

## **Summary**

The fetal liver (FL) is an important structure in expansion and differentiation of hematopoietic stem cells (HSC), but despite this little is known about the exact mechanisms in which FL hematopoiesis takes place. Primitive hematopoiesis gives way to definitive hematopoiesis at 12.5 dpc in mice and the process is regulated by a number of intrinsic and extrinsic factors. Intrinsic regulations are intracellular processes that have been reported to be important in the initiation of definitive hematopoiesis. Several structures are involved with extrinsic regulations of hematopoiesis within the FL, including hepatoblasts and liver sinusoidal endothelial cells (LSEC). Hepatoblasts and endothelial cells comprise separate niches involved in the extrinsic regulation of hematopoiesis. Studies have shown that co-cultures with fetal liver stromal cells can promote the expansion of erythroid cells, although the way in which stromal cells do this is still unknown. Understanding the mechanisms in which hematopoiesis is regulated in the FL could lead to the production of novel therapies involving the safe and reliable transplantation of HSCs to patients with blood and bone marrow complications. This review aims to summarize the current state of knowledge about the regulation of hematopoiesis specifically within the FL.

**Key words:** Fetal liver, Hematopoiesis, Niche regulation, Hematopoietic stem cells

## **Introduction**

Hematopoiesis is a process that occurs at several sites both intraembryonically and extraembryonically over approximately a 20 day gestation period in mice and 40 day gestation period in humans (Peault and Tavian 2003). The sites of hematopoiesis change over this gestation period. In a mouse hematopoiesis starts with the yolk sac (YS) where primitive erythroid cells form (7.5 days postcoitum (dpc)) followed by erythroid progenitor cells at approximately 8.25 dpc. The erythroid progenitor cells seed the fetal liver (FL) where at 12.5 dpc this primitive hematopoiesis changes to definitive hematopoiesis and begins to sustain the adult blood system through hematopoietic stem cells (HSCs). In contrast, hematopoiesis in a human begins at day 17 in the YS, and then undergoes two stages of hepatic colonization at 23 days and 30 days (Baron et al. 2012). There is some controversy about where exactly the HSCs are generated; be it sites in the extraembryonic mesoderm or the intraembryonic para-aortic splanchnopleural (P-Sp) mesoderm/aorta-gonad-mesonephros (AGM). Regardless, it is at this time where HSCs are believed to circulate and colonize the FL and rapidly expand and differentiate into several different hematopoietic cells (HCs), such as erythrocytes, megakaryocytes, lymphocytes, neutrophils or macrophages (Sugiyama and Tsuji 2006; Sugiyama et al. 2011 a).

It is considered that there are mainly two kinds of hematopoietic regulation systems that maintain HSC expansion; intrinsic and extrinsic. Intrinsic regulation is programmed primarily by autonomous gene expression and extrinsic regulation is governed by cells surrounding the HSCs that have come to be called “niche cells” (Sugiyama et al. 2011 a). There have been many studies that show evidence of different cell types that may regulate HSCs in the bone marrow (BM), although little is known about the cells that support HSC expansion in the fetal liver (Chou et al. 2013).

HSCs are currently used clinically in regenerative medicine and are good candidates for the in utero treatment of severe immunologic, hematologic and metabolic diseases (Fukudome and Esmon 1994; Iwasaki et al. 2010; Sugiyama et al. 2011 a). However, when transplanting HSCs, problems involved with the shortage of cells include donor risks, rejection, and graft-versus-host disease (GVHD). It is important to understand the mechanisms of the rapid expansion and differentiation of HSCs in the FL in order to better apply HSC transplantation therapy into clinical use and it is with this in mind that this review is written.

### **Fetal liver hematopoiesis**

Although hematopoiesis begins in the YS at 7.5 days, it is not until mid gestation that hematopoiesis occurs in the FL (Johnson and Jones 1973; Johnson and Moore 1975; Medvinsky and Dzierzak 1998; Sugiyama et al. 2011 a). FL hematopoiesis is detected at 10dpc in mice and 5 gestational weeks (gw) in humans and becomes the predominant hematopoietic site by 11.5dpc and 15gw, respectively, where it remains as such until the end of gestation. Between embryonic day 12.5 and 16.5, the definitive HSCs of the fetal liver self-renew and expand to great numbers and differentiate into a massive amount of hematopoietic progenitor cells (HPCs) (Dzierzak et al. 1997; Chou et al. 2013). It is during these 5 days that rapid expansion occurs where the repopulating units in the fetal liver undergo a 38-fold increase (Lansdorp et al. 1993; Chou et al. 2013). Ema and Nakauchi demonstrated this increase in using a competitive repopulation assay comparing repopulating units and competitive repopulating units in the FL cells of C57BL/6 mouse embryos (Ema and Nakauchi 2000). Fetal liver HSCs express the membrane glycoproteins AA4.1 and Mac-1 which are not present in bone marrow HSCs (Jordan et al. 1990; Morrison et al. 1995; Chagraoui et al. 2003). HSCs in the FL express the markers c-Kit, stem cell antigen-1 (Sca-1) and protein tyrosine phosphatase receptor type C (CD45, which is also known as common leukocyte antigen) (Sugiyama et al. 2011 a).

## **Intrinsic and Extrinsic Regulation of Hematopoiesis**

Intrinsic regulation of hematopoiesis in the FL is associated with the ETS family transcription factor PU.1 which is involved in the differentiation and expansion of HSCs. Although the exact action of PU.1 is not fully understood, it has been reported that PU.1 plays an important role in the initiation of definitive hematopoiesis (Kim et al. 2004). A number of genes that are responsible for encoding for a family of 39 transcription factors that play a role in specifying position and tissue fate in embryos are called homeobox genes. Homeobox genes, including Class I Hoxb4 and Hoxb3, have been shown to positively regulate HSC and HPC proliferation (Bjornsson et al. 2003). HoxB4 has been linked to the up regulation of *Myc*, *AP-1*, *Jun-N*, *Fra-1*, and *cyclin D1* which are associated with its proliferative effects (Pan and Simpson 1999; Krosl and Sauvageau 2000; Satoh et al. 2004; Forrester and Jackson 2012).

With regard to the extrinsic regulation of FL hematopoiesis, it is important to first look at the composition of the FL and observe the cells responsible for hematopoietic regulation.

## **Cells involved in the extrinsic regulation of hematopoiesis**

Previous research reported that cells that are important in the extrinsic regulation of hematopoiesis in the FL are hepatoblasts (hepatocyte precursors) and endothelial cells (Mouta Carreira et al. 2001; Tanimizu et al. 2003; Sugiyama et al. 2011 a) (Figure 1). Stromal cells are also a subject of interest within this field as it has been reported that these cells support hematopoiesis (although the exact mechanism in which they do so is largely unknown). It is these three types of cells which this review will expand upon in detail.

### **Hepatoblasts**

Hepatoblasts are the result of early hepatic differentiation of the endoderm. They are bipotent cells with the ability to give rise to mature hepatocytes and bile duct epithelial cells (Chagraoui et al. 2003). Hepatoblasts are a set of important cells comprising a niche regulating hematopoiesis within the fetal liver. Hepatoblasts expressing Dlk-1 function in HSC differentiation, particularly erythropoiesis and through the secretion of erythropoietin (EPO), a cytokine regulating erythroid function and stem cell factor (SCF) (Sugiyama et al. 2011 a; Sugiyama et al. 2011 b) (Figure 2). SCF is an important membrane-bound growth factor that mediates interaction between stromal cells and the c-kit receptors on the surfaces of HSCs (Avraham et al. 1992; Heissig et al. 2002; Wilson and Trumpp 2006; Chou et al. 2013). Enzyme-Linked

Immunosorbent assays (ELISA) have shown that EPO is expressed to a high level in hepatoblasts along with SCF protein (Sugiyama et al. 2011 b). SCF<sup>+</sup>DLK<sup>+</sup> hepatocytes at 15.5 dpc increase the number of HSCs in the FL through the production of cell supportive cytokines, including thrombopoietin (TPO), SCF, angiopoietin-like 3 (Angptl3), and insulin-like growth factor-2 (Chou and Lodish 2010; Chou et al. 2013). SCF<sup>+</sup>DLK<sup>+</sup> cells also express high levels of  $\alpha$ -fetoprotein (AFP) and albumin (ALB) which have been recognized as markers of fetal hepatic progenitor cells (Chou et al. 2013). In addition to cytokines in the extracellular matrix (ECM) which includes the interstitial matrix and basement membrane, there are interactions with the cell surface membranes to regulate hematopoiesis extrinsically in the FL. Integrins such as beta-1 integrin (fibronectin receptor, CD29) function as receptors for the ECM and are important effectors for adhesion, differentiation, and migration of HSCs (Hynes and Yamada 1982; Humphries et al. 1989; Hirsch et al. 1996; Frisch and Ruoslahti 1997; Sugiyama et al. 2011 a). Integrin heterodimers and the ECM are thought to interact and function as a homing mechanism to enable HSCs and HPCs to reside in the FL (Patel and Lodish 1987; Long and Dixit 1990; Strobel et al. 1997; Sugiyama et al. 2011 a). Cells are able to interpret cytokine signaling in particular contexts due to the proteins produced in different cell kinds in the ECM (Taipale and Keski-Oja 1997;

Sugiyama et al. 2011 a).

### **Endothelial cells**

Liver sinusoidal endothelial cells (LSEC) are the cells that make up the sinusoidal wall, also known as the endothelium or endothelial lining within the liver. When examined with flow cytometry LSEC cells were shown to express Lyve-1 (lymphatic vessel endothelial hyaluronan receptor 1) which is not expressed on any other hepatic cells or on conventional endothelium (Mouta Carreira et al. 2001; Sugiyama et al. 2011 b; Tan et al. 2013). They can be considered as unique capillaries but with the presence of open pores or fenestrae and lacking a diaphragm and basal lamina (Braet and Wisse 2002). These pores allow medium sized proteins such as albumin to be transported into the circulation but exclude larger particles such as blood cells, chylomicrons and platelets (Tan et al. 2013). The vascular niche involved in controlling the HSCs consists of LSEC and reticular cells that secrete the chemokine CXCL12, which promotes HSC maintenance through chemokine signaling (Kiel et al. 2005; Iwasaki et al. 2010). Iwasaki et al. (2013) recently released a study on the transmembrane glycoprotein endothelial protein C receptor (EPCR) (Fukudome and Esmon 1994; Iwasaki et al. 2010). In their study they used flow cytometry to find that cultured lineage-negative (Lin<sup>-</sup>) Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) EPCR<sup>+</sup> cells significantly decreased after



short-term culture but this reduction did not occur when they were co-cultured with FL-derived Lyve-1<sup>+</sup> cells, which suggests that FL HSC maintenance and self renewal is largely dependent on the perisinusoidal niche (Iwasaki et al. 2010). LSEC expressing Lyve-1 have also been shown to express TGF-beta-1 which has been shown to increase HSC adherence to the fetal liver and help support hematopoiesis in the fetal liver (Sugiyama et al. 2013).

### **Stromal Cells**

Stromal cells can be defined as cells from the fetal liver with hematopoietic supportive ability that have epithelial to mesenchymal features. Experiments on primary cultures and cell lines of florid phase fetal liver cells have shown that the stromal cells express markers present in mesenchymal cells (vimentin, osteopontin, collagen I, alpha smooth muscle actin, calponin, thrombospondin-1, EDa fibronectin, Stro-1 antigens, and MEF2C) and epithelial cells (alpha-fetoprotein, cytokeratins 8 and 18, albumin, E-cadherin, and HNF3 alpha) (Chagraoui et al. 2003). Stromal cells show hematopoietic supportive abilities during gestation, although that ability is lost as the stromal cells are replaced by hepatocytes as the liver matures (Chagraoui et al. 2003). The actual growth proteins that are produced by stromal cells that support

hematopoiesis have still not been identified; however coculturing experiments have been done on different fetal liver stromal cells (Xi et al. 2013). More recently experiments with human fetal liver stromal cells (hFLSCs) have also been successful, which could lead to the production of a safer culture through achieving satisfactory maturation of terminal erythroid cells (Xi et al. 2013). Although experiments have shown that co-culture with fetal liver stromal cells have demonstrated that the cells do support hematopoiesis and could be useful in producing cells that could have future clinical applications, the underlying mechanism of how the cells support hematopoiesis is still largely unknown. More work in this field is required in order to understand how to better manipulate this discovery (Xi et al. 2013). Some recent studies that have involved the use of fetal liver stromal cell lines can be seen in table 1.

### **Perspective**

The understanding of the mechanisms that govern the regulation of hematopoietic stem cells within the FL are still largely unknown. The fetal liver is the site where primitive hematopoiesis draws to a close and definitive hematopoiesis takes over, leading to it being the major site for HSC expansion and differentiation before migration to the bone marrow. Better understanding of hepatoblasts, endothelial cells and stromal cells will hopefully pave the way to the creation of novel therapies that can

help patients with blood and bone marrow related illnesses.

### **Acknowledgements**

The authors acknowledge grant support from the Special Coordination Funds for Promoting Science and Technology, a Grant-in-Aid for Young Scientists (B) from The Ministry of Education, Culture, Sports, Science and Technology, Bilateral Joint Projects for Japan Society for the Promotion of Science.

### **References**

- Avraham H., Scadden D. T., Chi S., Broudy V.C., Zsebo K. M. and Groopman J.E. (1992). Interaction of human bone marrow fibroblasts with megakaryocytes: role of the c-kit ligand. *Blood*. 80(7), 1679-1684.
- Baron M.H., Isern J. and Fraser S.T. (2012). The embryonic origins of erythropoiesis in mammals. *Blood*. 119(21), 4828-4837.
- Bjornsson J.M., Larsson N., Brun A.C., Magnusson M., Andersson E., Lundstrom P., Larsson J., Repetowska E., Ehinger M., Humphries R.K. and Karlsson S. (2003). Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4. *Mol Cell Biol*. 23(11), 3872-3883.
- Braet F. and Wisse E. (2002). Structural and functional aspects of liver sinusoidal

endothelial cell fenestrae: a review. *Comp Hepatol.* 1(1), 1.

Chagraoui J., Lepage-Noll A., Anjo A., Uzan G. and Charbord P. (2003). Fetal liver stroma consists of cells in epithelial-to-mesenchymal transition. *Blood.* 101(8), 2973-2982.

Chou S., Flygare J. and Lodish H.F. (2013). Fetal hepatic progenitors support long-term expansion of hematopoietic stem cells. *Exp Hematol.* 41(5), 479-490.e4.

Chou S. and Lodish H.F. (2010). Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. *Proc Natl Acad Sci U S A.* 107(17), 7799-7804.

Chung Y.S., Choi B., Kwon C.H., Joh J.W. and Kim S.J. (2010). AFT024 cell line in co-culture system using high pore density insert (HPDI) maintains hematopoietic stem/progenitor cells (HSCs/HPCs) as more primitive state through histone modification. *Transplant Proc.* 42(10), 4611-4618.

Deurholt T., van Til N.P., Chhatta A.A., ten Bloemendaal L., Schwartlander R., Payne C., Plevris J.N., Sauer I.M., Chamuleau R. A., Elferink R.P., Seppen J. and Hoekstra R. (2009). Novel immortalized human fetal liver cell line, cBAL111, has the potential to differentiate into functional hepatocytes. *BMC Biotechnol.* 9, 89.

Dzierzak E., Sanchez M.J., Muller A., Miles C., Holmes A., Tidcombe H. and Medvinsky A. (1997). Hematopoietic stem cells: embryonic beginnings. *J Cell Physiol.* 173(2),

216-218.

Ema, H. and Nakauchi H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood*. 95(7), 2284-2288.

Forrester L.M. and Jackson M. (2012). Mechanism of action of HOXB4 on the hematopoietic differentiation of embryonic stem cells. *Stem Cells*. 30(3), 379-385.

Frisch S.M. and Ruoslahti E. (1997). Integrins and anoikis. *Curr Opin Cell Biol*. 9(5), 701-706.

Fukudome K. and Esmen C.T. (1994). Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem*. 269(42), 26486-26491.

Heissig B., Hattori K., Dias S., Friedrich M., Ferris B., Hackett N.R., Crystal R.G., Besmer P., Lyden D., Moore M.A., Werb Z. and Rafii S. (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 109(5), 625-637.

Hirsch E., Iglesias A., Potocnik A.J., Hartmann U. and Fassler R. (1996). Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature*. 380(6570), 171-175.

Humphries M.J., Obara M., Olden K. and Yamada K.M. (1989). Role of fibronectin in

- adhesion, migration, and metastasis. *Cancer Invest.* 7(4), 373-393.
- Hynes R.O. and Yamada K.M. (1982). Fibronectins: multifunctional modular glycoproteins. *J Cell Biol.* 95(2 Pt 1), 369-377.
- Iwasaki H., Arai F., Kubota Y., Dahl M. and Suda T. (2010). Endothelial protein C receptor-expressing hematopoietic stem cells reside in the perisinusoidal niche in fetal liver. *Blood.* 116(4), 544-553.
- Johnson G.R. and Jones R.O. (1973). Differentiation of the mammalian hepatic primordium in vitro. I. Morphogenesis and the onset of haematopoiesis. *J Embryol Exp Morphol.* 30(1), 83-96.
- Johnson G.R. and Moore M.A. (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature.* 258(5537), 726-728.
- Jordan C.T., McKearn J.P. and Lemischka I.R. (1990). Cellular and developmental properties of fetal hematopoietic stem cells. *Cell.* 61(6), 953-963.
- Joshi M., Patil B.P., He Z., Holgersson J., Olausson M. and Sumitran-Holgersson S. (2012). Fetal liver-derived mesenchymal stromal cells augment engraftment of transplanted hepatocytes. *Cytotherapy.* 14(6), 657-669.
- Kiel M.J., Yilmaz O.H., Iwashita T., Terhorst C. and Morrison S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal

endothelial niches for stem cells. *Cell*. 121(7), 1109-1121.

Kim H.G., de Guzman C.G., Swindle C.S., Cotta C.V., Gartland L., Scott E.W. and Klug C.A. (2004). The ETS family transcription factor PU.1 is necessary for the maintenance of fetal liver hematopoietic stem cells. *Blood*. 104(13), 3894-3900.

Krosl J. and Sauvageau G. (2000). AP-1 complex is effector of Hox-induced cellular proliferation and transformation. *Oncogene* 19(45), 5134-5141.

Lansdorp P.M., Dragowska W. and Mayani H. (1993). Ontogeny-related changes in proliferative potential of human hematopoietic cells. *J Exp Med*. 178(3), 787-791.

Long M.W. and Dixit V.M. (1990). Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. *Blood*. 75(12), 2311-2318.

Medvinsky A.L. and Dzierzak E.A. (1998). Development of the definitive hematopoietic hierarchy in the mouse. *Dev Comp Immunol*. 22(3), 289-301.

Morrison S.J., Hemmati H.D., Wandycz A.M. and Weissman I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 92(22), 10302-10306.

Mouta Carreira C., Nasser S.M., di Tomaso E., Padera T.P., Boucher Y., Tomarev S.I. and Jain R.K. (2001). LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and

- cirrhosis. *Cancer Res.* 61(22), 8079-8084.
- Pan Q. and Simpson R.U. (1999). *c-myc* intron element-binding proteins are required for 1, 25-dihydroxyvitamin D<sub>3</sub> regulation of *c-myc* during HL-60 cell differentiation and the involvement of HOXB4. *J Biol Chem.* 274(13), 8437-8444.
- Patel V.P. and Lodish H.F. (1987). A fibronectin matrix is required for differentiation of murine erythroleukemia cells into reticulocytes. *J Cell Biol.* 105(6 Pt 2), 3105-3118.
- Peault B. and Tavian M. (2003). Hematopoietic stem cell emergence in the human embryo and fetus. *Ann N Y Acad Sci.* 996, 132-140.
- Satoh Y., Matsumura I., Tanaka H., Ezoe S., Sugahara H., Mizuki M., Shibayama H., Ishiko E., Ishiko J., Nakajima K. and Kanakura Y. (2004). Roles for *c-Myc* in self-renewal of hematopoietic stem cells. *J Biol Chem.* 279(24), 24986-24993.
- Strobel E.S., Mobest D., von Kleist S., Dangel M., Ries S., Mertelsmann R. and Henschler R. (1997). Adhesion and migration are differentially regulated in hematopoietic progenitor cells by cytokines and extracellular matrix. *Blood.* 90(9), 3524-3532.
- Sugiyama D., Inoue-Yokoo T., Fraser S.T., Kulkeaw K., Mizuochi C. and Horio Y. (2011). Embryonic regulation of the mouse hematopoietic niche. *ScientificWorldJournal.* 11, 1770-1780.



- Sugiyama D., Kulkeaw K. and Mizuochi C. (2013). TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts. *Mech Dev.* 130(2-3), 195-206.
- Sugiyama D., Kulkeaw K., Mizuochi C., Horio Y. and Okayama S. (2011). Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production. *Biochem Biophys Res Commun.* 410(2), 301-306.
- Sugiyama D. and Tsuji K. (2006). Definitive hematopoiesis from endothelial cells in the mouse embryo; a simple guide. *Trends Cardiovasc Med.* 16(2), 45-49.
- Taipale J. and Keski-Oja J. (1997). Growth factors in the extracellular matrix. *FASEB J.* 11(1), 51-59.
- Tan K.S., Tamura K., Lai M.I., Veerakumarasivam A., Nakanishi Y., Ogawa M. and Sugiyama D. (2013). Molecular pathways governing development of vascular endothelial cells from ES/iPS cells. *Stem Cell Rev.* 9(5), 586-598.
- Tanimizu N., Nishikawa M., Saito H., Tsujimura T. and Miyajima A. (2003). Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J Cell Sci.* 116(Pt 9), 1775-1786.
- Toksoz D., Zsebo K.M., Smith K. A., Hu S., Brankow D., Suggs S.V., Martin F.H. and Williams D.A. (1992). Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and

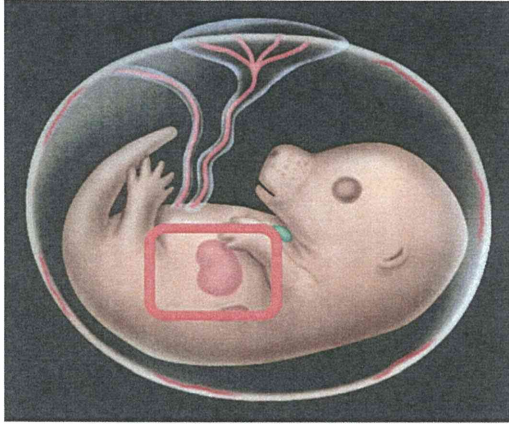
secreted forms of the human homolog of the steel gene product, stem cell factor.

Proc Natl Acad Sci U S A. 89(16), 7350-7354.

Wilson A. and Trumpp A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol. 6(2), 93-106.

Xi J., Li Y., Wang R., Wang Y., Nan X., He L., Zhang P., Chen L., Yue W. and Pei X. (2013). In vitro large scale production of human mature red blood cells from hematopoietic stem cells by coculturing with human fetal liver stromal cells. Biomed Res Int. 2013, 807863.

**a**



**b**

