# Hematopoietic Stem Cell Development and Transcriptional Regulation

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ACADEMIC DISSERTATION

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***To my family***

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# 1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their roman numerals.

I. **Gekas C, Dieterlen-Liévre F, Orkin SH, Mikkola HK.** The placenta is a niche for hematopoietic stem cells. **Dev Cell**. 2005 Mar;8(3):365-75.

II. **Rhodes K, Gekas C, Wang Y, Lux C, Francis CS, Yoder MC, Mikkola HK.** Hematopoietic stem cells emerge in the placental vasculature in the absence of circulation. **Cell Stem Cell**. 2008 Mar 6; Vol 2, 252-263

III. **Schlaeger TM, Mikkola HK, Gekas C, Helgadottir HB, Orkin SH.** Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (Scl/tal1)-dependent window during hematopoietic stem-cell development**. Blood**. 2005, 105(10): 3871-4

IV. **Gekas C, Rhodes K, Gereige LM, Helgadottir HB, Ferrari R, Kurdistani SK, Montecino-Rodriquez E, Bassel-Duby RS, Olson EN, Orkin SH, Pellegrini M, Mikkola HK.** Mef2C is a lineage-restricted target of Scl/Tal1 and regulates megakaryopoiesis and B-cell homeostasis. (Submitted)

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## 2. ABBREVIATIONS

| | |
|---|---|
| AGM | Aorta-Gonad-Mesonephros Region |
| AML | Acute Myeloid Leukemia |
| Aml1 | Acute Myeloid Leukemia 1 |
| Ang1 | Angiopoetin1 |
| BM | Bone Marrow |
| BMEC | Bone Marrow Endothelial Cell |
| Bmi1 | B Lymphoma Mo-MLV Insertion Region 1 |
| BMP | Bone Morphogenic Protein |
| C/EBPα | CCAAT/Enhancer Binding Protein |
| CaR | Calcium-sensing Receptor |
| CFU | Colony Forming Unit |
| ChIP-on-chip | Chromatin Immunoprecipitation on chip |
| c-kit | Receptor for c-kit Ligand/SCF |
| CLP | Common Lymphoid Progenitor |
| CMP | Common Myeloid Progenitor |
| c-Myb | Myeloblastosis Oncogene |
| c-Myc | Myelocytomatosis Oncogene |
| CpG | Cytosine followed by Guanine |
| CSF | Colony Stimulating Factor |
| DC | Dendritic Cell |
| DNA | Deoxyribonucleic Acid |
| DNMT | DNA Methyl Transferase |
| E | Embryonic Day |
| EBF | Early B-cell Factor 1 |
| ECM | Extracellular Matrix |
| ee | Embryo Equivalent |
| ELP | Early Lymphoid Progenitor |
| Epo | Erythropoietin |
| Ezh2 | Enhancer of Zeste Homolog 2 |
| FACS | Fluorescence Activated Cell Sorter |
| FGF | Fibroblast Growth Factor |
| FL | Fetal Liver |
| Flt3 | Fms-related Tyrosine Kinase 3 |
| Fog-1 | Friend of Gata-1 |
| GMP | Granulocyte Macrophage Progenitors |
| HDAC | Histone Deacetylase |
| HPC | Hematopoietic Progenitor Cell |
| HSC | Hematopoietic Stem Cell |

| | |
|---|---|
| Ihh | Indian Hedgehog |
| IL | Interleukin |
| LacZ | β-galactosidase |
| Lin | Lineage |
| Lmo2 | LIM domain only 2 |
| LoxP | Locus of Crossover of P1 |
| LSK | Lineage$^-$ Sca-1$^+$ c-kit$^+$ cells |
| LT-HSC | Long-Term HSC |
| MBP | Methyl-CpG Binding Proteins |
| Mef2C | Myocyte Enhancer Factor 2 C |
| MEP | Megakaryocyte Erythroid Progenitor |
| Mll | Mixed-Lineage Leukemia |
| MPP | Multipotent Progenitor |
| mRNA | Messenger RNA |
| Ncx1 | Sodium-Calcium Exchanger 1 |
| NK | Natural Killer |
| OB | Osteoblast |
| OCL | Osteoclast |
| OPN | Osteopontin |
| Pax-5 | Paired box gene 5 |
| p-SP | Para-aortic Splanchnopleura |
| RNA | Ribonucleic Acid |
| RU | Repopulating Unit |
| Runx1 | Runt-related Transcription Factor 1 |
| Sca-1 | Stem Cell Antigen 1 |
| SCF | Stem Cell Factor/c-kit Ligand |
| Scl | Stem Cell Leukemia Gene |
| SDF | Stromal Derived Factor |
| Shh | Sonic Hedgehog |
| SLAM | Signaling Lymphocyte-Activating Molecule |
| SP | Side Population |
| ST-HSC | Short-Term HSC |
| Tal1 | T-cell acute Leukemia Gene 1 |
| TF | Transcription Factor |
| Tgf-β | Transforming Growth Factor-beta |
| Tie2 | Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains–2 |
| TPO | Thrombopoietin |
| YS | Yolk Sac |

# 3. SUMMARY

Hematopoietic stem cells (HSCs) have the unique abilities of self-renewal and potential to generate all blood cell types throughout an individual's lifetime (Weissman, 2000). In the adult, HSCs reside in specialized HSC microenvironments within the bone marrow. During fetal development, hematopoiesis occurs at multiple temporally and anatomically restricted sites, including the yolk sac (YS), the aorta-gonad-mesonephros region (AGM) and the fetal liver (FL). Due to a discrepancy in the total number of HSCs in the fetus before and after hematopoietic seeding of the fetal liver (E11.5-12.5) we sought to identify other sources for HSCs in the embryo.

In avian embryos the allantois is a hematopoietic site and in mammals it participates in the formation of the placental labyrinth. Therefore, we asked whether HSCs could be found in the mouse placenta (**Paper I**). Importantly, we could show that placenta is a major fetal hematopoietic niche that harbors a large number of HSCs during midgestation (Gekas et al., 2005). Furthermore, as HSCs in placenta appear as early as in the AGM (E10.5), our data suggested that placenta could potentially serve as a site of *de novo* generation of HSCs. However, since blood circulation is initiated already at E8.5, cells from other sites could theoretically migrate via the bloodstream to any other site. In order to address this we utilized the Runx1-LacZ knock-in and Ncx1 knockout mouse models (**Paper II**). Importantly, we could show that HSCs emerge *de novo* in the placental vasculature in the absence of circulation (Rhodes et al., 2008). Furthermore, we could identify defined microenvironmental niches within the placenta with distinct roles in hematopoiesis: the large vessels of the chorioallantoic mesenchyme serve as sites of HSC generation whereas the placental labyrinth is a niche supporting HSC expansion (Rhodes et al., 2008). Overall, these studies illustrate the importance of distinct milieus in the emergence and subsequent maturation of HSCs.

To ensure proper function of HSCs several regulatory mechanisms are in place. The microenvironment in which HSCs reside provides soluble factors and cell-cell interactions. In the cell-nucleus, these cell-extrinsic cues are interpreted in the context of cell-intrinsic developmental programs which are governed by transcription factors. An essential transcription factor for initiation of hematopoiesis is Scl/Tal1 (stem cell

leukemia gene/T-cell acute leukemia gene 1). Loss of Scl results in early embryonic death and total lack of all blood cells. Surprisingly, however, deactivation of Scl in the adult does not affect HSC function, but leads to impaired erythroid and platelet development (Mikkola et al., 2003b). In order to define the temporal window of Scl requirement during fetal hematopoietic development, we deactivated Scl in all hematopoietic lineages shortly after hematopoietic specification in the embryo (**Paper III**). Interestingly, maturation, expansion and function of fetal HSCs was unaffected, and, as in the adult, red blood cell and platelet differentiation was impaired (Schlaeger et al., 2005). These findings highlight that, once specified, the hematopoietic fate is stable even in the absence of Scl and is maintained through mechanisms that are distinct from those required for the initial fate choice.

As the critical downstream targets of Scl remain unknown, we sought to identify and characterize target genes of Scl (**Paper IV**). We could identify transcription factor Mef2C (myocyte enhancer factor 2 C) as a novel direct target gene of Scl specifically in the megakaryocyte lineage which largely explains the megakaryocyte defect observed in Scl deficient mice. In addition, we observed an Scl-independent requirement of Mef2C in the B-cell compartment, as loss of Mef2C leads to accelerated B-cell aging (Gekas *et al.* Submitted). Taken together, these studies identify key extracellular microenvironments and intracellular transcriptional regulators that dictate different stages of HSC development, from emergence to lineage choice to aging.

# 4. INTRODUCTION

The continuous production of blood cells, a process termed hematopoiesis, is sustained throughout the lifetime of an individual by a relatively small population of cells known as hematopoietic stem cells (HSCs). HSCs are unique cells characterized by their ability to self-renew and give rise to all types of mature blood cells. Given their high proliferative potential, HSCs need to be tightly regulated on the cellular and molecular levels or could otherwise turn malignant. On the other hand, the tight regulatory control of HSC function also translates into difficulties in culturing and expanding HSCs *in vitro*. In fact, it is currently not possible to maintain or expand HSCs *ex vivo* without rapid loss of self-renewal. Increased knowledge of the unique features of important HSC niches and of key transcriptional regulatory programs that govern HSC behavior is thus needed. Additional insight in the mechanisms of stem cell formation could enable us to recapitulate the processes of HSC formation and self-renewal/expansion *ex vivo* with the ultimate goal of creating an unlimited supply of HSCs from e.g. human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPS) to be used in therapy. We thus asked: How are hematopoietic stem cells formed and in what cellular niches does this happen (**Papers I, II**)? What are the molecular mechanisms that govern hematopoietic stem cell development and differentiation (**Papers III, IV**)?

# 5. REVIEW OF THE LITERATURE

## 5.1. HEMATOPOIESIS

The process of hematopoiesis ensures the daily production of the over a thousand billion ($1 \times 10^{12}$) blood cells needed for the survival of an adult (Ogawa, 1993). The reason of this high demand is mainly due to the short life-span of neutrophils and platelets that need to be continuously replaced, but also that of other myeloid and lymphoid cells. To achieve this incredible feat, the bone marrow develops early on in life into a specialized *niche* that can support hematopoietic stem cells (see (Stier et al., 2005; Zhu and Emerson, 2004)), which are formed during fetal development. Although HSCs are rare in numbers, comprising about 1 out of $10^5$ BM cells (Harrison et al., 1988; Morrison et al., 1995), all blood cells originate from HSCs. In the right environment, these cells have the remarkable properties to give rise to all types of blood cells, termed *multipotentiality*, and their ability to divide into daughter cells where either one or both remain HSCs, termed *self-renewal capacity* (Siminovitch et al., 1963). A functional requirement used to define true HSCs is their unique ability to long-term reconstitute the entire hematopoietic system of a myeloablated host (Dick et al., 1985; Lemischka et al., 1986). For this reason, HSCs have been under immense experimental scrutiny for the past few decades, since this also means that healthy HSCs can be used to treat human blood disorders. There was also initial promise that HSCs could change their cell-fate, through the process of *transdifferentiation* (e.g. (Krause et al., 2001; Lagasse et al., 2000; Weissman et al., 2001), and be used to treat neurological, heart and other non-blood disorders. However, most such studies were shown to be either too inefficient for treatment or explained by processes other than transdifferentiation, such as cell-fusion, and will not be discussed further here (Nygren et al., 2004; Orkin and Zon, 2002; Wagers et al., 2002).

## 5.2. THE HEMATOPOIETIC HIERARCHY

Since all mature blood cells and their progenitors originate from hematopoietic stem cells, the HSC is on the top of the hierarchy of adult hematopoiesis. The most widely accepted model of hematopoiesis is depicted in Figure 1. The most immature cells in the

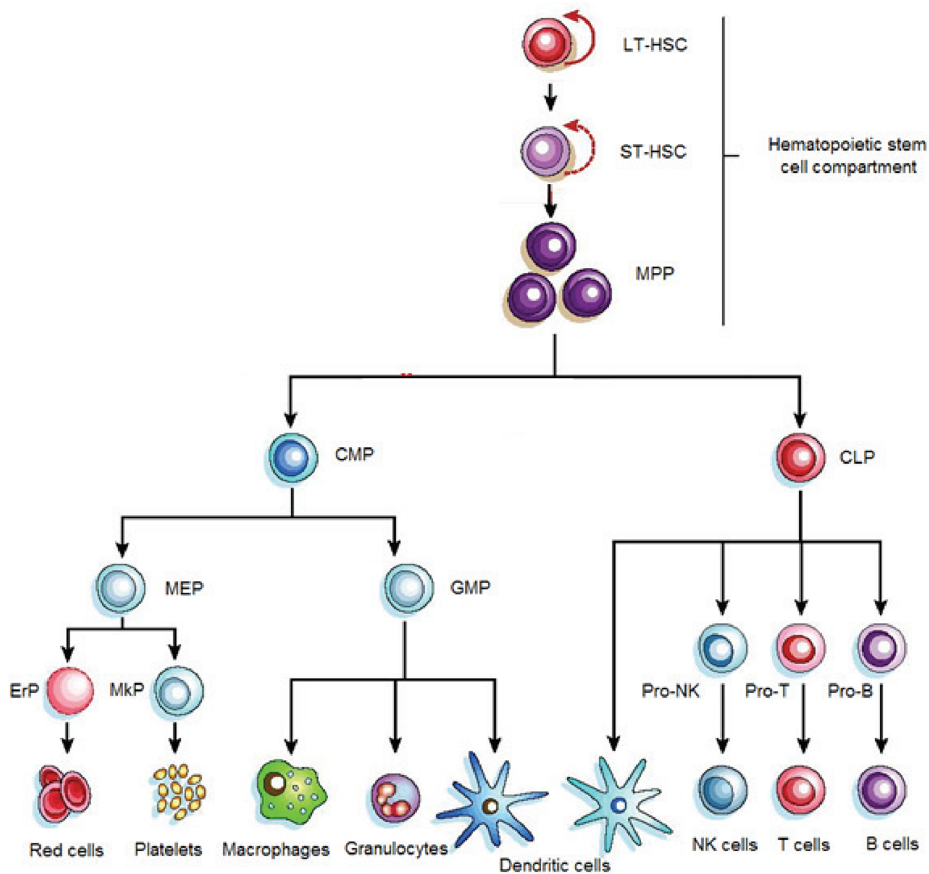


**Figure 1. The hematopoietic hierarchy.** Schematic drawing of the hematopoietic tree (adapted from (Reya et al., 2001)), showing involved cell types and their hierarchical relationships.

stem cell compartment are the long-term, or LT-, HSCs, named after their capacity to give rise to and maintain hematopoiesis for over six-eight weeks and up to lifetime after transplantation in mice (Dick et al., 1985; Lemischka et al., 1986). These cells have the most extensive self-renewal capacity, which can be assessed through serial transplantations. Briefly, a cell capable of extensive self-renewal will be able to propagate itself and its progeny also after a secondary, tertiary and so on, serial transplantation to new hosts (Keller and Snodgrass, 1990). The short-term (ST-) HSC is multipotential, but has lower self-renewal potential and thus capable only of transient hematopoietic reconstitution that gets lost after 6-8 weeks (Morrison and Weissman,

1994). The MPP, or multipotential progenitor, is a cell downstream of the LT- and ST-HSCs that has the same multilineage differentiation capacity, but is not defined as a stem cell as it lacks self-renewal ability (Morrison et al., 1997b; Weissman, 2000). Up to this point all cells have the ability to differentiate to all mature lineages. However, the next differentiation step involves a lineage choice and a restriction in potential as, according to the commonly accepted model of hematopoiesis, either a CMP (common myeloid progenitor) or a CLP (common lymphoid progenitor) is formed. The CMP can give rise to two oligopotential cell types, the megakaryocytic/erythroid (MEP) and granulocyte/monocyte (GMP) progenitors, each retaining the ability to differentiate to platelets and red cells and granulocytes, macrophages and dendritic cells, respectively (Akashi et al., 1999; Akashi et al., 2000). The lymphoid branch of the hematopoietic tree arises at the level of the CLP, which has the potential to form B-, T-, natural killer (NK-), and dendritic (DC) lymphoid cells (Kondo et al., 1997). Of note, whereas all other differentiation steps and the formation of mature cells in the adult occur in the bone marrow, the differentiation and maturation of T-cells occurs in the thymus, a primary lymphoid organ in the chest. It should be mentioned that recent evidence point to the CLP as mainly a B-cell poised progenitor with no significant T-cell potential, indicating that the T-cell lineage branches off at a separate point during differentiation (reviewed in (Montecino-Rodriguez and Dorshkind, 2003)).

## 5.3. REGULATION OF HEMATOPOIESIS

Given the incredible repopulation potential of HSCs, in order to avoid hyper-proliferation or dysfunctional differentiation that could lead to blood disorders such as leukemia, it is evident that HSCs need to be tightly regulated (Domen and Weissman, 1999). Indeed, this is ensured by maintaining most of HSCs in the BM residing in the $G_0$-, or dormant, phase of the cell cycle (Bradford et al., 1997; Cheshier et al., 1999). Upon the reception of an appropriate signal, the HSC will enter the cell cycle and either divide *symmetrically*, giving rise to two daughter cells both being either HSCs, or *asymmetrically*, giving rise to a progenitor, which will subsequently divide and differentiate into mature cells, and to an

HSC, thus retaining the numbers of HSCs (Morrison et al., 1997a). In *steady-state* hematopoiesis asymmetric cell division is most common, whereas during *hematopoietic stress*, such as during the repopulation of a host, most HSCs will divide symmetrically to initially expand their own numbers before prioritizing asymmetrical divisions (Osawa et al., 1996). In certain disease states, such as bone marrow failure, HSCs divide symmetrically in a non-self-renewal manner, thus progressively diminishing in numbers and eventually becoming extinct. Fate decisions of HSCs are the outcome of the interplay between cell-extrinsic and cell-intrinsic signals and will be discussed more next.

### 5.3.1. CELL-EXTRINSIC REGULATORY MECHANISMS

#### 5.3.1.1. THE HEMATOPOIETIC MICROENVIRONMENT

As can be appreciated, the factors regulating the above mentioned processes of stem cell proliferation, self-renewal and differentiation, are more complex than can be included in this text and not fully known. However, several key notions could be worth discussing.

The concept that the nature of the physical residence of cells is important is implied by the Greek term *anoikis* – homelessness – which is used to describe cell death in the absence of appropriate cell-extracellular matrix interactions. In recent years, it has become clear that probably the single most important factor that governs also hematopoietic stem cell behavior is the characteristics of the microenvironment, or *niche*, in which the HSC resides (Adams and Scadden, 2006; Suda et al., 2005). It is an unfortunate fact that removing HSCs from their *in vivo* places of residence causes rapid loss of self-renewal capacity and subsequent terminal differentiation.

Although mostly undefined, the hematopoietic niche in the bone marrow has been shown to be comprised of several key cellular and molecular components (Zhu and Emerson, 2004). Notably, the osteoblast (OB), a non-hematopoietic bone-forming cell, was shown to be directly implicated in positively regulating HSC numbers (Calvi et al., 2003). Osteoblasts line the endosteal surface of trabecular bone, a region within the bone marrow which was known to be preferentially inhabited by immature hematopoietic cells,

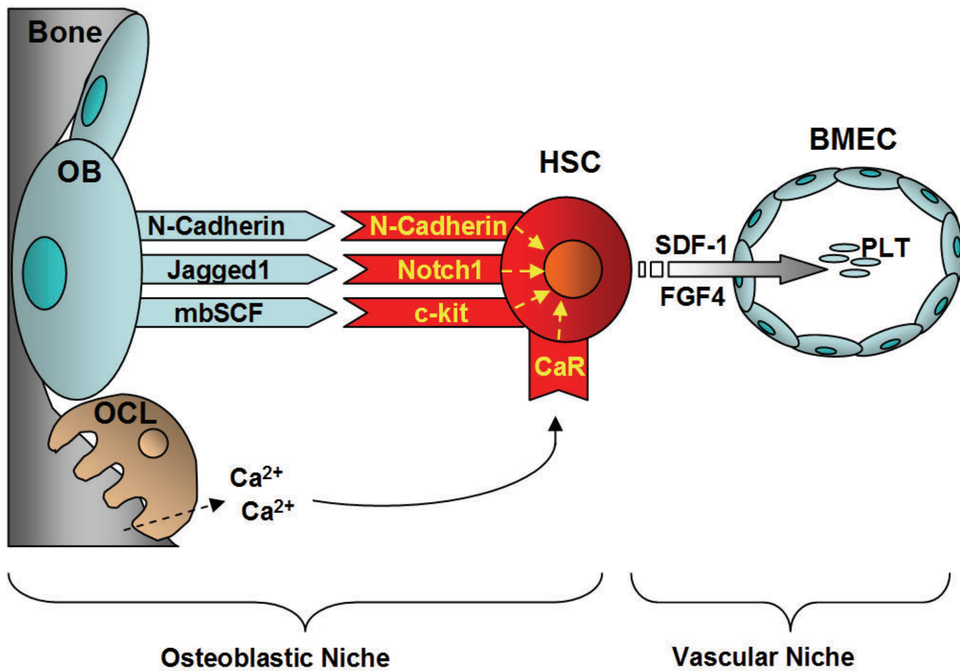


**Figure 2. The bone marrow hematopoietic stem cell niche.** Schematic drawing of the HSC microenvironment within the bone marrow comprised of the osteoblastic and vascular niches. The key cellular components of the niche are osteoblasts (OB), which express several ligands important for HSC function, and osteoclasts (OCL) whose bone resorbing activity has been implicated in HSC engraftment through signaling of the calcium receptor. The vascular niche, although less defined, is comprised of bone marrow endothelial cells (BMEC), and has been shown to regulate HSC maintenance and platelet (PLT) development through expression of SDF-1 and FGF4. mbSCF: membrane bound SCF, CaR: Calcium-Sensing Receptor.

whereas more differentiated hematopoietic cells are located in the BM cavity further away from the endosteum (See Figure 2) (Taichman and Emerson, 1998). Among the key signaling components proposed for osteoblast-HSC interaction are adhesion molecules N-cadherin, which upon activation releases β-catenin, a potent signaling molecule of the wnt-pathway, and Notch1 receptors on HSCs that interact with Jagged1 ligands on OBs to signal through Notch activation pathways. An interesting finding, implicating the role of cytokines in HSC-niche interactions, is the preferential adhesion of HSCs on osteoblastic regions that express membrane bound SCF (Driessen et al., 2003; Taichman and Emerson, 1998). Osteoclasts (OCLs), specialized bone-resorbing macrophage-like

cells of hematopoietic descent, were recently implicated as an additional cellular component of the endosteal HSC niche. Notably, induction of osteoclastogenesis by RANK-ligand (RANKL), a member of the tumor necrosis factor (TNF) family of cytokines and normally produced by OBs, led to mobilization of HSCs and progenitors away from the BM (Kollet et al., 2006). The proposed mechanism of RANKL-induced HSC mobilization includes upregulation of protease MMP-9 and cathepsin K on OCLs which cleave membrane-bound SCF on OBs, as well as reduction in production of osteopontin, which regulates HSC numbers (Nilsson et al., 2005; Stier et al., 2005). In addition, it was shown that loss of Calcium-sensing Receptor (CaR) on HSCs resulted in a defect in HSC homing to the endosteal niche, which correlated with diminished binding to collagen type 1 (Adams et al., 2006). This suggests that the calcium gradient within the remodeling bone, a direct outcome of OCL activity, is important for engraftment of HSCs into the endosteal niche. Worth mentioning is the osteopetrotic op/op mouse model, which due to lack of functional macrophage-colony stimulating factor-1 (M-CSF) leads to severe deficiencies of macrophages and OCLs resulting in excessive bone formation. These mice exhibit occlusion of the marrow cavity and reduced hematopoietic activity (Nilsson and Bertoncello, 1994). However, a stromal cell line isolated from op/op bone marrow, termed OP9, displays remarkable hematopoietic-promoting potential and has been widely used by us and others as an invaluable tool for *in vitro* culture of HSCs and progenitors (see **Papers II, IV** and (Carlyle et al., 1997; Kodama et al., 1994)).

In addition to the osteoblastic niche found exclusively in the adult bone marrow, there exist specialized vascular HSC niches within bone marrow and other adult tissues, such as the spleen and liver, which are known sites of extramedullary hematopoiesis (reviewed in (Zhang and Li, 2008)). The vascular niche in the BM is even less defined than the osteoblastic niche, but its functional components appear to include reticular cells, which express high levels of the chemokine SDF-1, important for HSC maintenance (Sugiyama et al., 2006). In addition, hematopoietic cell interactions with vascular endothelium are likely to be vital as exemplified by studies showing association of a large population of HSCs with sinusoidal endothelium in multiple organs, and the role of BM endothelial cells (BMECs) in sinusoidal spaces in promoting megakaryopoiesis and platelet production through SDF-1 and FGF-4 (See Figure 2) (Avecilla et al., 2004; Kiel et al.,

2005). Importantly, as HSCs are formed during fetal development prior to bone formation, fetal hematopoietic niches are exclusively vascular niches. From the perspective of this thesis, the variety of the distinct microenvironmental niches provided by fetal tissues during the initial stages of hematopoiesis is likely to contain important clues as to the signals required for HSC emergence, maturation and expansion and would have major clinical applications for *de novo* generation of HSCs. Fetal hematopoietic niches will be discussed in more detail later.

#### 5.3.1.2. Hematopoietic Cytokine Signaling

In addition to the above mentioned extrinsic cues regulating hematopoietic stem cells, the HSC niche and other cell types secrete soluble regulatory factors. One class of regulators that has been shown to affect hematopoietic decisions throughout the blood hierarchy is the hematopoietic *cytokine* family (Metcalf, 1998). Cytokines are extracellular molecules that exert their function through interaction with cell membrane-bound cytokine receptors which send their message to the cell nucleus where it is interpreted to yield a cellular response. One of the important receptor regulating hematopoiesis is c-kit, a member of the platelet-derived growth factor receptor (PDGFR) group, belonging to the protein receptor tyrosine kinase (RTK) family of genes (Chabot et al., 1988; Geissler et al., 1988; Matthews et al., 1991; Rosnet et al., 1991a; Rosnet et al., 1991b). The common feature of the genes in this group is that they encode for proteins that span the cellular membranes. The extracellular domain acts as a receptor for the corresponding ligand, and the intracellular portion consists of regions with inducible enzymatic activity capable of phosphorylation of tyrosine residues on specific target amino-acid sequences. Thus, an extracellular signal can be propagated to the interior of the cell upon ligand binding and subsequent phosphorylation and activation of the receptor. This means that only cells expressing the receptor will understand the cytokine message. Indeed, whereas the ligand of c-kit, stem cell factor (SCF), is widely expressed in many tissues, the expression of the receptor is much more limited to specific cellular subsets of the body (reviewed in (Lyman and Jacobsen, 1998)). This fact has not only facilitated the identification of the cells where each ligand confers its biological activity, but also provided for a tool to functionally dissect different subsets of cells based on the

relative or absolute levels of receptor expression. In fact, expression of c-kit was found to be predominantly restricted to immature progenitor/stem cell subsets (Metcalf and Nicola, 1991; Rasko et al., 1995). Notably, c-kit is expressed on LT- and ST-HSCs, where SCF has been shown to be a critical regulator controlling their proliferation and differentiation, as naturally occurring and engineered mutations and deletions of c-kit seriously affects hematopoiesis and stem cell function (Ogawa et al., 1991; Ogawa et al., 1993; Okada et al., 1991; Russell, 1979; Silvers, 1979).

Other cytokines worth mentioning in this regard are the members of hematopoietin family, which include receptors for interleukins (IL), erythropoietin (EPO), thrombopoietin (TPO) and colony stimulating factors (e.g. granulocyte-macrophage colony stimulating factor; GM-CSF). A couple of these deserve a brief introduction as they have been used as growth factors for *in vitro* culture assays in the studies presented herein. Firstly, interleukins 3 (IL3) and 6 (IL6) are widely used for *in vitro* cultures of hematopoietic stem and progenitor cells, as they play important roles in cell growth and support a variety of myelo-erythroid differentiation pathways. Supplementing myeloerythroid cultures with TPO and IL11 promotes megakaryocytic development and platelet release, whereas G-, M- or GM-CSF drives differentiation towards the granulocyte and macrophage lineages. It is worth noting that the receptor of TPO, c-mpl, acts both as a late-acting factor to stimulate megakaryocyte and platelet formation, and is expressed on immature stem and progenitor cells. Concordantly, loss of TPO or c-mpl leads to a deficiency in megakaryopoiesis as well as hematopoietic stem cell numbers (Kimura 1998). The importance of growth factor EPO in erythropoiesis is made evident by the EPO and EPO-receptor knockout mice, which are embryonic lethal and display severe anemia (Lin et al., 1996). Interleukin 7 (IL7) has been shown to play a non-redundant and key role in lymphocyte development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Specifically, mice lacking either IL7 or its receptor, IL7Rα, display a profound reduction of both thymic and peripheral thymocyte numbers and a near complete absence of B-cell progenitors in the adult (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). IL7 is thus widely used as a promoter of B- and T-cell differentiation *in vitro*.

Finally, an important notion of cytokine signaling is cytokine *synergy*, as many cytokine signaling pathways have redundant and additive functions. As an example, PDGFR family member flt-3 has been shown to synergize with IL7Rα in regulating lymphoid development. Flt3 is expressed on early lymphoid progenitors (ELPs), common lymphoid progenitors (CLPs) and early B- and T-cell progenitors (Igarashi et al., 2002; Rasko et al., 1995; Sitnicka et al., 2002). Mice deficient in flt3 or its ligand (flt3-L), have near normal levels of mature B-cells and T-cells, but have largely reduced early B- and T-cell progenitors (McKenna et al., 2000; Sitnicka et al., 2002). However, mice deficient in both flt3-ligand and IL7Rα exhibit a complete block of all stages of adult and fetal B-cell development as well as extensive reductions in fetal and postnatal thymic progenitors that result in a loss of active thymopoiesis in adult mice (Sitnicka et al., 2003; Sitnicka et al., 2007). Thus, the combined phenotype of loss of flt3 and IL7R signaling is more drastic than the phenotypes of either one, revealing synergy between these signaling pathways.

### 5.3.2. CELL-INTRINSIC REGULATORY MECHANISMS

Having briefly outlined some important cell-extrinsic regulatory mechanisms mediated through cell-niche interactions and soluble factors such as cytokines we are now ready to enter the hematopoietic cell nucleus and discuss the cell-intrinsic fate decision machinery. For the purposes of this text, this section will be broadly divided into the functions of key hematopoietic transcription factors and a brief introduction to epigenetic regulation of transcription. Not discussed in detail are regulators of cell-cycle and apoptosis (programmed cell-death), which despite their important roles in HSC homeostasis fall largely outside of the scope of this thesis (Kondo et al., 2003; Teitell and Mikkola, 2006).

#### 5.3.2.1. TRANSCRIPTIONAL REGULATION OF HSCS

Gene regulatory programs are governed by transcription factors (TFs), which are proteins with sequence-specific DNA-binding activity that recruit co-factors and/or other TFs and initiate transcription of specific genes. Of note, TF recruitment of histone acetyltransferases (HATs) or histone deacetylases (HDACs) is associated with transcriptional activation or repression, respectively, though processes described below. Not surprisingly, TFs play a major role in all stages of hematopoiesis, from commitment to the hematopoietic lineage, to emergence and maturation of HSCs and finally lineage choice (Orkin, 2000; Sieweke and Graf, 1998; Teitell and Mikkola, 2006). As suggested previously, transcriptional dysregulation of hematopoiesis often leads to leukemia, indicating not only the potency but also the fine balance between a physiological or pathological action of a certain transcription factor. In fact, many genes determined as essential regulatory factors in hematopoiesis were first identified from chromosomal translocations or as being misexpressed in human leukemias.

One such transcription factor, as implied by its name, is stem cell leukemia/T-cell acute leukemia 1 (Scl/Tal1) whose aberrant activation is found in over 60% of childhood and adult T-ALL cases (Bash et al., 1995). Scl is a member of the basic helix-loop-helix (bHLH) family of transcription factors, is known to heterodimerize with other bHLH factors including E12 and E47, and in hematopoietic cells is part of a large transcriptional complex that includes Gata-1 and LIM-only proteins Lmo2 and Ldb-1 (Hsu et al., 1991;

Wadman et al., 1997; Voronova and Lee, 1994). The genetic deactivation, or knockout, of Scl in mice leads to a total lack of blood cell formation in the embryo, and vascular and cardiac defects that together lead to embryonic death at embryonic day (E) 9.5 (Shivdasani et al., 1995; Visvader et al., 1998). Hence, it is evident that Scl is essential for establishment of hematopoiesis and therefore was postulated to be required also for proper HSC function. However, deactivation of Scl in the adult, using the Cre/LoxP recombination system which enables tissue- and/or time specific conditional deactivation of genes, showed that, surprisingly, Scl is dispensable for adult HSC function (Mikkola et al., 2003b). On the other hand, a requirement of Scl in erythroid and megakaryocyte differentiation was revealed, as inducible deactivation of Scl in adult mice leads to severe anemia and thrombocytopenia (Mikkola et al., 2003b). These data suggest distinct roles of Scl during different developmental stages and in different cellular contexts.

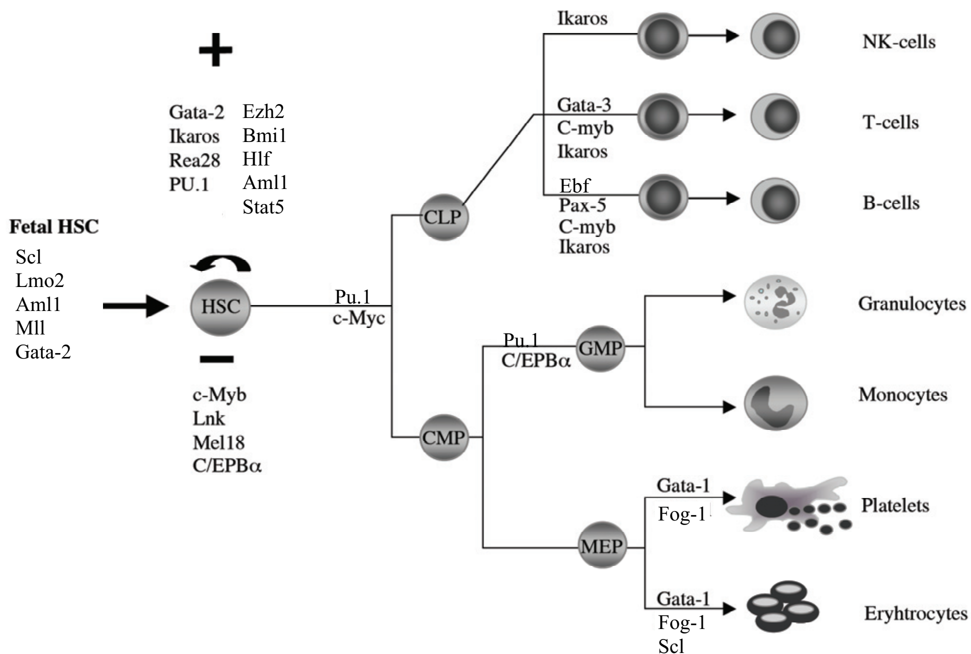


**Figure 3. Transcription factors regulating HSC fate and lineage commitment.** The picture shows TFs crucial for fetal hematopoiesis and either positively (+) or negatively (-) regulate adult HSC self-renewal. Furthermore, TFs important for different stages in the hematopoietic development are shown throughout the hierarchy.

In particular, Scl is critical for HSC generation in the fetus but not required for HSC maintenance in the adult. The requirements of Scl during HSC development and efforts to identify its target genes will be discussed in greater detail below (**Papers III** and **IV**).

Other transcription factors important for fetal hematopoiesis include Lmo2, Gata-2, Aml1/Runx1, and Mll (see Figure 3). Mice deficient in Lmo2, a binding partner of Scl, display largely similar defects in primitive and definitive hematopoiesis as Scl (Yamada et al., 1998). Loss of Gata-2 leads to proliferation defects of nascent HSCs, whereas loss of mixed leukemia factor (Mll) has no effect on primitive hematopoiesis or the emergence of definitive hematopoietic cells, but rather leads to loss of HSCs (Ernst et al., 2004; Tsai et al., 1994). A transcription factor displaying an intermediate phenotype is Runx1/Aml1, which has been shown to be essential for the development of all definitive but not primitive hematopoietic cells during ontogeny (North et al., 1999; Okuda et al., 1996). Interestingly, as Runx1 is specifically and continuously expressed in hematopoietic stem and progenitor cells, a mouse model where LacZ is knocked in to the runx1-locus (Runx1-LacZ) was engineered (North et al., 2002) and is a valuable tool for the localization of emerging hematopoietic cells during ontogeny, as we shall see in **Paper II**.

Once the hematopoietic system is established, transcription factors play a crucial role in lineage specification, as demonstrated by gain- or loss of function studies (Orkin, 2000). For our purposes, some key concepts will be briefly introduced. Firstly, transcription factors can be intuitively regarded as ‘master regulators’ that govern lineage programs. Such a transcription factor would have to be able to enforce its lineage program on cells of a different lineage. Notably, Gata-1, a TF essential for the development of both primitive and definitive erythropoiesis and megakaryopoiesis was shown to reprogram CLPs into progenitors with erythropoietic and megakaryopoietic potential (Fujiwara et al., 1996; Iwasaki et al., 2003; Pevny et al., 1995; Pevny et al., 1991). Secondly, the actual concentration of a TF has been shown to influence lineage choice, as exemplified by Pu.1, a TF important for myeloid maturation, whose high expression leads to macrophage differentiation whereas low expression leads to B-cell differentiation (DeKoter and Singh, 2000). It is likely that Pu.1 negatively regulates key

B-cell factors, and illustrates the concept of lineage antagonism: for a specific lineage program to be turned on others have to be shut down. Indeed, Pu.1 was shown to physically interact with and repress transactivation of key transcription factor Pax5, which is crucial for B-cell development (Nutt et al., 1999). Given the fact that HSCs are multipotential, step-wise lineage commitment is in fact as much a question of lineage restriction as lineage choice.

Lastly, the DNA itself or proteins associated with it act as an additional transcriptional regulatory entity, which is comprised of several layers of regulatory elements loosely termed 'epigenetics', and will be discussed next.

#### 5.3.2.2. EPIGENETICS

Historically, the meaning of the term epigenetics has had both currently misleading and confusing meanings, from "how genotypes give rise to phenotypes during development" to "heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996; Waddington, 1957). By definition, both terms are correct, as the Greek prefix *epi-* implies features that are 'on top of' or 'in addition to' genetics. For the sake of specificity, the term epigenetics will here be used to describe three aspects of chromatin organization: DNA methylation, genomic imprinting and histone modifications (Kouzarides, 2007). In recent years, the importance of epigenetic modifications for cell identity, cellular memory and fate choice has been made evident; however, currently very little is known about these processes.

The first of the epigenetic marks to be discovered was DNA methylation, which involves methylation of cytosine bases by DNA methyltransferases (DNMTs) within gene promoter regions (Methyl-CpG) and is associated with a repressed chromatin state and inhibition of gene expression (reviewed in (Klose and Bird, 2006)). It has been shown that a class of proteins (methyl-CpG-binding proteins, MBPs) that recognize and bind methyl-CpG can recruit transcriptional co-repressors, such as HDACs, as well as modify surrounding chromatin, thereby silencing transcription (Boyes and Bird, 1991; Jones et al., 1998). Thus, the level of promoter methylation, as can be assessed through bisulphite sequencing, can be used to predict availability of the promoter and transcriptional potential of a certain gene.

DNA is at any given moment, except during mitosis or meiosis, organized into units of nucleosomes which are each composed by a globular octamer of four core histones (H3, H4, H2A and H2B). It was shown that most amino-acids on the N-terminal tails of histones carry a vast variety and number of post-translational modifications, including acetylation, methylation and phosphorylation and are important epigenetic regulators (reviewed in (Kouzarides, 2007). Functionally, histone modifications act either on a global chromatin level to establish zones of DNA accessibility (euchromatin) or inaccessibility/protection (heterochromatin), or on DNA-based processes such as transcription of individual genes. Briefly, acetylation of specific lysines (e.g. H3K9Ac) correlates with transcriptional activation, whereas lysine methylation can be either activating (e.g. H3K4me) or repressing (e.g. H3K9me) depending on the lysine. Interestingly, a recent study shows that the differential expression of dimethylation or trimethylation on Histone 3 Lysine 4 (H3K4me2+/H3K4me3-) on lineage-specific promoters marks developmentally 'poised' genes, i.e. genes that are currently silenced but have the potential to become expressed (Orford et al., 2008). On the other hand, genes displaying both marks (me2+/me3+) are transcriptionally active (Orford et al., 2008). As such, a so-called "histone-code" emerges that together with DNA methylation patterns contributes to environmentally responsive gene expression programs.

Most of the knowledge of epigenetic modifications comes from studies in lower eukaryotes, such as the budding yeast. Importantly, also in vertebrate biology and within the hematopoietic system, HSC function has been shown to be linked to epigenetic modifications (Teitell and Mikkola, 2006). For example, the polycomb-group protein Ezh2 regulates HSC self-renewal capacity and was shown to bind DNMTs, thus controlling gene silencing (Vire et al., 2006). Also transcription factor Pu.1 mentioned above, and oncogene c-myc, an essential proliferation factor and inducer of apoptosis, have the ability to associate with DNMTs and direct gene repression at specific loci (Brenner et al., 2005; Kihara-Negishi et al., 2001). A further elucidation of both global and site-specific epigenetic marks will undoubtedly become a valuable tool in developmental hematopoiesis, as progenitors poised towards certain lineages, or emerging HSCs could be identified as they become specified. A valuable tool for site-specific epigenetic mark analysis that deserves mention is the ChIP-on-chip technique

(Buck and Lieb, 2004). Briefly, as the name implies, it combines traditional chromatin immunoprecipitation (ChIP) techniques with modern microarray technology and can be used for genome-wide analysis of TF-to-DNA binding. In its simplest form it can reveal whether, and on what gene promoters, a certain transcription factor binds in any given cellular context. In combination with interrogation of specific epigenetic marks using antibodies to modified histones, this technique is very informative as it tells not only if a transcription factor binds to a gene, but also whether the gene itself is activated or repressed (see **Paper IV**).

### 5.3.3. HEMATOPOIETIC FATE DETERMINATION

In summary, HSC fate decisions, such as quiescence, self-renewal and differentiation, are the outcome of a tightly orchestrated interplay between cell-extrinsic cues such as cytokine signalling and cell-cell interactions imparted from the niche, and cell-intrinsic regulatory programs in the nucleus governed by transcription factors that act in the context of epigenetic modifications. How the interpretation process in stem cells works is still not fully understood, but two models have been proposed (Enver et al., 1998; Metcalf, 1998). The first model suggests that lineage choice has already been decided intrinsically by chance (Enver et al., 1998). The role of the external cues imparted by the niche in this model would be to regulate the survival and expansion of a cell that is responsive for the cytokine message, while having no effect on a cell of a non-compatible fate, thus selecting for the responsive cell subset over the other. This model, due to the effect of chance in cell fate decision, is called the *stochastic* or permissive model.

On the other hand, a model that ascribes a more direct role of cell-extrinsic factors in the regulation of cellular decisions is the *instructive* model. In this model, transcriptional regulatory programs are induced as a result of external signaling (Metcalf, 1998). Hence, the stem or progenitor cells would be waiting for the appropriate extrinsic signals to stimulate or inhibit different cellular programs, such as to differentiate to certain lineages, undergo self-renewal etc. As experimental support exists for both models (e.g. (Fairbairn et al., 1993; Kondo et al., 2000)) they could both be correct, operating at different levels of hematopoiesis or even simultaneously. Theoretically, a stochastic decision could be reversed or nullified by an instructive signal, or due to a stochastic over-advantage, an

instructive signal is rendered insufficient. Pertaining to this thesis is the conundrum that the same transcription factor can direct a cell towards distinct fate outputs in different cellular systems (addressed in **Papers III, IV**). The advent of epigenetic research will add to our understanding of these processes and will allow not only a more accurate prediction of cell-fate determination but will likely open up for engineering the epigenome towards specific lineage or 'stemness' programs of choice.

## 5.4. FETAL HEMATOPOIETIC DEVELOPMENT

As mentioned earlier, in the adult HSCs reside primarily in the bone marrow. However, during fetal development, when the hematopoietic program is initiated and the first HSCs are formed, hematopoiesis occurs at multiple temporally and anatomically restricted sites (Mikkola and Orkin, 2006). These sites likely impart unique cues critical for the proper development of the emerging hematopoietic cells. The first step towards the initiation of hematopoiesis is the specification of a subset of cells belonging to the mesodermal germ layer, termed hemangioblast cells for their potential to give rise to both blood and endothelial cells (Choi et al., 1998; Huber et al., 2004; Robertson et al., 2000; Sabin, 1920). This process depends critically on a number of genes, such as Scl as mentioned above (Porcher et al., 1996; Shivdasani et al., 1995).

In the mouse, the first hemangioblasts arise in the primitive streak of the embryo and migrate to the *yolk sac* as early as embryonic day (E) 7.0-7.5 to form so called *blood islands* (Palis et al., 1999). Thus the yolk sac becomes the first site where blood is formed. However, this so called *primitive* hematopoiesis differs from adult-type, or *definitive* hematopoiesis in several ways. First of all, the erythrocytes being formed at this stage are nucleated, and express embryonic globins. Secondly, only these primitive erythrocytes and a small fraction of early macrophages are formed, compared to the many blood lineages in the adult (Palis et al., 1999). Finally, and importantly, the primitive wave of hematopoiesis occurs independently of HSCs, as indicated by the fact that loss of Runx1 or Mll leads to loss of definitive but not primitive hematopoiesis, as mentioned above. In fact, it is not until three days later at E10.5 that the first definitive HSCs can be found, as assessed by transplantation experiments. The main site where *de novo* generation of the

first HSCs occurs is called the aorta-gonad-mesonephros region (AGM) where rare HSCs bud out from so called hemogenic endothelium and into the vessel lumen (Cumano et al., 1996; de Bruijn et al., 2000; Godin et al., 1999; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Alternatively, these nascent HSCs could theoretically arise in the stroma below the endothelial lining and transverse into the lumen of the aorta. However, currently the hemogenic endothelium theory is the most favored one. Interestingly, the newly formed HSCs cannot be retained in the AGM and instead migrate to the fetal liver, (FL), which they seed starting at E11 – E11.5 (Kumaravelu et al., 2002). In the fetal liver, HSCs are able to expand exponentially and also undergo differentiation into multiple lineages, and thus the fetal liver serves as the main hematopoietic site until shortly before birth when hematopoiesis shifts to the bone marrow (Ema and Nakauchi, 2000). It is important to note that despite of the great capacity of the fetal liver niche to expand and differentiate HSCs, no *de novo* formation of HSCs takes place there. Thus, as previously hinted, HSCs and definitive progenitors from the AGM have been assumed to be responsible for the colonization of FL (Houssaint, 1981; Johnson and Moore, 1975). However, the exceptional increase of HSC numbers between E11.5 – E12.5 taken together with the length of the cell cycle, have raised the question whether also immature HSC precursors from the yolk sac, or yet another, unknown, site could possibly contribute to establishing the fetal liver HSC pool (Kumaravelu et al., 2002).

In the avian system there is an additional mesodermal appendage that is active in hematopoiesis, namely the allantois, which was shown to contain hemangioblasts and where *de novo* hematopoiesis occurs (Caprioli et al., 1998; Caprioli et al., 2001). In mice, the allantois, which develops from the epiblast, grows out as an extension of the posterior primitive streak into the exocoelomic cavity to fuse at E8-8.5 with the chorion, thus forming the chorio-allantoic portion of the placenta (Adamson et al., 2002; Downs, 2002; Downs and Gardner, 1995; Rossant and Cross, 2001). As mentioned, the allantois has a high vascular potential and, indeed, the fusion product soon gives rise to a complex vascular network of endothelial cell-lined vessels called the placental labyrinth. This vascular compartment allows for the exchange of gases and nutrients between the trophoblast-lined maternal blood spaces and the fetal blood system, thus ensuring survival for the fetus (Adamson et al., 2002).

In addition to these well established roles of the placenta there were preliminary findings reporting hematopoietic potential in the placenta (Till and McCulloch, 1961) (Melchers, 1979). These initial studies were not followed up until our collaborators assessed hematopoietic potential in the placenta by *in vitro* cultures (Alvarez-Silva et al., 2003). However, since hematopoietic progenitors arise prior to and independently from the genesis of definitive HSCs, it was important to define whether the placenta supports the development of definitive HSCs (see **Paper I**), and whether hematopoietic cells in the placenta are generated there *de novo* (**Paper II**).

# 6. AIMS OF THE PRESENT STUDY

The general questions that inspired this study were: How are hematopoietic stem cells formed and in what cellular niches does this happen? What are the gene transcriptional mechanisms that govern stem cell development and differentiation?

## 6.1. SPECIFIC AIMS

To address these questions the specific aims of my thesis are:

1) To investigate whether the placenta is a hematopoietic organ (I)
2) To investigate whether HSCs in the placenta are generated *de novo* and, if so, in what microenvironmental niches HSC generation and maturation occurs. (II)
3) To define the temporal window of Scl requirement for HSC formation (III)
4) To identify and characterize target genes of Scl in hematopoiesis (IV)

# 7. MATERIALS AND METHODS

A summary of the methods used in the work presented in this thesis can be found in Table 1. The details of each method are presented in the original publications.

| Methods Used | Papers |
|---|---|
| Dissection of fetal hematopoietic and other organs | I-IV |
| Preparation of single-cell suspension of organs for further analysis | I-IV |
| In vitro stroma-free (methylcellulose cultures) for assessment of hematopoietic clonogenic potential | I-IV |
| In vitro stroma co-culture (on OP9-GFP, OP9-DL1 assays for assessment of hematopoietic potential | II-IV |
| In vivo transplantation assays for assessment of HSC activity and numbers | I, III, IV |
| Multi-color flow-cytometry (FACS) for phenotypical identification of hematopoietic and other populations | I-IV |
| Multi-color FACS cell sorting for functional assessment of hematopoietic populations | I-II, IV |
| Immunohistochemical analysis of tissues | II |
| Microarray (Affymetrix) analysis for genome-wide gene identification | IV |
| PCR and real-time Q-PCR for analysis of gene expression | I-IV |
| Complete blood count (CBC) test for blood composition analysis | IV |
| Chromatin immunoprecipitation on chip (ChIP on chip) for genome-wide identification of protein to DNA binding | IV |

**Table 1**. Outline of methods used in the work presented in this thesis.

# 8. RESULTS AND DISCUSSION

## 8.1. PLACENTA IS A NOVEL HEMATOPOIETIC ORGAN THAT HARBORS A LARGE POOL OF HSCS DURING MIDGESTATION (I)

Hematopoietic stem cells emerge during fetal development in temporally and anatomically restricted sites. Although the placenta was not generally considered as a hematopoietic organ, a study by the group of Dieterlen-Liévre suggested the presence of hematopoietic progenitors in midgestation placenta (Alvarez-Silva et al., 2003). Thus, the aim of this study was to investigate the possible involvement of the placenta in the genesis, expansion and/or differentiation of hematopoietic stem cells during fetal development.

In order to accomplish this, we performed a spatial and temporal analysis of HSC activity, using the limiting dilution long-term competitive repopulation assay (Harrison, 1980; Jones et al., 1996), the most reliable and stringent assay for detection and quantification of HSCs. Briefly, hematopoietic tissues including the placenta, fetal liver, yolk sac, the AGM, fetal blood and others, from E10.5 – E18.5 mouse embryos were dissected. Single-cell suspensions were performed by treatment with collagenase. Clonogenic progenitors were assayed on *in vitro* methylcellulose colony forming assays, and hematopoietic stem cell activity was assessed by transplantation of limiting dilutions of fetal tissue samples into lethally irradiated recipients. The use of the congenic CD45 model allowed us to distinguish between fetal and maternal cells, as well as support, endogenous and donor cells in the transplantation setup. Blood from transplanted mice was analyzed for multilineage reconstitution by multi-color FACS at 4 – 6, 10 – 12 weeks and 5 – 12 months, and serial transplantations of primary recipient BM was performed at 10 – 12 weeks or 5 – 8 months; secondary recipients were analyzed the same way.

It has been shown that the first cells capable of reconstituting adult hematopoiesis are found in the AGM region as early as E10.5 – E11.0, albeit at very low levels (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Interestingly, we could detect rare (0.1 RUs/ee; repopulating units/embryo equivalent)

transplantable hematopoietic cells in the placenta already at E10.5, or 33-36 somite pairs (sp), as well as in the caudal half of the embryo, which includes the P-Sp, and in the yolk sac, but never in the rostral half of the embryo, decidua or blood. However, hematopoietic reconstitution at this stage was very low (>1 – 5%) and often transient, even upon transplantation of 3ee of each tissue, indicating the rarity and/or immaturity of these cells throughout the conceptus. In support of the latter, it has been shown that cells incapable of hematopoietic reconstitution of normal adult mice, could still possess robust hematopoietic potential when transplanted into lymphoid deficient, e.g. Rag2$^{-/-}$ γc$^{-/-}$ (Cumano et al., 2001), or newborn (Yoder et al., 1996) mice. These studies suggest that certain maturation events, such as upregulation of MHC class I antigens (Cumano et al., 2001), need to occur in order for these cells to engraft and survive in an adult wild-type host.

At E11.0 (37-40 sp) the first cells capable of sustained multilineage high-level (>10%) reconstitution were found in the AGM as well as in the placenta, which contained roughly half of all long-term repopulating units (LT-RUs) in the embryo. Importantly, although the circulating blood and fetal liver rudiment contained abundant clonogenic progenitors, no HSCs were present in circulation or fetal liver until 12 hours later, at E11.5 (41-45 sp), consistent with other reports (Kumaravelu et al., 2002). During this period of time, both numbers and reconstitution levels increased for HSCs in all hematopoietic sites except in the blood where no >1% reconstitution was noted. Taken together, these data show that onset of HSC activity during fetal development occurs not only in the AGM and yolk sac, as previously thought, but also coincides in the placenta. Importantly, HSC activity in the placenta precedes the appearance of HSCs in the fetal liver and circulation.

In spite of the similar temporal onset and level of hematopoietic activity in the AGM and the placenta, the placenta HSC pool was found to expand drastically after E11.5 to contain ~50 LT-RUs by E12.5, in striking contrast to the AGM and yolk sac which increased from ~2 to 3 RUs during the same 24-hour period (Figure 4A). The placental HSC expansion is similar in magnitude to that in the fetal liver (~25x and ~30x, respectively), which by E12.5 was found to contain ~100 RUs/ee. Importantly, in contrast to the fetal liver where HSC expansion was paralleled with a major (~100x) expansion

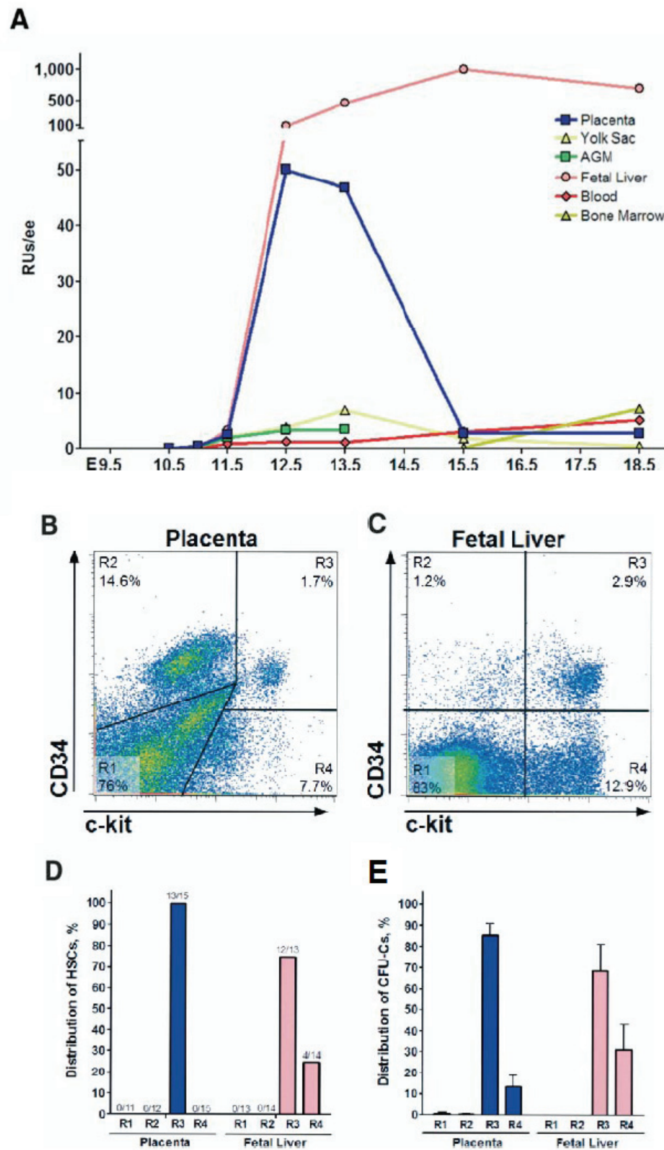


**Figure 4. Developmental Kinetics and Phenotype of Placental HSCs and Clonogenic Progenitors.** (A) Number of total LT-RUs in fetal hematopoietic sites representing ability to repopulate peripheral blood >10–12 weeks after transplantation. (B-C) Surface phenotype analysis of placental and fetal liver HSCs and progenitors by flow cytometry for expression of CD34 and c-kit. (D-E) Distribution of (D) HSCs and (E) clonogenic progenitors between the four isolated populations indicated in (B) and (C) (n = 3 experiments). The numbers in (D) indicate the number of engrafted mice/total recipients transplanted with 0.05–0.3 ee from each population.

of clonogenic progenitors, only a very modest (~2x) expansion of progenitors took place in the placenta. These results show not only that the placenta, in contrast to the yolk sac and AGM, can support the expansion of HSCs, but also suggest that the placental microenvironment differs from the fetal liver in that it can maintain an undifferentiated state of HSCs. Furthermore, we found that the placental HSC pool, which was maintained by E13.5, was reduced drastically to E15.5 and remained very low until E18.5 and birth, in contrast to the fetal liver in which HSCs continued to expand. Interestingly, the number of circulating HSCs also increased steadily and surpassed the number of HSC in placenta at E15.5, showing that the kinetics of HSC activity in the placenta is unique and doesn't merely represent HSCs in fetal circulation. In agreement with other reports (Christensen et al., 2004), we observed a major increase of clonogenic progenitors in blood around E15.5, while progenitors in the placenta were instead diminishing.

We next went on to define the immunophenotype of placental HSCs. Adult LT-HSCs are found within the lineage-negative ($Lin^-$) $c\text{-}kit^{hi}sca\text{-}1^+$ (LSK) compartment, where expression of CD34 marks short-term HSCs. However, fetal HSCs express CD34 indicating a non-quiescent active cycling state (Sanchez et al., 1996). In accordance with this, we found that all HSCs and most clonogenic progenitors in the placenta were $CD34^+c\text{-}kit^{hi}$, whereas some progenitors were $ckit^+$-single positive (Figure 4B-D). In comparison to the placenta, some fetal liver HSCs and a larger fraction of progenitors also resided in the relatively more abundant $c\text{-}kit^+$-single positive fraction. Our data have been confirmed by others (Ottersbach and Dzierzak, 2005). In that study, a sca-1 transgenic mouse (Ly6A-GFP) was used to identify the presence of $sca\text{-}1^+$ cells in the placenta, which in a previous study indicated all HSC activity in the AGM (de Bruijn et al., 2002). Although sca-1 expression is not confined solely to hematopoietic lineages in the placenta, but is rather widely expressed in endothelial cells and maternal decidua, $GFP^+$ cells in placenta were evident as early as E9.5 and increased towards E12.5 (Ottersbach and Dzierzak, 2005). Co-expression analysis revealed that most GFP+ hematopoietic activity was found within the $c\text{-}kit^+CD34^+$ subset, which is well in line with our own results.

In summary, we identified the placenta as a novel fetal niche for hematopoietic stem cells. The placenta displays unique developmental kinetics of definitive HSCs and supports the presence of a considerable pool of HSCs during midgestation.

## 8.2. HEMATOPOIETIC STEM CELLS EMERGE IN THE PLACENTAL VASCULATURE IN THE ABSENCE OF CIRCULATION (II)

The findings presented in **Paper I** nominate the placenta as an important hematopoietic organ, unique in its capacity to sustain a large pool of HSCs while segregating them from signals that promote differentiation. However, as discussed above, it was not possible to determine whether the placenta is capable of producing HSCs *de novo* or whether it functions solely as a niche for the maturation and expansion of HSCs originating from other sites. Defining the origin of HSCs *in vivo* has been complicated by mainly two factors. Firstly, as developing HSCs prior to E10.5 are unable to engraft in lethally irradiated adult bone marrow, an assessment of HSC potential through standard *in vivo* transplantation assays is not possible, and the culture requirements of emerging HSCs remain undefined. Secondly, as blood circulation is initiated already at E8.5, theoretically any cell forming in any one site could migrate via the bloodstream to any other site, and this has been a major obstacle for developmental studies. In order to address this question we utilized the Runx1-LacZ knock-in (North et al., 2002) and Ncx1 knockout (Koushik et al., 2001) mouse models to find out whether HSCs are formed in the placenta, and, in that case, in what specific niches HSC emergence, maturation and expansion takes place.

As mentioned earlier, expression of LacZ in $Runx1^{LacZ/+}$ and $Runx1^{LacZ/LacZ}$ embryos was used to visualize sites of definitive hematopoiesis in the placenta. Importantly, since $Runx1^{LacZ/LacZ}$ mice have both Runx1 alleles targeted and thus lack expression of Runx1, definitive hematopoietic cells never develop. However, cells that would have developed into hematopoietic cells and therefore had activated the Runx1 locus can still be visualized by LacZ. Thus, we were able to discern between sites of emergence (LacZ-positive cells (LacZ+) in both $Runx1^{LacZ/+}$ and $Runx1^{LacZ/LacZ}$ embryos) and sites that contain nascent or migrated definitive hematopoietic cells (LacZ+ only in $Runx1^{LacZ/+}$ embryos). This was confirmed by analysis of sections of AGM, known to generate HSCs *de novo*, and fetal liver which contains blood cells exclusively from other sources.

Interestingly, LacZ+ cells where found in the chorioallantoic vessel walls of $Runx1^{LacZ/+}$ placentas (Figure 5B). As Runx1 expression is not restricted to HSCs, the hematopoietic identity of these cells was confirmed by co-localization with CD41 which is known to be a marker for nascent HSCs and progenitors (Corbel and Salaun, 2002; Mikkola et al., 2003a). Importantly, similar LacZ+ cells were observed also in $Runx1^{LacZ/LacZ}$ placentas, indicating that Runx1-positive cells emerge *de novo* in the walls of chorioallantoic vessels (Figure 5C). In addition, other LacZ+ cell types were found in the chorioallantoic mesenchyme of $Runx1^{LacZ/+}$ and $Runx1^{LacZ/LacZ}$ placentas, such as cuboidal endoderm derived cells forming clusters known as Crypts of Duval. Moreover, round LacZ+ cells reminiscent of HSCs were found in the lumen of the placental labyrinth only in $Runx1^{LacZ/+}$ but not $Runx1^{LacZ/LacZ}$ placentas (Figures 5D-E). These data nominate the chorioallantoic vessels and possibly the chorioallantoic mesenchyme as sites of HSC emergence and the labyrinthine vessels as sites of residence, maturation or expansion of ready-made HSCs. In addition the data suggest that Runx1+ endodermal structures in placenta could play a role in the process of HSC emergence. This notion could be supported by the fact that Crypts of Duval express an important signaling molecule, Indian hedgehog (Ihh), and surrounding mesenchymal cells its receptor Patched-1 (Ptch1), thus forming a functional signaling pathway which in yolk sac and avian allantois is important for formation of blood from mesoderm (Baron, 2003).

Next, to investigate whether the placental microenvironment promotes proliferation of hematopoietic cells, we performed co-staining of $Runx1^{LacZ/+}$ placental sections with a mitosis marker (PH3S10). We found that a large proportion of the LacZ+ cells in the lumen of the labyrinth vessels were mitotically active whereas only few LacZ+ cells were PH3S10+ in the chorioallantoic mesenchyme. This finding suggested that the placental labyrinth provides a microenvironment where hematopoietic cells actively proliferate, rather than serving as a passive retainer of blood cells. The unique microenvironmental characteristics of the labyrinth and its surrounding cell types, including trophoblast cells not found anywhere else in the embryo are currently under closer study in our lab.

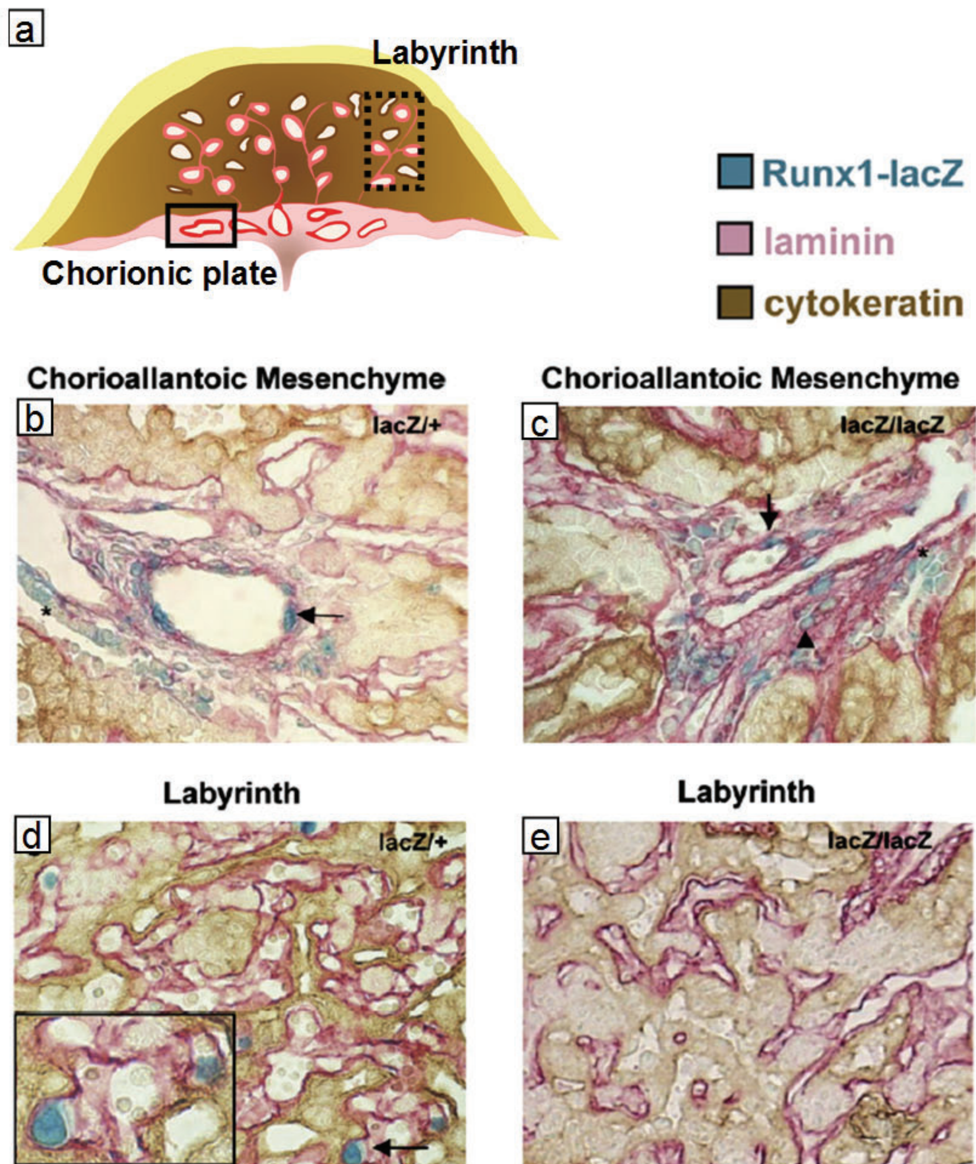


**Figure 5. Runx1 Expression Marks the Sites of Definitive Hematopoiesis in the Placenta**. (a) Schematic of cross-section of placenta. (b-e) Paraffin sections stained with β-galactosidase (lacZ, blue), laminin (mesodermal derivatives, red), and cytokeratin (trophoblast and epithelial cells, brown**).** (b-c) Chorioallantoic vessels of Runx1$^{lacZ/+}$ and Runx1$^{lacZ/lacZ}$ placentas harbor lacZ+ candidate HSCs (arrow) within the vessel walls. Asterisk (*), Crypt of Duval. (d) Labyrinth vessels of the Runx1$^{lacZ/+}$ placenta harbored a number of lacZ+ cells. (e) However, Runx1lacZ/lacZ labyrinth vessels never contained lacZ+ cells.

Finally, we utilized the Ncx1 knockout mouse model in order to answer whether the HSCs found in the placenta are generated *in situ* or are imported via circulation. $Ncx1^{-/-}$ embryos have no heartbeat due to a defect in the sodium-calcium exchange pump 1, and trafficking of hematopoietic cells via the bloodstream is abolished (Koushik et al., 2001). Interestingly $CD41^{+}ckit^{+}$ cells were found in $Ncx1^{-/-}$ placentas (Figure 6A). Due to the

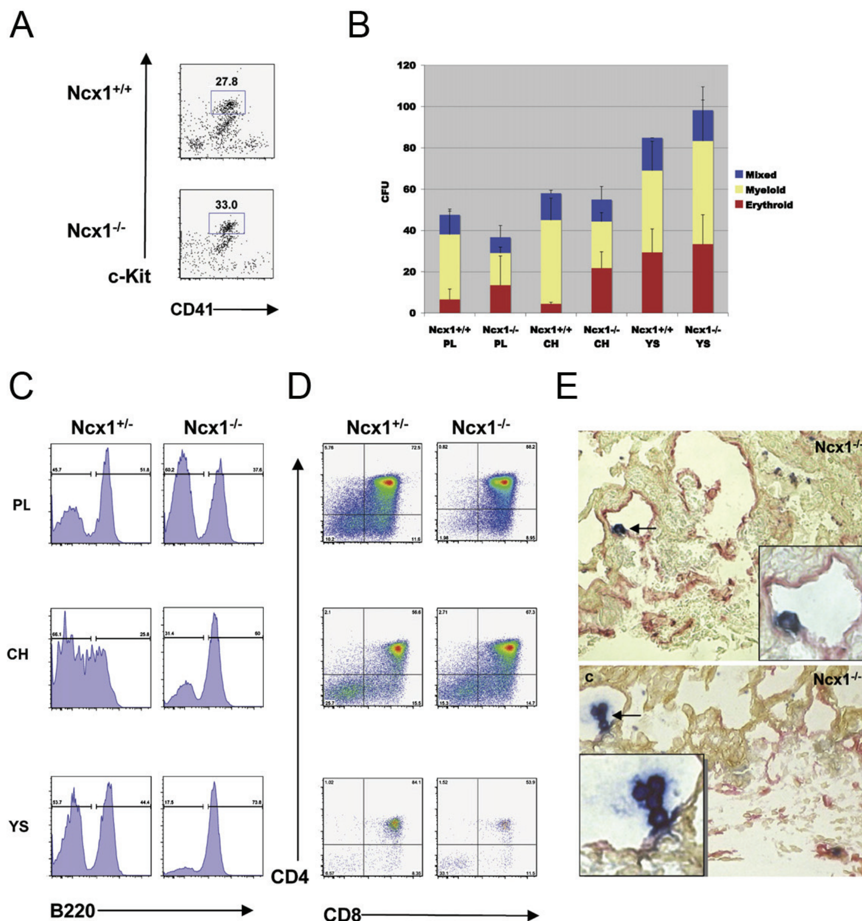


**Figure 6. Definitive Hematopoietic Cells Derived from $Ncx1^{-/-}$ Placentas Have Myelo-Erythroid and Lymphoid Potential.** (A) Definitive hematopoietic progenitors, marked by expression c-kit and CD41 double positive progenitors were present in $Ncx1^{-/-}$ placentas. (B-D) Placenta, yolk sac, and caudal half of the embryos were dissected, and the tissue explants were cultured on OP9 and OP9-DL1 stroma. Myelo-erythroid (B) and B-lymphoid potential (C) was assessed by FACS after plating of the cells from the OP9 stroma on methylcellulose culture, whereas T lymphoid potential was assessed directly on OP9-DL1 stroma (D). (E) Sections of $Ncx1^{-/-}$ placenta stained with CD41 revealing presence of budding hematopoietic cells from chorioallantoic vessels.

loss of heart function Ncx1$^{-/-}$ embryos do not survive past E10.5, meaning that the function of HSCs cannot be studied through *in vivo* transplantation experiments, as described earlier. However, by culturing fetal Ncx1$^{-/-}$ tissue explants *in vitro* on OP9 and OP9-DL1 stroma and methylcellulose cultures, we could show that cells indicative of HSCs with the potential to give rise to definitive myelo-erythroid, B-lymphoid and T-lymphoid progeny were present in Ncx1$^{-/-}$ placenta (Figure 6B-D). Of note, similar to the aorta where hemogenic clusters expressing Runx1 are observed (North et al., 1999), clusters of round hematopoietic cells expressing CD41 were observed in the chorio-allantoic vasculature of E10.5 Ncx$^{-/-}$ placenta (Figure 6E). Thus, our study suggests that in addition to the AGM and the yolk sac, the placenta is capable of *de novo* generation of HSCs (Rhodes et al., 2008).

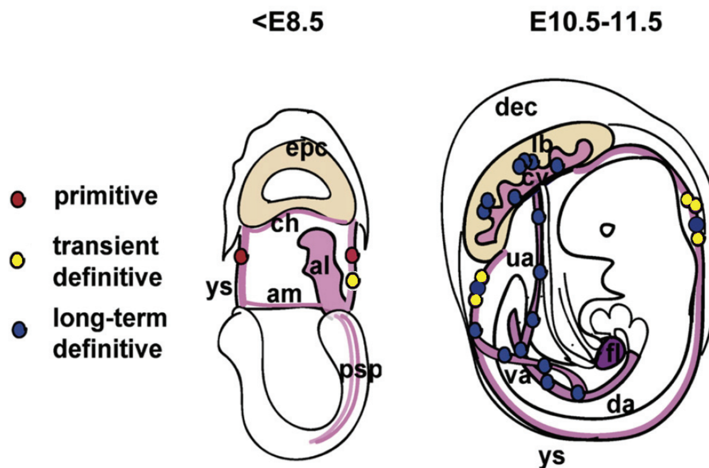


**Figure 7. Revised model of fetal HSC development.** Fetal hematopoiesis can be divided into at least three different waves. The first waves of hematopoiesis occur in the yolk sac: the primitive wave, which gives rise to primitive erythroblasts, and the transient definitive wave, which generates myelo-erythroid progenitors that first colonize the liver. In contrast, the emergence of adult repopulating HSCs occurs in multiple sites yet is confined to the major blood vessels: the dorsal aorta, the adjacent vitelline, umbilical vessels, and as shown in this study, the large vessels of the placenta. Subsequently, HSCs generated in the placenta and possibly the AGM and yolk sac are directed via blood flow to the placental labyrinth, which may provide a unique environment for HSC expansion/maturation prior to seeding of the fetal liver. Pink marks mesodermal tissues with hematopoietic potential (al, allantois; ch, chorion; ys, yolk sac; and p-sp, para-aortic splanchopleura) and their derivatives (ua, umbilical artery; cv, chorio-allantoic vessels; va, vitelline artery; and da, dorsal aorta), and brown marks trophoectodermal tissues (epc, ectoplacental cone and lb, placental labyrinth). Dec, maternal decidua and fl, fetal liver.

In summary we have identified in **Papers I** and **II** an important, previously unknown site for HSC development, and therefore the current model of fetal HSC development needs to be revised (see Figure 7). We propose that the placenta is required for the *de novo* generation and initial expansion of an undifferentiated HSC pool. The placental HSC pool is transient, as HSCs in the placenta diminish in numbers probably due to migration to the fetal liver and other sites, such as the fetal bone marrow. In contrast to the fetal liver, the placenta HSC niche does not serve as a site for myeloerythroid or lymphoid differentiation. As such, it would be of great general interest to further characterize the stem-cell promoting properties of the placenta microenvironment. It is worth mentioning that ongoing work in our laboratory has led to the establishment of stroma cell lines from human first trimester placenta that support the remarkable expansion of human $CD34^+CD38^-CD90^+$ cells (i.e. the most immature HSC phenotype in human) *ex vivo*.

## 8.3. SCL/TAL1 IS REQUIRED ONLY TRANSIENTLY FOR HEMATOPOIETIC SPECIFICATION (III)

As described above, the stem cell-leukemia gene (Scl/Tal1) is essential for the initiation of hematopoiesis and loss of Scl leads to total lack of all blood lineages in the embryo (Shivdasani et al., 1995). As Scl is and remains expressed in fetal and adult HSCs it was postulated that the continuous presence of Scl was required to maintain stem cell function in HSCs. However, the inducible deactivation of Scl in the adult did not lead to loss of HSC numbers or defects in HSC engraftment and long-term repopulating ability upon transplantation to irradiated hosts (Mikkola et al., 2003b). On the other hand, erythroid and megakaryocyte progenitors in the bone marrow and platelets in the blood were severely affected (Hall et al., 2003; Mikkola et al., 2003b). Thus, these findings showed that Scl is dispensable for HSC maintenance but remains critical for erythroid and megakaryocyte development in the adult BM, yet did not reveal at what stage HSC development becomes Scl-independent.

In order to better understand the roles of Scl in the establishment of the hematopoietic system, we sought to define the temporal window of Scl requirement during fetal HSC development. To address this question, we crossed a conditionally targeted $Scl^{fl/fl}$ mouse strain with a Tie2-Cre mouse strain (Kisanuki et al., 2001; Mikkola et al., 2003b). Tie2 (Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains–2) is the receptor for angiopoietin 1 (Ang1), an important signaling factor for vasculogenesis. As such, expression of Tie2 marks endothelial cells in hemogenic and vasculogenic sites in the embryo, and has been shown to be expressed on HSCs (Hsu et al., 2000; Schlaeger et al., 1997). Upon crossing of the $Scl^{fl/fl}$ and Tie2-Cre mouse strains, expression of Cre recombinase is driven by the Tie-promoter and leads to excision and deactivation of the conditionally targeted Scl locus in endothelial and hematopoietic stem cells.

We could show that expression of Scl was completely abolished in hematopoietic cells of the fetal liver by embryonic day E12.5. In contrast to $Scl^{-/-}$ embryos that die before E9.5 due to absence of blood cells and disrupted vasculature, $Scl^{fl/fl}$ x Tie2Cre embryos survived until E13.5-14.5 and had circulating primitive erythrocytes. However,

morphological and FACS analysis of the cellular composition of E12.5-13.5 peripheral blood and fetal liver exposed a maturation defect of primitive erythroid cells at the level of $CD71^{+}ter119^{+}$ basophilic erythroblasts (Figure 8A). Furthermore, acetylcholine-esterase (AchE) staining of E12.5 fetal liver cultures revealed a severe reduction of mature megakaryocytes (Figure 8B). Therefore, as in the adult, we concluded that Scl is critically required for fetal erythroid and megakaryocyte development.

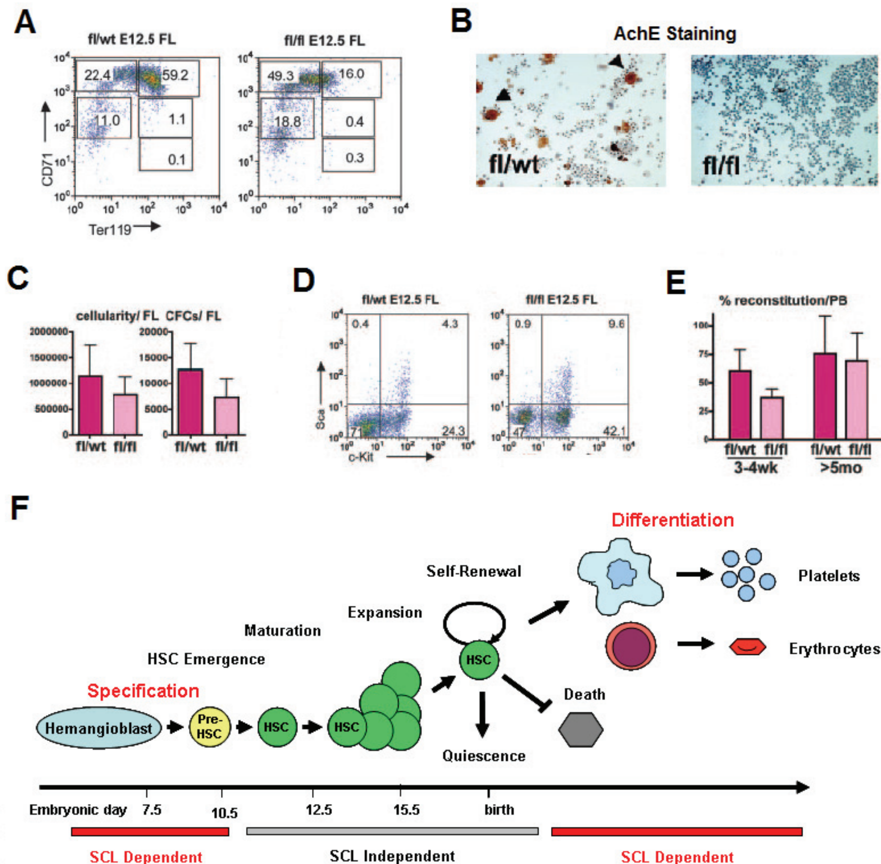


**Figure 8. Effect of Tie2Cre-mediated deletion of Scl gene in the development and function of primitive and definitive hematopoietic cells.** (A) FACS analysis of CD71 and ter119 expression on primitive erythrocytes from E12.5 fetal liver (FL). (B) Acetylcholine-esterase staining of E12.5 FL. (C) Total cellularity and colony-forming cells (CFCs) in E12.5 fetal liver. (D) FACS analysis of c-kit and sca-1 expression on definitive progenitor populations in fetal liver. (E) Short- and long term analysis of competitive repopulation after transplantation of fetal liver derived HSCs into adult hosts. (F) Model of Scl requirement during hematopoiesis. Scl is essential for specification of hematopoiesis but not for HSC emergence, maturation, expansion or self-renewal. However, during fetal and adult hematopoiesis, Scl is critical for proper megakaryocyte and erythroid development.

To investigate whether early loss of Scl affected the formation and function of definitive hematopoietic progenitors, we assessed the colony-forming unit (CFU) capacity of fetal liver on methylcellulose culture assays. Of note, the number of colony-forming progenitors in Tie2Cre$^{+}$Scl$^{fl/fl}$ fetal liver as well as the total feta liver cellularity were only slightly reduced (Figure 8C). Furthermore, FACS analysis of known progenitor and stem cell markers revealed the presence of c-kit$^{+}$sca-1$^{+}$ and ckit$^{+}$sca1$^{-}$ cells, indicating that definitive progenitors form and display no proliferation or maturation defects in the absence of Scl (Figure 8D). Finally, to assess whether also functional adult-type HSCs were present, fetal liver cells were transplanted into irradiated adult hosts. Importantly, we found that Tie2Cre$^{+}$Scl$^{fl/fl}$ cells were capable of short (3-4 wks) and long-term (>5 months) reconstitution of the recipient blood system arguing that ongoing expression of Scl is not required for function of fetal liver LT-HSCs (Figure 8E).

Our data thus suggest that the developmental time window during which cells require Scl in order to establish the hematopoietic program is prior to and overlapping with the onset of Tie2 expression. Importantly, in contrast to its transient requirement in hematopoietic specification, Scl remains essential for proper development of erythroid and megakaryocytic cells throughout embryonic, fetal, and adult hematopoiesis (Figure 8F). In summary these findings highlight that, once specified, the hematopoietic fate is stable and is maintained through yet undefined mechanisms that are distinct from those required for the initial fate choice.

## 8.4. MEF2C IS A LINEAGE-RESTRICTED TARGET OF SCL/TAL1 AND REGULATES MEGAKARYOPOIESIS AND B-CELL HOMEOSTASIS. (IV)

As shown by our previous study (**Paper III**), in spite of the pivotal role of Scl in the initiation of the blood program, Scl becomes dispensable for further development and function of HSCs shortly after hematopoietic specification (Schlaeger et al., 2005). Although the entire hematopoietic transcriptional program can be regarded as being downstream of Scl, the direct target genes of Scl during hematopoietic specification and differentiation are not known. Neither has it been defined whether Scl regulates shared or unique gene expression programs depending on its cellular context. Our results suggest that Scl utilizes different mechanisms for regulating at least a subset of target genes during hematopoietic specification vs. lineage differentiation (Schlaeger 2005, Mikkola 2003).

To understand how the same transcription factor regulates different developmental fates within the hematopoietic hierarchy, we sought to identify Scl target genes in the different cell types. We thus generated hematopoietic progenitor cell lines from E12.5 $Scl^{fl/fl}$ fetal liver cells by immortalization with Hox11 retrovirus (Keller et al., 1998). The cell lines were cultured in the presence of IL-3 and selected for megakaryocyte specific activity of acetylcholine esterase (AchE). Scl expression was then abolished ($Scl^{\Delta/\Delta}$ cell line) and subsequently rescued ($Scl^{\Delta/\Delta}$/+Scl cell line) through transduction with Cre-GFP and Scl retroviruses, respectively, thus establishing serial cell lines. Importantly, the $Scl^{\Delta/\Delta}$ cell line exhibited an arrest in megakaryocyte differentiation as demonstrated by loss of $AchE^+$ cells, whereas AchE-activity was rescued in the $Scl^{\Delta/\Delta}$/+Scl cell line, confirming that the cell lines remained responsive to megakaryocytic induction in the presence of Scl. The differential gene expression between the three cell lines was analyzed by Affymetrix microarray after culture with IL3 and TPO for 5 days and enabled identification of Scl-dependent genes in the megakaryocytic lineage.

Among the top downregulated genes in the absence of Scl were known megakaryocytic, erythroid or other hematopoietic or vascular cell genes, as well as miscellaneous genes and as yet unknown ESTs/cDNAs not previously linked to blood development. One of the top downregulated genes was the transcription factor Mef2C. Importantly, we could show by ChIP-on-chip analysis on the megakaryocytic cell line L8057 that Scl binds to the Mef2C promoter directly. Moreover, binding of Scl was associated with acetylation of histone 3 lysine 9 (H3K9Ac), which as mentioned earlier is an indicator of gene activation. Importantly, in erythroid cells, where Mef2C is not expressed, no significant levels of Scl binding or H3K9Ac were observed on the promoter of Mef2C. This suggests that Scl uses distinct target genes in different cell types to regulate differentiation. Thus, we conclude that Mef2C is a novel direct target gene of Scl in megakaryocytes.

Mef2C (myocyte enhancer factor 2 C) is a member of the MADS-box transcription factor family, which were originally discovered as important regulators of skeletal, smooth and cardiac muscle development (Black and Olson, 1998; Lin et al., 1998). Homozygous deletion of Mef2C was reported to result in cardiac and vascular defects that together lead to early embryonic lethality at E9.5 (Black and Olson, 1998; Lin et al., 1998). Until recently, a role of Mef2C in hematopoiesis was unknown; however, implications of Mef2C in various types of leukemias, and expression of Mef2C in hematopoietic populations suggested a role of Mef2C also during physiological hematopoietic processes (Fuhrken et al., 2008; Krivtsov et al., 2006).

We therefore sought to investigate a role of Mef2C in hematopoiesis. First, we found that Mef2C is downstream of Scl also during embryonic development, through Q-PCR analysis of Mef2C mRNA expression in fetal hematopoietic and vascular tissues of E9.0 $Scl^{-/-}$ embryos. Mef2C expression was reduced in the yolk sac and caudal half of the embryo which contains the para-aortic splanchnopleura (p-Sp), a site of hematopoietic stem cell emergence and somite development (Cumano et al., 2001; Godin et al., 1995). On the other hand, expression of Mef2C in the heart was unaltered compared to controls. Taken together, these data imply that Mef2C is either directly regulated by Scl within the same cell, or that cells in which Mef2C would be expressed are reduced by loss of Scl.

However, loss of Mef2C in conventional knockout Mef2C$^{-/-}$ embryos did not recapitulate the profound hematopoietic defects observed in Scl$^{-/-}$ embryos, namely total loss of blood cells (Shivdasani et al., 1995; Visvader et al., 1998). Interestingly, although the yolk sac vasculature appeared perturbed, the yolk sacs of Mef2C$^{-/-}$ embryos contained pools of blood (Figure 9A), suggesting that Mef2C is not essential for primitive erythropoiesis, which was confirmed by FACS analysis of yolk sac for presence of CD71$^{+}$ and ter119$^{+}$ erythroblasts.

To assess a role of Mef2C in establishing definitive hematopoiesis we analyzed the *in vitro* hematopoietic potential of fetal hematopoietic tissues of Mef2C$^{-/-}$ E8.5-9.5 embryos. Multi-lineage definitive clonogenic were present at levels comparable to controls in the placenta and yolk sac of *Mef2C*$^{-/-}$ embryos, whereas only very rare functional hematopoietic progenitors were found in the caudal half of *Mef2C*$^{-/-}$ embryos. In order to assess whether the poor capacity to generate hematopoietic progenitors in *Mef2C*$^{-/-}$ embryo proper was due to a cell intrinsic requirement for Mef2C in hematopoietic cells in the embryo proper, cell suspensions were initially co-cultured on a supportive OP9-stroma before being transferred to methylcellulose, as shown in **Paper II** (Rhodes et al., 2008). Interestingly, the ability to generate hematopoietic progenitors in the caudal half of the *Mef2C*$^{-/-}$ embryos was partially rescued after 4 days of OP9 co-culture, indicating that there is no cell-autonomous block in specification of definitive progenitors in the embryo proper. Importantly, all organs displayed B-lymphoid hematopoietic potential after culture on OP9 stroma, as assessed by FACS.

Thus, we could conclude that in contrast to Scl, Mef2C is not absolutely required for hematopoietic specification and emergence of primitive and definitive hematopoietic progenitors. As Mef2C$^{-/-}$ mice die in utero, analysis of a potential role of Mef2C in post-natal life using this model was precluded. In order to study the potential requirement of Mef2C in adult hematopoiesis, we utilized a conditionally targeted Mef2C$^{fl/fl}$ mouse strain, which we crossed with a VavCre mouse strain (Arnold et al., 2007; Stadtfeld and Graf, 2005). As the Vav promoter drives Cre recombinase expression exclusively in hematopoietic lineages shortly after the emergence of HSCs, Mef2C was specifically targeted for deletion in all fetal and adult hematopoietic lineages.

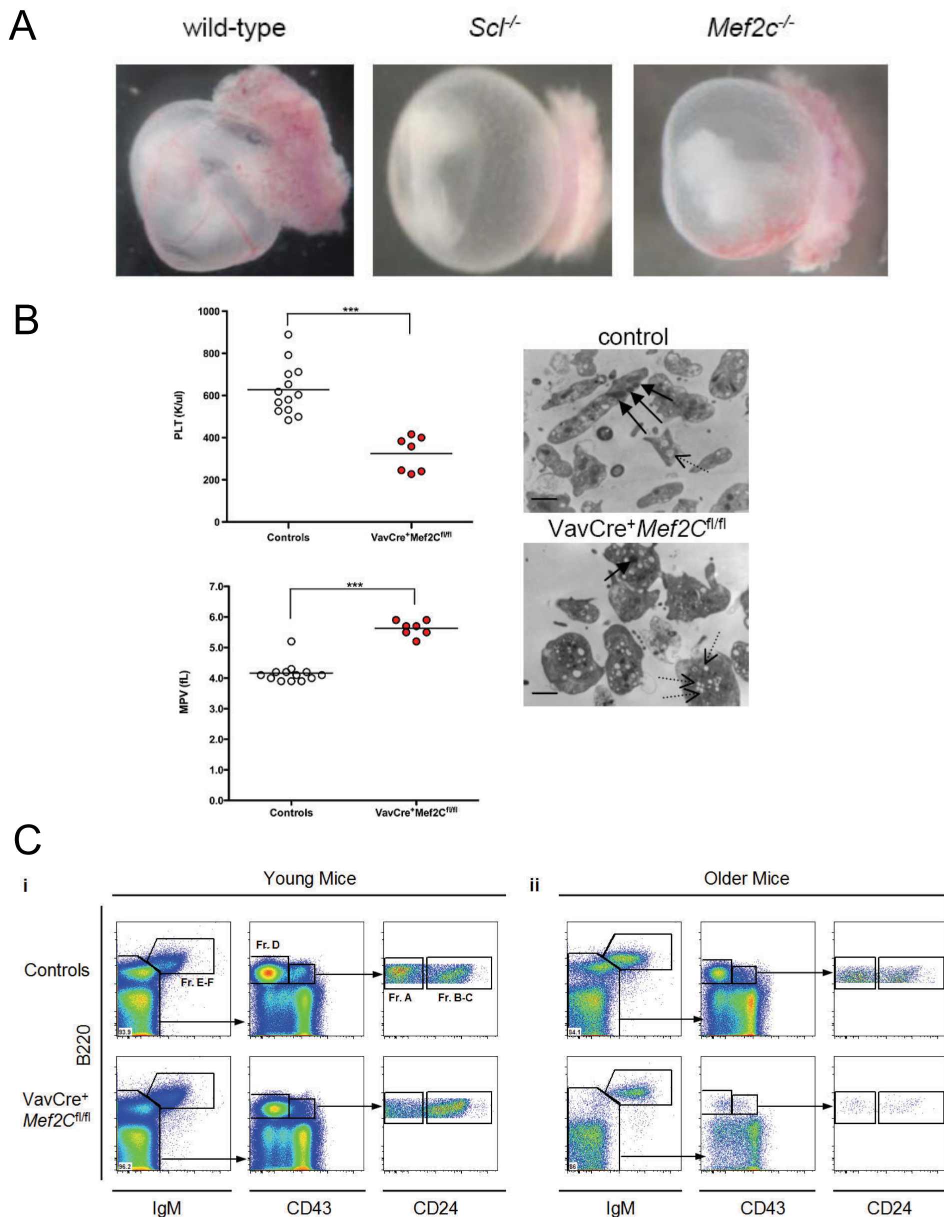


**Figure 9. Mef2C is not critical for fetal hematopoiesis, but in the adult Mef2C is important for platelet development and B cell homeostasis.** (A) Pictures of wild-type, $Scl^{-/-}$ and $Mef2C^{-/-}$ embryos, showing the presence of blood in $Mef2C^{-/-}$ yolk sac. (B) Complete blood cytometry (CBC) analysis of peripheral blood showed that $VavCre^{+}Mef2C^{fl/fl}$ mice exhibit marked thrombocytopenia and increased platelet size. Circles indicate analysis of individual mice. (PLT; platelet count, MPV; mean platelet volume) (*** indicates $p<0.0005$). Electron microscopy imaging of platelets reveals severe ultrastructural

**Figure 9. Continued:** defects of Mef2C-deficient platelets, including enlarged size and loss of elongated form. In addition, Mef2C deficient platelets demonstrated a loss of α-granules (solid arrows) and an increase in depleted granules (dashed arrows). (C) FACS analyses of frequencies of B cell subsets in bone marrow of VavCre$^+$*Mef2C*$^{fl/fl}$ and age-matched littermate control mice, for expression of B220, IgM, CD43 and CD24. Panels i-ii display representative examples of young (1 month) and older (15 month) mice, respectively. Frequency of B220$^+$IgM$^+$ B cells was not significantly altered regardless of age. Subgating on IgM$^-$ cells visualizes pre-B and pro-B populations. Strikingly, older, but not young, mice exhibited severe reductions of pre-B cells. Subgating on B220$^+$CD43$^+$ cells visualizes pre-pro-B (Fr. A) and pro-B cells (Fr. B-C). Of note, a slight reduction in the frequency of pre-pro-B cells was noted in both young and older mice.

Of note, VavCre$^+$Mef2C$^{fl/fl}$ survived into adulthood demonstrating that loss of Mef2C during fetal hematopoiesis or adult steady state hematopoiesis does not result in profound hematopoietic failure. Also most peripheral blood parameters were unperturbed, as analyzed by complete blood cytometry (CBC). However, we found that VavCre$^+$Mef2C$^{fl/fl}$ mice, as Scl-deficient mice, displayed thrombocytopenia and enlarged platelet size (Figure 9B). Moreover, electron microscopy of Mef2C-deficient platelets revealed ultrastructural defects, including loss of elongated forms and reduction in platelet granularity (Figure 9B), highly similar to Scl-deficient platelets (McCormack et al., 2006).

To understand the requirement of Mef2C for megakaryocyte development we next turned to the bone marrow to assess the state of ongoing megakaryopoiesis. We could conclude that frequencies of MEPs and single-lineage megakaryocyte committed progenitors (CFU-Mk) were increased in VavCre$^+$Mef2C$^{fl/fl}$ BM. Interestingly, mature megakaryocytes as defined by acetylcholinesterase staining (AchE$^+$), DNA content, and high expression of CD41 and CD31 megakaryocytes were present despite loss of Mef2C. These data suggest that the reduction in platelet count due to absence of Mef2C is not caused by decreased commitment to the megakaryocyte lineage or initial maturation of megakaryocytes, but rather due to defects in platelet formation. Interestingly, when Mef2C deficient BM was cultured *in vitro* in megakaryopoietic conditions, we observed a severe reduction in formation of AchE$^+$ cells from VavCre$^+$Mef2C$^{fl/fl}$ BM. This phenotype is highly reminiscent to the megakaryocyte defect observed in cultures of Scl-

deficient bone marrow, where no AchE+ megakaryocytes could be observed. These data clearly document a cell autonomous requirement of Mef2C for proper megakaryocyte/platelet development, and nominate the Scl-Mef2C axis as an important regulatory entity utilized specifically in the megakaryocyte lineage.

In addition to the defects in the platelet compartment, we observed reductions in peripheral blood B220$^{+}$IgM$^{+}$ B-cells, which were not correlated with a decrease in mature IgM$^{+}$ B cells in the spleen. Analysis of BM of both young and older VavCre$^{+}$*Mef2C*$^{fl/fl}$ mice revealed a reduction in the frequency of pre-pro-B cells (Figure 9C). Strikingly, older VavCre$^{+}$*Mef2C*$^{fl/fl}$ mice exhibited a severe reduction of the pre-B cell compartment, in stark contrast to their age-matched littermate controls (Figure 9C). Even so, the frequency of mature IgM$^{+}$ B-cells was not altered compared to age-matched controls, regardless of age. This phenotype is very similar to the defects associated with B-cell aging (reviewed in (Riley et al., 2005)), Taken together, these data indicate a previously unappreciated role for Mef2C in bone marrow B-cell homeostasis.

In summary, our data demonstrate that within the adult hematopoietic system Mef2C, as Scl, is required for proper platelet development (Figure 10). In addition, our data indicate an Scl-independent requirement of Mef2C in B-cell homeostasis (Figure 10). As Scl is not expressed in B-cells, Mef2C expression in B-cells is regulated proposedly by other bHLH factors, such as E12/E47 known to be implicated in B-cell aging. Furthermore, Scl's role in hematological malignancies and Mef2C's recent implication as a cooperating oncogene in leukemia (Du et al., 2005; Krivtsov et al., 2006; Nagel et al., 2007; Suzuki et al., 2002) raises the interesting hypothesis that Mef2C may be regulated by Scl or other bHLH factors also during leukemogenesis. However, the fact that Scl was unable to bind and activate the Mef2C promoter in erythroid cells suggests that additional prerequisites have to be fulfilled to activate Mef2C expression. Therefore, unraveling the mechanisms by which Mef2C expression is regulated, and how Mef2C subsequently controls cell proliferation and survival in different tissues may shed light not only on these physiological developmental processes but also on the pathogenesis of malignant blood diseases.

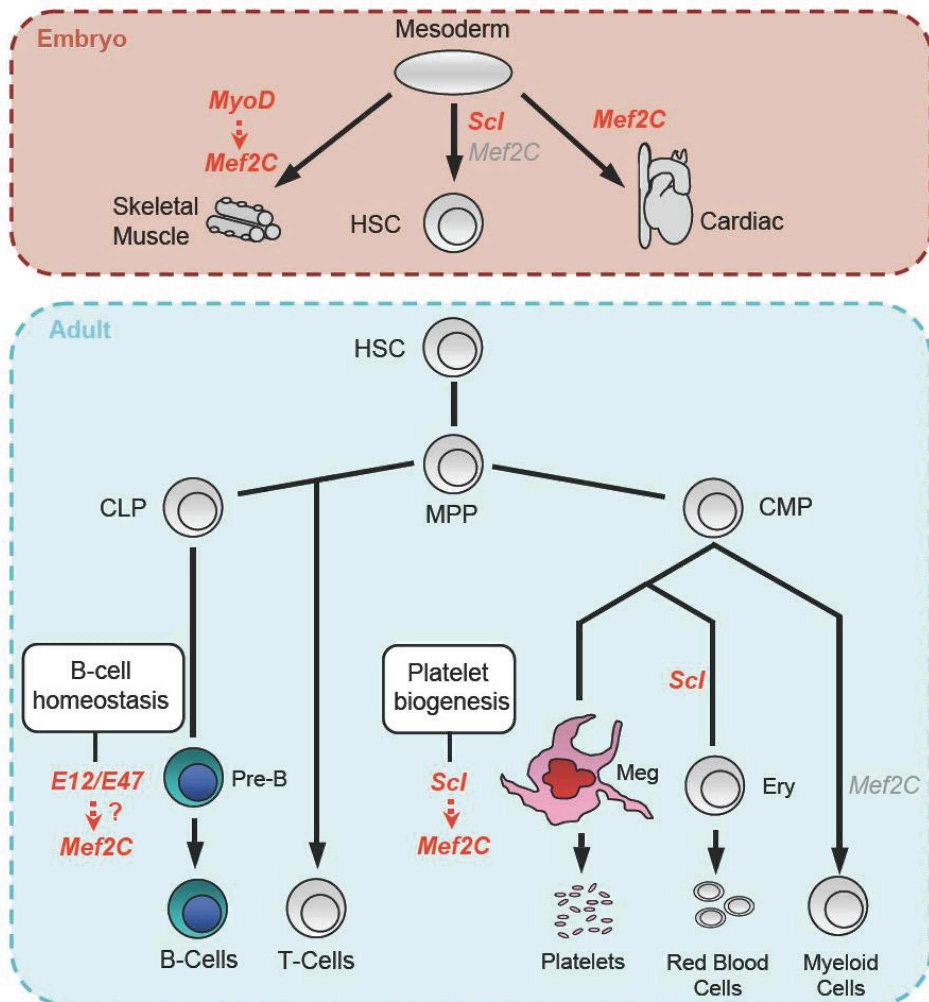


**Figure 10. Model of Mef2C gene regulation in the hematopoietic hierarchy.** Unlike Scl, which is absolutely required for the specification of mesoderm to blood and emergence of HSCs, Mef2C is not critically involved in embryonic hematopoiesis. Mef2C is however required for skeletal muscle and cardiac lineage differentiation. In the adult, we discovered novel requirements for Mef2C in two hematopoietic lineages. In B cells, where Scl is not expressed, Mef2C likely acts downstream of other bHLH factors (e.g. E12/E47) to counteract B lymphocyte aging, especially within the pre-B cell fraction. Our data indicate that in the megakaryocytic lineage, that Mef2C is a direct target gene of Scl and, as Scl, is required for proper megakaryopoiesis and platelet biogenesis. Although Scl is required for erythroid differentiation, Scl is not bound to the Mef2C promoter in erythroid cells. Finally, Mef2C was recently shown to be expressed in myeloid cells where it acts to modulate myeloid cell fates (Schuler et al., 2008). Bold text in red indicates expression and functional requirement, grey text indicates expression but no functional requirement in the specific lineages. HSC; hematopoietic stem cell, MPP; multipotent progenitor, CLP; common lymphoid progenitor; CMP, common myeloid progenitor, Meg; megakaryocyte, Ery; erythroid progenitor.

# 9. ACKNOWLEDGEMENTS

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# 10. PUBLICATIONS NOT INCLUDED IN THIS THESIS

**1.** **Gekas C,** Rhodes K, Mikkola HKA. Isolation and visualization of placental hematopoietic stem cells. **Current Protocols in Stem Cell Biology. In Press**

**2.** Schenke-Layland K, Rhodes KE, Angelis E, Butylkova Y, Heydarkhan-Hagvall S, **Gekas C,** Zhang R, Goldhaber JI, Mikkola HK, Plath K, MacLellan WR. Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. **Stem Cells, 2008.**

**3.** **Gekas C.,** E. Rhodes K., K. A. Mikkola H. (2008). Isolation and Analysis of Hematopoietic Stem Cells from the Placenta. **Journal of Visualized Experiments** http://www.jove.com/index/Details.stp?ID=742

**4.** Sitnicka E, Buza-Vidas N, Ahlenius H, Cilio CM, **Gekas C,** Nygren JM, Svensson M, Agace WW, Jacobsen SEW. Critical role of FLT3 ligand in IL-7 receptor-independent T lymphopoiesis and regulation of lymphoid-primed multipotent progenitors. **Blood, 2007**

**5.** Mikkola HK, **Gekas C**, Orkin SH, Dieterlen-Lievre F. Placenta as a site for hematopoietic stem cell development. **Experimental Hematology, 2005**

**6.** Svensson E, Eriksson H, **Gekas C,** Olofsson T, Richter J, Gullberg U. DNA-binding dependent and independent functions of WT1 protein during human hematopoiesis. **Experimental Cell Research, 2005**

## 11. References

**Adams, G. B., Chabner, K. T., Alley, I. R., Olson, D. P., Szczepiorkowski, Z. M., Poznansky, M. C., Kos, C. H., Pollak, M. R., Brown, E. M. and Scadden, D. T.** (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* **439**, 599-603.

**Adams, G. B. and Scadden, D. T.** (2006). The hematopoietic stem cell in its place. *Nat Immunol* **7**, 333-7.

**Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C. and Cross, J. C.** (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* **250**, 358-73.

**Akashi, K., Traver, D., Kondo, M. and Weissman, I. L.** (1999). Lymphoid development from hematopoietic stem cells. *Int J Hematol* **69**, 217-26.

**Akashi, K., Traver, D., Miyamoto, T. and Weissman, I. L.** (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-7.

**Alvarez-Silva, M., Belo-Diabangouaya, P., Salaun, J. and Dieterlen-Lievre, F.** (2003). Mouse placenta is a major hematopoietic organ. *Development* **130**, 5437-44. Epub 2003 Sep 24.

**Arnold, M. A., Kim, Y., Czubryt, M. P., Phan, D., McAnally, J., Qi, X., Shelton, J. M., Richardson, J. A., Bassel-Duby, R. and Olson, E. N.** (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev Cell* **12**, 377-89.

**Avecilla, S. T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D. K., Dias, S., Zhang, F., Hartman, T. E. et al.** (2004). Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* **10**, 64-71.

**Baron, M. H.** (2003). Embryonic origins of mammalian hematopoiesis. *Exp Hematol* **31**, 1160-9.

**Bash, R. O., Hall, S., Timmons, C. F., Crist, W. M., Amylon, M., Smith, R. G. and Baer, R.** (1995). Does activation of the TAL1 gene occur in a majority of patients with T-cell acute lymphoblastic leukemia? A pediatric oncology group study. *Blood* **86**, 666-76.

**Black, B. L. and Olson, E. N.** (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol* **14**, 167-96.

**Boyes, J. and Bird, A.** (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* **64**, 1123-34.

**Bradford, G. B., Williams, B., Rossi, R. and Bertoncello, I.** (1997). Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol* **25**, 445-53.

**Brenner, C., Deplus, R., Didelot, C., Loriot, A., Vire, E., De Smet, C., Gutierrez, A., Danovi, D., Bernard, D., Boon, T. et al.** (2005). Myc represses transcription through recruitment of DNA methyltransferase corepressor. *Embo J* **24**, 336-46.

**Buck, M. J. and Lieb, J. D.** (2004). ChIP-chip: considerations for the design, analysis, and application of genome-wide chromatin immunoprecipitation experiments. *Genomics* **83**, 349-60.

**Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringhurst, F. R. et al.** (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-6.

**Caprioli, A., Jaffredo, T., Gautier, R., Dubourg, C. and Dieterlen-Lievre, F.** (1998). Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc Natl Acad Sci U S A* **95**, 1641-6.

**Caprioli, A., Minko, K., Drevon, C., Eichmann, A., Dieterlen-Lievre, F. and Jaffredo, T.** (2001). Hemangioblast commitment in the avian allantois: cellular and molecular aspects. *Dev Biol* **238**, 64-78.

**Carlyle, J. R., Michie, A. M., Furlonger, C., Nakano, T., Lenardo, M. J., Paige, C. J. and Zuniga-Pflucker, J. C.** (1997). Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J Exp Med* **186**, 173-82.

**Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. and Bernstein, A.** (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* **335**, 88-9.

**Cheshier, S. H., Morrison, S. J., Liao, X. and Weissman, I. L.** (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A* **96**, 3120-5.

**Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G.** (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-32.

**Christensen, J. L., Wright, D. E., Wagers, A. J. and Weissman, I. L.** (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol* **2**, E75.

**Corbel, C. and Salaun, J.** (2002). AlphaIIb integrin expression during development of the murine hemopoietic system. *Dev Biol* **243**, 301-11.

**Cumano, A., Dieterlen-Lievre, F. and Godin, I.** (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-16.

**Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P. and Godin, I.** (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-85.

**de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E.** (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-83.

**de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E.** (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *Embo J* **19**, 2465-74.

**DeKoter, R. P. and Singh, H.** (2000). Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* **288**, 1439-41.

**Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. and Bernstein, A.** (1985). Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. *Cell* **42**, 71-9.

**Domen, J. and Weissman, I. L.** (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol Med Today* **5**, 201-8.

**Downs, K. M.** (2002). Early placental ontogeny in the mouse. *Placenta* **23**, 116-31.

**Downs, K. M. and Gardner, R. L.** (1995). An investigation into early placental ontogeny: allantoic attachment to the chorion is selective and developmentally regulated. *Development* **121**, 407-16.

**Driessen, R. L., Johnston, H. M. and Nilsson, S. K.** (2003). Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Exp Hematol* **31**, 1284-91.

**Du, Y., Spence, S. E., Jenkins, N. A. and Copeland, N. G.** (2005). Cooperating cancer gene identification via oncogenic retrovirus-induced insertional mutagenesis. *Blood*.

**Ema, H. and Nakauchi, H.** (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284-8.

**Enver, T., Heyworth, C. M. and Dexter, T. M.** (1998). Do stem cells play dice? *Blood* **92**, 348-51; discussion 352.

**Ernst, P., Fisher, J. K., Avery, W., Wade, S., Foy, D. and Korsmeyer, S. J.** (2004). Definitive hematopoiesis requires the mixed-lineage leukemia gene. *Dev Cell* **6**, 437-43.

**Fairbairn, L. J., Cowling, G. J., Reipert, B. M. and Dexter, T. M.** (1993). Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell* **74**, 823-32.

**Fuhrken, P. G., Chen, C., Apostolidis, P. A., Wang, M., Miller, W. M. and Papoutsakis, E. T.** (2008). Gene-Ontology driven transcriptional analysis of CD34+-cell initiated megakaryocytic cultures identifies new transcriptional regulators of megakaryopoiesis. *Physiol Genomics*.

**Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C. and Orkin, S. H.** (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* **93**, 12355-8.

**Geissler, E. N., Ryan, M. A. and Housman, D. E.** (1988). The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* **55**, 185-92.

**Gekas, C., Dieterlen-Lievre, F., Orkin, S. H. and Mikkola, H. K.** (2005). The placenta is a niche for hematopoietic stem cells. *Dev Cell* **8**, 365-75.

**Godin, I., Dieterlen-Lievre, F. and Cumano, A.** (1995). Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci U S A* **92**, 773-7.

**Godin, I., Garcia-Porrero, J. A., Dieterlen-Lievre, F. and Cumano, A.** (1999). Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites

Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *J Exp Med* **190**, 43-52.

**Hall, M. A., Curtis, D. J., Metcalf, D., Elefanty, A. G., Sourris, K., Robb, L., Gothert, J. R., Jane, S. M. and Begley, C. G.** (2003). The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proc Natl Acad Sci U S A* **100**, 992-7.

**Harrison, D. E.** (1980). Lifespans of immunohemopoietic stem cell lines. *Adv Pathobiol* **7**, 187-99.

**Harrison, D. E., Astle, C. M. and Lerner, C.** (1988). Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. *Proc Natl Acad Sci U S A* **85**, 822-6.

**Houssaint, E.** (1981). Differentiation of the mouse hepatic primordium. II. Extrinsic origin of the haemopoietic cell line. *Cell Differ* **10**, 243-52.

**Hsu, H. C., Ema, H., Osawa, M., Nakamura, Y., Suda, T. and Nakauchi, H.** (2000). Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. *Blood* **96**, 3757-62.

**Hsu, H. L., Cheng, J. T., Chen, Q. and Baer, R.** (1991). Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. *Mol Cell Biol* **11**, 3037-42.

**Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. and Keller, G.** (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-30.

**Igarashi, H., Gregory, S. C., Yokota, T., Sakaguchi, N. and Kincade, P. W.** (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* **17**, 117-30.

**Iwasaki, H., Mizuno, S., Wells, R. A., Cantor, A. B., Watanabe, S. and Akashi, K.** (2003). GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity* **19**, 451-62.

**Johnson, G. R. and Moore, M. A.** (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature* **258**, 726-8.

**Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. and Wolffe, A. P.** (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**, 187-91.

**Jones, R. J., Collector, M. I., Barber, J. P., Vala, M. S., Fackler, M. J., May, W. S., Griffin, C. A., Hawkins, A. L., Zehnbauer, B. A., Hilton, J. et al.** (1996). Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. *Blood* **88**, 487-91.

**Keller, G. and Snodgrass, R.** (1990). Life span of multipotential hematopoietic stem cells in vivo. *J Exp Med* **171**, 1407-18.

**Keller, G., Wall, C., Fong, A. Z., Hawley, T. S. and Hawley, R. G.** (1998). Overexpression of HOX11 leads to the immortalization of embryonic precursors with both primitive and definitive hematopoietic potential. *Blood* **92**, 877-87.

**Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., 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**, 1109-21.

**Kihara-Negishi, F., Yamamoto, H., Suzuki, M., Yamada, T., Sakurai, T., Tamura, T. and Oikawa, T.** (2001). In vivo complex formation of PU.1 with HDAC1 associated with PU.1-mediated transcriptional repression. *Oncogene* **20**, 6039-47.

**Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M.** (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* **230**, 230-42.

**Klose, R. J. and Bird, A. P.** (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31**, 89-97.

**Kodama, H., Nose, M., Niida, S., Nishikawa, S. and Nishikawa, S.** (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp Hematol* **22**, 979-84.

**Kollet, O., Dar, A., Shivtiel, S., Kalinkovich, A., Lapid, K., Sztainberg, Y., Tesio, M., Samstein, R. M., Goichberg, P., Spiegel, A. et al.** (2006). Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* **12**, 657-64.

**Kondo, M., Scherer, D. C., Miyamoto, T., King, A. G., Akashi, K., Sugamura, K. and Weissman, I. L.** (2000). Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* **407**, 383-6.

**Kondo, M., Wagers, A. J., Manz, M. G., Prohaska, S. S., Scherer, D. C., Beilhack, G. F., Shizuru, J. A. and Weissman, I. L.** (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* **21**, 759-806.

**Kondo, M., Weissman, I. L. and Akashi, K.** (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-72.

**Koushik, S. V., Wang, J., Rogers, R., Moskophidis, D., Lambert, N. A., Creazzo, T. L. and Conway, S. J.** (2001). Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. *FASEB J* **15**, 1209-11.

**Kouzarides, T.** (2007). Chromatin modifications and their function. *Cell* **128**, 693-705.

**Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S. J.** (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369-77.

**Krivtsov, A. V., Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., Levine, J. E., Wang, J., Hahn, W. C., Gilliland, D. G. et al.** (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* **442**, 818-22.

**Kumaravelu, P., Hook, L., Morrison, A. M., Ure, J., Zhao, S., Zuyev, S., Ansell, J. and Medvinsky, A.** (2002). Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* **129**, 4891-9.

**Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I. L. and Grompe, M.** (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* **6**, 1229-34.

**Lemischka, I. R., Raulet, D. H. and Mulligan, R. C.** (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45**, 917-27.

**Lin, C. S., Lim, S. K., D'Agati, V. and Costantini, F.** (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev* **10**, 154-64.

**Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A. and Olson, E. N.** (1998). Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development* **125**, 4565-74.

**Lyman, S. D. and Jacobsen, S. E. W.** (1998). c-kit Ligand and Flt3 Ligand: Stem/Progenitor Cell Factors With Overlapping Yet Distinct Activities. *Blood* **91**, 1101-1134.

**Matthews, W., Jordan, C. T., Wiegand, G. W., Pardoll, D. and Lemischka, I. R.** (1991). A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell* **65**, 1143-52.

**McCormack, M. P., Hall, M. A., Schoenwaelder, S. M., Zhao, Q., Ellis, S., Prentice, J. A., Clarke, A. J., Slater, N. J., Salmon, J. M., Jackson, S. P. et al.** (2006). A critical role for the transcription factor Scl in platelet production during stress thrombopoiesis. *Blood* **108**, 2248-56.

**McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C. R., Lynch, D. H., Smith, J., Pulendran, B. et al.** (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95**, 3489-97.

**Medvinsky, A. and Dzierzak, E.** (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.

**Melchers, F.** (1979). Murine embryonic B lymphocyte development in the placenta. *Nature* **277**, 219-21.

**Metcalf, D.** (1998). Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation. *Blood* **92**, 345-7; discussion 352.

**Metcalf, D. and Nicola, N. A.** (1991). Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors. *Proc Natl Acad Sci U S A* **88**, 6239-43.

**Mikkola, H. K., Fujiwara, Y., Schlaeger, T. M., Traver, D. and Orkin, S. H.** (2003a). Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* **101**, 508-16.

**Mikkola, H. K., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y. and Orkin, S. H.** (2003b). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* **421**, 547-51.

**Mikkola, H. K. and Orkin, S. H.** (2006). The journey of developing hematopoietic stem cells. *Development* **133**, 3733-44.

**Montecino-Rodriguez, E. and Dorshkind, K.** (2003). To T or not to T: reassessing the common lymphoid progenitor. *Nat Immunol* **4**, 100-1.

**Morrison, S. J., Shah, N. M. and Anderson, D. J.** (1997a). Regulatory mechanisms in stem cell biology. *Cell* **88**, 287-98.

**Morrison, S. J., Uchida, N. and Weissman, I. L.** (1995). The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* **11**, 35-71.

**Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. and Weissman, I. L.** (1997b). Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**, 1929-39.

**Morrison, S. J. and Weissman, I. L.** (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**, 661-73.

**Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. and Dzierzak, E.** (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301.

**Nagel, S., Meyer, C., Quentmeier, H., Kaufmann, M., Drexler, H. G. and Macleod, R. A.** (2007). MEF2C is activated by multiple mechanisms in a subset of T-acute lymphoblastic leukemia cell lines. *Leukemia*.

**Nilsson, S. K. and Bertoncello, I.** (1994). The development and establishment of hemopoiesis in fetal and newborn osteopetrotic (op/op) mice. *Dev Biol* **164**, 456-62.

**Nilsson, S. K., Johnston, H. M., Whitty, G. A., Williams, B., Webb, R. J., Denhardt, D. T., Bertoncello, I., Bendall, L. J., Simmons, P. J. and Haylock, D. N.** (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **106**, 1232-9.

**North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A.** (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-75.

**North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A.** (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-72.

**Nutt, S. L., Heavey, B., Rolink, A. G. and Busslinger, M.** (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* **401**, 556-62.

**Nygren, J. M., Jovinge, S., Breitbach, M., Sawen, P., Roll, W., Hescheler, J., Taneera, J., Fleischmann, B. K. and Jacobsen, S. E.** (2004). Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat Med* **10**, 494-501.

**Ogawa, M.** (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-53.

**Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H. and Nishikawa, S.** (1991). Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med* **174**, 63-71.

**Ogawa, M., Nishikawa, S., Yoshinaga, K., Hayashi, S., Kunisada, T., Nakao, J., Kina, T., Sudo, T., Kodama, H. and Nishikawa, S.** (1993). Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo. *Development* **117**, 1089-98.

**Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Nishikawa, S., Miura, Y. and Suda, T.** (1991). Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* **78**, 1706-12.

**Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G. and Downing, J. R.** (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321-30.

**Orford, K., Kharchenko, P., Lai, W., Dao, M. C., Worhunsky, D. J., Ferro, A., Janzen, V., Park, P. J. and Scadden, D. T.** (2008). Differential H3K4 methylation identifies developmentally poised hematopoietic genes. *Dev Cell* **14**, 798-809.

**Orkin, S. H.** (2000). Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* **1**, 57-64.

**Orkin, S. H. and Zon, L. I.** (2002). Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol* **3**, 323-8.

**Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H.** (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-5.

**Ottersbach, K. and Dzierzak, E.** (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* **8**, 377-87.

**Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G.** (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-84.

**Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B. et al.** (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-60.

**Pevny, L., Lin, C. S., D'Agati, V., Simon, M. C., Orkin, S. H. and Costantini, F.** (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development* **121**, 163-72.

**Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H. and Costantini, F.** (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257-60.

**Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H.** (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* **86**, 47-57.

**Rasko, J. E., Metcalf, D., Rossner, M. T., Begley, C. G. and Nicola, N. A.** (1995). The flt3/flk-2 ligand: receptor distribution and action on murine haemopoietic cell survival and proliferation. *Leukemia* **9**, 2058-66.

**Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L.** (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-11.

**Rhodes, K. E., Gekas, C., Wang, Y., Lux, C. T., Francis, C. S., Chan, D. N., Conway, S., Orkin, S. H., Yoder, M. C. and Mikkola, H. K.** (2008). The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell* **2**, 252-63.

**Riley, R. L., Blomberg, B. B. and Frasca, D.** (2005). B cells, E2A, and aging. *Immunol Rev* **205**, 30-47.

**Robertson, S. M., Kennedy, M., Shannon, J. M. and Keller, G.** (2000). A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development* **127**, 2447-59.

**Rosnet, O., Marchetto, S., deLapeyriere, O. and Birnbaum, D.** (1991a). Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. *Oncogene* **6**, 1641-50.

**Rosnet, O., Mattei, M. G., Marchetto, S. and Birnbaum, D.** (1991b). Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. *Genomics* **9**, 380-5.

**Rossant, J. and Cross, J. C.** (2001). Placental development: lessons from mouse mutants. *Nat Rev Genet* **2**, 538-48.

**Russell, E. S.** (1979). Hereditary anemias of the mouse: a review for geneticists. *Adv Genet* **20**, 357-459.

**Russo, V. E. A., Martienssen, R. A. and Riggs, A. D.** (1996). Epigenetic Mechanisms of Gene Regulation. Woodbury: Cold Spring Harbor Laboratory Press.

**Sabin, F. R.** (1920). Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during second day of incubation. *Contrib. Embryol.* **9**, 213–262.

**Sanchez, M. J., Holmes, A., Miles, C. and Dzierzak, E.** (1996). Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* **5**, 513-25.

**Schlaeger, T. M., Bartunkova, S., Lawitts, J. A., Teichmann, G., Risau, W., Deutsch, U. and Sato, T. N.** (1997). Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci U S A* **94**, 3058-63.

**Schlaeger, T. M., Mikkola, H. K., Gekas, C., Helgadottir, H. B. and Orkin, S. H.** (2005). Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development. *Blood* **105**, 3871-4.

**Schuler, A., Schwieger, M., Engelmann, A., Weber, K., Horn, S., Muller, U., Arnold, M. A., Olson, E. N. and Stocking, C.** (2008). The MADS transcription factor Mef2c is a pivotal modulator of myeloid cell fate. *Blood* **111**, 4532-41.

**Shivdasani, R. A., Mayer, E. L. and Orkin, S. H.** (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-4.

**Sieweke, M. H. and Graf, T.** (1998). A transcription factor party during blood cell differentiation. *Curr Opin Genet Dev* **8**, 545-51.

**Silvers, W. K.** (1979). In the Coat Colors of Mice: A Modelfor Mammalian Gene Actions and Interaction. *New York, NY: Springer Verlag*, 243-267.

**Siminovitch, L., McCulloch, E. A. and Till, J. E.** (1963). The Distribution Of Colony-Forming Cells Among Spleen Colonies. *J Cell Physiol* **62**, 327-36.

**Sitnicka, E., Brakebusch, C., Martensson, I. L., Svensson, M., Agace, W. W., Sigvardsson, M., Buza-Vidas, N., Bryder, D., Cilio, C. M., Ahlenius, H. et al.** (2003). Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. *J Exp Med* **198**, 1495-506.

**Sitnicka, E., Bryder, D., Theilgaard-Monch, K., Buza-Vidas, N., Adolfsson, J. and Jacobsen, S. E.** (2002). Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity* **17**, 463-72.

**Sitnicka, E., Buza-Vidas, N., Ahlenius, H., Cilio, C. M., Gekas, C., Nygren, J. M., Mansson, R., Cheng, M., Jensen, C. T., Svensson, M. et al.** (2007). Critical role of FLT3 ligand in IL-7 receptor independent T lymphopoiesis and regulation of lymphoid-primed multipotent progenitors. *Blood* **110**, 2955-64.

**Stadtfeld, M. and Graf, T.** (2005). Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. *Development* **132**, 203-13.

**Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L. M., Rittling, S. R. et al.** (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med* **201**, 1781-91.

**Suda, T., Arai, F. and Hirao, A.** (2005). Hematopoietic stem cells and their niche. *Trends Immunol* **26**, 426-33.

**Sugiyama, T., Kohara, H., Noda, M. and Nagasawa, T.** (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-88.

**Suzuki, T., Shen, H., Akagi, K., Morse, H. C., Malley, J. D., Naiman, D. Q., Jenkins, N. A. and Copeland, N. G.** (2002). New genes involved in cancer identified by retroviral tagging. *Nat Genet* **32**, 166-74.

**Taichman, R. S. and Emerson, S. G.** (1998). The role of osteoblasts in the hematopoietic microenvironment. *Stem Cells* **16**, 7-15.

**Teitell, M. A. and Mikkola, H. K.** (2006). Transcriptional activators, repressors, and epigenetic modifiers controlling hematopoietic stem cell development. *Pediatr Res* **59**, 33R-9R.

**Till, J. E. and McCulloch, E.** (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213-22.

**Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H.** (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-6.

**Waddington, C. H.** (1957). The Strategy of the Genes. London: Allen&Unwin.

**Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A. and Rabbitts, T. H.** (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *Embo J* **16**, 3145-57.

**Wagers, A. J., Sherwood, R. I., Christensen, J. L. and Weissman, I. L.** (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* **297**, 2256-9.

**Weissman, I. L.** (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100**, 157-68.

**Weissman, I. L., Anderson, D. J. and Gage, F.** (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol* **17**, 387-403.

**Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J. M. et al.** (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**, 871-4.

**Visvader, J. E., Fujiwara, Y. and Orkin, S. H.** (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev* **12**, 473-9.

**von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. and Murray, R.** (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-26.

**Voronova, A. F. and Lee, F.** (1994). The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. *Proc Natl Acad Sci U S A* **91**, 5952-6.

**Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R. and Rabbitts, T. H.** (1998). The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc Natl Acad Sci U S A* **95**, 3890-5.

**Yoder, M. C., Cumming, J. G., Hiatt, K., Mukherjee, P. and Williams, D. A.** (1996). A novel method of myeloablation to enhance engraftment of adult bone marrow cells in newborn mice. *Biol Blood Marrow Transplant* **2**, 59-67.

**Zhang, J. and Li, L.** (2008). Stem cell niche: microenvironment and beyond. *J Biol Chem* **283**, 9499-503.

**Zhu, J. and Emerson, S. G.** (2004). A new bone to pick: osteoblasts and the haematopoietic stem-cell niche. *Bioessays* **26**, 595-9.

## 12. ARTICLES I-IV