PI-3K isoforms and adapter subunits as well as complementary signaling pathways induced by multiple signals that hematopoietic cells in vivo are normally exposed to. Future studies designed to further reduce class Ia PI-3K activity in vivo would be required to completely unravel PI-3K-dependent signal transduction and its biological function in different hematopoietic cell types. Finally,
why HSCs would use the alternative mode of PI-3K-Raf-1 cross-talk is not yet clear, but it might be a way to enhance their survival and assure expansion in response to the combined action of SF and other stimuli, without accompanying differentiation (Figure 15). Increased apoptosis, accelerated erythroid differentiation and depletion of hematopoietic progenitors in Raf1<sup>-/-</sup> embryos support this hypothesis (Kolbus et al., 2002). A model proposing the putative biological role of PI-3K-Raf-1 cross-talk in HSCs is presented in figure below.

Figure 15. Possible biological role of PI-3K-Raf1 cross-talk in HSCs. A, PI-3K-dependent activation of Raf1-Mek-Erk cascade and PKB in response to SF promotes survival of HSCs as well as their self-renewal when SF acts together with other factors (X). In the HPC line X might correspond to the Lhx2-dependent factors. PI-3K-Raf-1 cross-talk might prevent pre-mature differentiation of HSCs, perhaps by making them less sensitive to differentiation stimuli. B, in committed mast cell precursors that already have initiated differentiation, activation of Erk is not PI-3K dependent and is most likely associated with proliferation and differentiation.
7. CONCLUSIONS

- Lhx2 is expressed in the septum transversum mesenchyme surrounding the outgrowing liver bud. During liver formation, a subpopulation of Lhx2-expressing mesenchymal cells becomes integrated into the liver and develops into hepatic stellate cells that continue to express Lhx2 in the adult liver.

- Functional Lhx2 expression in the septum transversum mesenchyme and hepatic stellate cells is required for normal liver expansion and differentiation. Lhx2 function in the developing stellate cells is to suppress genes associated with stellate cell activation and fibrogenesis.

- Liver fibrosis and ductular reaction observed in Lhx2<sup>-/-</sup> embryos display many features in common with adult hepatic fibrosis and liver regeneration described in studies based on animal models and patient material. Since transfection of Lhx2 cDNA into the human hepatic stellate cell line LX-2 leads to downregulation of genes associated with hepatic fibrosis, Lhx2 is likely to play a role in the pathogenesis of this disorder in humans.

- In agreement with the cell nonautonomous phenotype of the lethal anemia observed in Lhx2<sup>-/-</sup> mice, Lhx2-immortalized HPC lines self-renew <em>in vitro</em> by a mechanism involving Lhx2-regulated secreted factor(s). This observation supports the hypothesis that Lhx2 might control expression of paracrine factor(s) important for expansion of the hematopoietic system in fetal liver.

- Using Lhx2-immortalized HSC-like lines as an <em>in vitro</em> model system to study self-renewal and differentiation of HSCs, we have shown that HSCs require PI-3K dependent activation of both PKB and Erk for their survival and self-renewal, whereas in more committed myeloid and mast cells activation of Erk is PI-3K independent. Thus, the mode of SF/c-Kit signaling is dependent on the differentiation status of the cells.
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8. ACKNOWLEDGEMENTS

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9. REFERENCES


REFERENCES


REFERENCES


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