Hematopoietic Progenitor and Stem Cell Regulation during Development: Hypoxia and Niches

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Cover is a symbolic presentation of the state of hematopoietic stem cells in low-oxygen and high-oxygen niches. The color gradient represents the change in oxygen level.

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Hematopoietic Progenitor and Stem Cell Regulation during Development: Hypoxia and Niches

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Chapter 1

General Introduction

Sections of this chapter will appear in a review article in a special issue of Blood Cells, Molecules, Diseases (BCMD) on “Embryonic and Fetal Hematopoiesis.”

The hematopoietic system

In a healthy adult person, almost 10^{11} - 10^{12} new blood cells are generated daily in order to maintain the steady state in peripheral circulation (Paul, 2008). To this end, a high level of self-renewal and differentiation of Hematopoietic stem cells (HSCs) is required. HSCs have the unique ability of self-renewal to maintain the stem cell pool. They also differentiate to more committed progenitors which produce all mature blood cell lineages (Lemischka, 1992; Medvinsky and Dzierzak, 1998; Orkin, 2000). In general, there are two main branches in the adult hematopoietic hierarchy: The lymphoid branch is derived from common lymphoid progenitors that mature to B-cells and T-cells. Lymphocytes are involved in the adaptive immune system. The myeloid branch is derived from common myeloid progenitor cells that give rise to more lineage-restricted precursors. These progenitors differentiate into the following cell types: erythrocytes, the most abundant terminally differentiated cells in the blood, which are required for oxygen transport, as well as megakaryocytes, involved in blood clotting and granulocytes and macrophages, which are involved in innate immunity.

Recent insights into understanding HSC regulation have facilitated HSC clinical therapies and trials for hematological disorders. Despite progress in this field, there remain difficulties in *ex vivo* expansion of HSCs on a scale that ensures efficient regeneration of blood system. Overcoming this challenge calls for a more information on the intrinsic and extrinsic factors that regulate development, maintenance, and function of HSCs.

Adult hematopoietic system

Bone marrow (BM) is the predominant hematopoietic tissue during adult life, harboring HSCs and a hierarchy of different hematopoietic cells. The hierarchy includes HSCs at the base and its derivatives such as multipotent progenitors, committed progenitors, and differentiated cells (Lemischka, 1992; Medvinsky and Dzierzak, 1998; Orkin, 2000). Blood contains more than ten different mature cell types including erythrocytes, megakaryocytes/platelets, myeloid cells (monocyte/macrophages and granulocytes), mast cells, T- and B-lymphocytes, natural killer cells, and dendritic cells. Spleen, thymus, and lymph nodes also contain hematopoietic cells. BM, spleen, and blood vessels harbor hematopoietic cells of myeloid, erythroid, and lymphoid lineages, whereas thymus and lymph nodes are lymphoid tissues. Spleen harbors B- and T-cells, myeloid cells and holds a reserve of blood in case of hemorrhagic shock. In thymus, T-cell precursors mature to functional T-cells. Lymph nodes are replete with lymphocytes and macrophages which play a role in adaptive immune response (Paul, 2008).

BM HSCs are rare and are phenotypically identified by markers present on their surface. There is no unique marker to exclusively identify HSCs; however, they can be highly enriched using a combination of several markers. HSCs are functionally identified by in vivo transplantation assay which verifies their multipotency and self-renewal activity (Boggs et al., 1982; Harrison et al., 1988; Harrison et al., 1993). The HSCs in adult BM are self-renewing; however, their

origin goes back to the embryonic hematopoietic tissues, where they are generated, expand, and migrate to the BM prior to the birth. Hence, studying different hematopoietic sites during development and the factors regulating their generation, maintenance, and function is crucial to have a clear picture of hematopoiesis and HSC biology.

Hematopoietic development in the mouse embryo

The first wave of embryonic hematopoiesis occurs in the yolk sac (YS) at embryonic day (E) 7.5 of mouse development. Short-lived primitive erythrocytes are generated in the YS to provide oxygen for the rapidly growing embryo, as well as primitive macrophages and megakaryocytes (Figure 1) (Ferkowicz and Yoder, 2005; Moore and Metcalf, 1970; Palis et al., 1999; Xu et al., 2001). The temporal and spatial association of both endothelial and hematopoietic cells in the YS of chick embryos led to the proposition of a common precursor for these two lineages called "hemangioblasts" (Murray, 1932; Sabin, 1920). The idea was later supported by studying some transgenic animals in which both hematopoietic and endothelial cells were affected (reviewed in Boisset and Robin, 2012; Kaimakis et al., 2012). Hemangioblasts are formed in the primitive streak, migrate to the YS, and differentiate into endothelial, hematopoietic, and vascular smooth muscle cells (Huber et al., 2004; Ueno and Weissman, 2006).

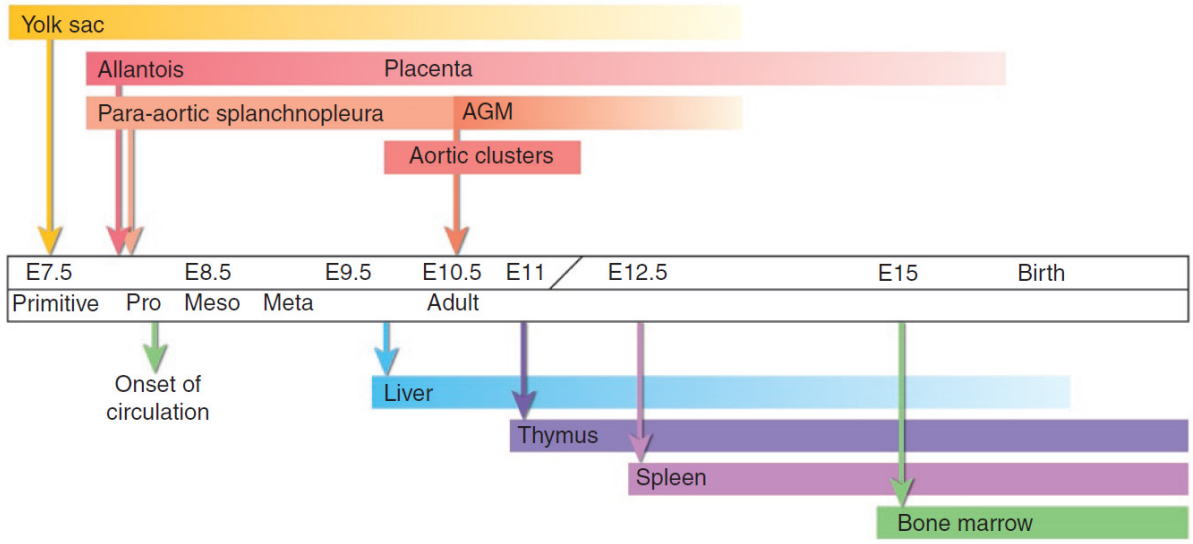

Figure 1. Timeline of hematopoietic progenitor and stem cells appearance in the developing mouse embryo. Arrows on top shows the onset of hematopoietic cell generation or appearance in the certain tissues. Arrows on bottom indicate the start time of colonization in the secondary hematopoietic tissues (adopted from Dzierzak and Speck, 2008).

The second wave of hematopoiesis begins at E8-8.5 when definitive hematopoietic progenitors are generated de novo in the YS, chorion, allantois (which form the placenta and

umbilical cord later) and para-aortic splanchnopleura (PSp) (Lux et al., 2008; Palis et al., 1999; Rhodes et al., 2008). Explant culture of YS and PSp before establishment of circulation showed the presence of progenitors in these tissues, indicating the de novo generation of hematopoietic progenitors (Cumano et al., 1996). Moreover, transplantation assays into neonatal recipients with the E9 yolk sac cells from Ncx1-/- mice (which lack the heart beat) showed that the YS de novo generates hematopoietic progenitors with B and T lymphoid potentials (Yoshimoto et al., 2011; Yoshimoto et al., 2012). Next at E10, the spleen colony forming unit (CFU-S) progenitors appear in the YS and intra-embryonic aorta-gonad-mesonephros (AGM) region (Medvinsky et al., 1993).

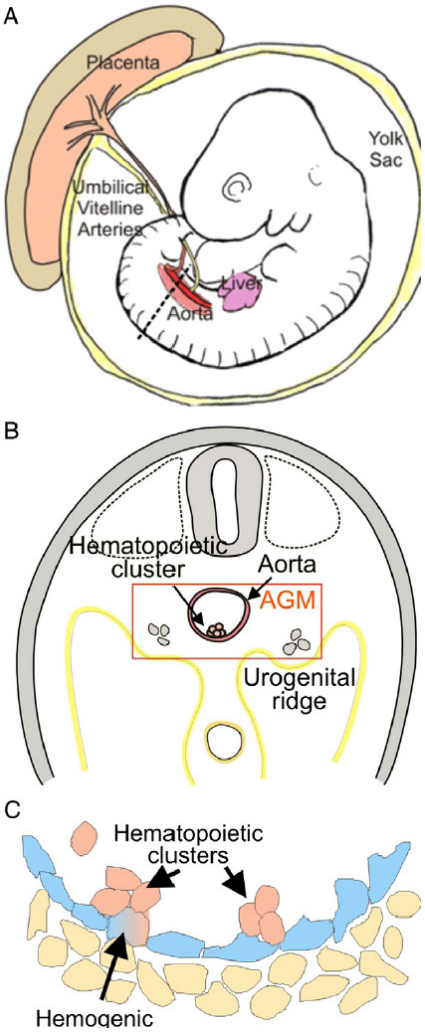

Figure 2. E10.5 mouse embryo harbors hematopoietic stem cells. (A) HPSCs appear or emerge in different anatomical sites, including aorta, fetal liver, yolk sac, placenta, and vitelline and umbilical arteries. Definitive HSCs first appear in aorta. The dotted line through the embryo indicates the transverse section depicted in panel B. (B) Tranverse section through embryo: Aorta-gonad-mesonephros (AGM) region is depicted in a red box. AGM is surrounded by neural tube and somites on the dorsal side and by gut on the ventral side. Hematopoietic clusters are shown on the ventral endothelial wall of the aorta. (C) Close-up of the region in the box. Hematopoietic clusters emerge from hemogenic endothelium lining the dorsal aorta (adopted from Kaimakis et al., 2012)

The third and most important wave of hematopoiesis during mouse embryonic development is the generation of the definitive HSCs. The first transplantable HSCs are detected and generated in the AGM at E10.5 (Medvinsky and Dzierzak, 1996; Muller et al., 1994) (Figure 2A). In addition, HSCs are found in vitelline and umbilical arteries at this stage (de Bruijn et al., 2000). Confocal live-imaging of the dorsal aorta demonstrated the emergence of hematopoietic cells from endothelial cells lining the ventral part of dorsa aorta called "hemogenic endothelium" (Boisset et al., 2010) (Figure 2B and C). Later at E11-11.5, HSCs are detected in the YS, placenta, and fetal liver (FL) (Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). Thereafter, FL remains the main tissue for colonization and expansion of HSCs. The spleen starts to be colonized at E12.5, harboring mostly multipotent hematopoietic cells (Delassus and Cumano, 1996; Godin et al., 1995). Hematopoietic cells colonize the thymus at E12-13 (Godin et al., 1995). At E17, HSCs start to migrate to the BM, the main hematopoietic tissue for the entirety of adult life (Christensen et al., 2004).

Bone marrow HSC microenvironment

The bone marrow is a complex microenvironment composed of different kinds of cells contributing to the "niche" that supports HSC regulation, myelopoiesis and lymphoiesis (Calvi et al., 2003; Kiel et al., 2005; Mendez-Ferrer et al., 2010). In adults, 75% of HSCs are quiescent (G0), and 8% enter the DNA synthesis (S) and self-renew or differentiate to progenitor cells (Cheshier et al., 1999; Wilson et al., 2008). Because of the limited life span of circulating hematopoietic cells, some HSCs are active and contribute to the replace old hematopoietic cells with new ones. Such a demanding job may lead to exhaustion of HSCs; however, the balance between the quiescent and proliferative states of HSCs is tightly regulated by intrinsic and extrinsic factors of the surrounding niche (Figure 3).

The HSC niche is composed of endothelial cells, osteoblasts, mesenchymal cells, reticular cells, etc. Among these components, endothelial cells and osteoblasts have been demonstrated to regulate HSC function. The use of SLAM ($CD150^+$ $CD48^-$ $CD244^-$) markers in identifying HSCs showed that 60% of these cells are located close to sinusoidal endothelium, while 14% of them are located in endosteal zone (Kiel et al., 2007; Kiel et al., 2005). It has been suggested that HSCs are located in hypoxic zones close to endosteal areas where they are maintained in a quiescent state to avoid their exhaustion and differentiation (Jang and Sharkis, 2007; Kubota et al., 2008; Parmar et al., 2007; Zhang et al., 2003), a characteristic that is crucial for their long-term repopulating activity (Cheshier et al., 1999; Wilson et al., 2008). However, more committed progenitors and cycling HSCs localize in close proximity to vasculature in the BM (Ding et al., 2012; Kiel et al., 2005; Kopp et al., 2005; Li and Li, 2006) (Figure 4). Time lapse observation of the BM with multi-photon microscopy demonstrated that transplanted HSCs detach from the endothelium and penetrate deep into the BM, which is a hypoxic zone (Lo Celso et al., 2009). Hence, it has been proposed that the vascular niche, which is rich in nutrient and oxygen by sinusoidal blood, is the proliferative niche, whereas endosteal niche,

which is poor in nutrient and oxygen, represents the quiescent niche (Hidalgo et al., 2002; Katayama et al., 2003; Schweitzer et al., 1996; Sipkins et al., 2005; Xie et al., 2009). Below, we briefly discuss the HSC niche components and some of their key regulatory factors.

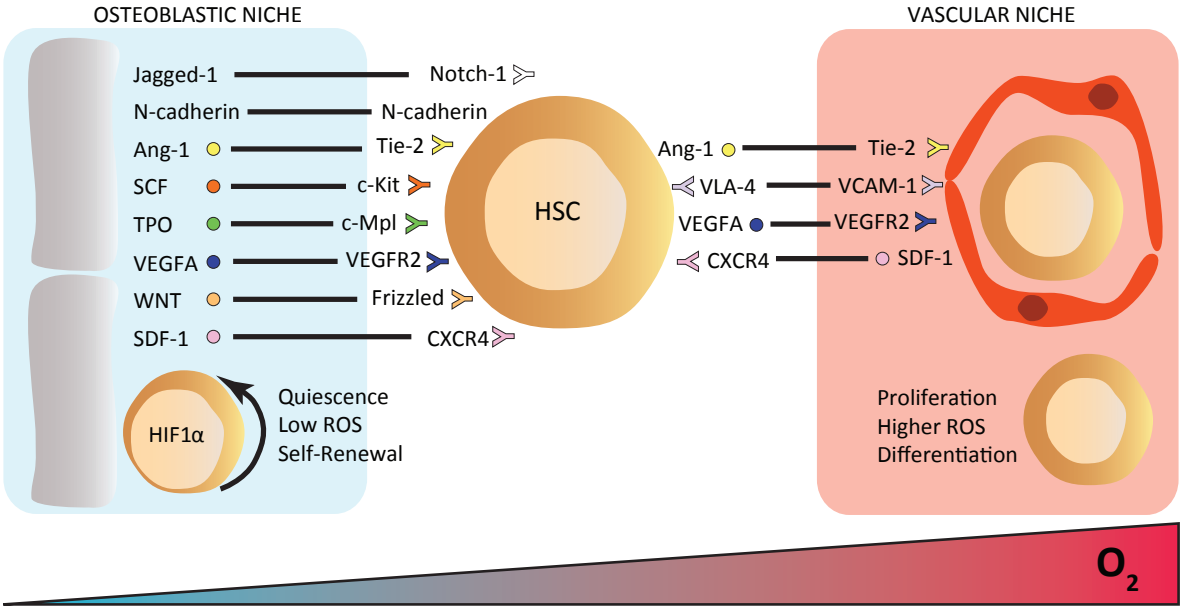

Figure 3. Bone marrow niche regulates self-renewal and differentiation activities of the hematopoietic stem cells. Osteoblasts and vascular cells constitute the two main HSC niches that tightly regulate HSC behavior through the indicated receptors and soluble factors.

Osteoblastic niche

Some HSCs localize close to endosteal surface, which suggests that the osteoblasts play a role in maintenance and regulation of HSCs (Lord and Hendry, 1972; Patt and Maloney, 1972). Osteoblasts are differentiated from mesenchymal precursors in response to bone morphogenetic protein (BMP) signaling. Manipulation of BMP signaling via the BMP receptor type IA (BMPRIA) revealed an increase in the number of osteoblastic cells, which resulted in ~2.4-fold increase in the HSC number (Zhang et al., 2003). In another study, overexpression of parathyroid hormone (PTH) and PTH receptor (PPR) in osteoblasts led to an increase in the osteoblast number, which in turn resulted in ~2-fold increase in HSC number (Calvi et al., 2003). Additionally, osteoblasts regulate the maturation and differentiation of B-lymphocytes (Wu et al., 2008). These observations suggest that osteoblastic cells constitute a supportive niche for HSCs in the BM. The interaction of HSCs with osteoblasts is mediated by many cell adhesion molecules and soluble factors (Figure 3) (reviewed in Coskun and Hirschi, 2010; Eliasson and Jonsson, 2010), some are briefly described here.

Notch receptor signaling between osteoblasts and HSCs regulates the maintenance of HSCs. Attachment of HSC to osteoblast via Notch ligand (Jagged-1) activates the PTH receptor and thereby results in an increase of HSC numbers (Calvi et al., 2003). Furthermore, it has been shown that Notch signaling prevents HSC differentiation and promotes its self-renewal and expansion (Weber and Calvi, 2010). Notch signaling is upregulated in HSCs interacting with the endosteal niche and is downregulated in differentiated hematopoietic cells (Duncan et al., 2005). Also Notch-1 is upregulated under hypoxic conditions (Gustafsson et al., 2005; Jogi et al., 2002).

N-Cadherin is expressed in both HSCs and osteoblasts, suggesting its role in homotypic interactions between these cells (Zhang et al., 2003). Like Notch, N-Cadherin has a function in HSC self-renewal and quiescence. Quiescent HSCs with moderate expression of N-Cadherin interact with highly N-Cadherin expressing osteoblasts (Haug et al., 2008). Moreover, when N-Cadherin is overexpressed in osteoblasts, the HSC-osteoblast adhesion increases, resulting in more quiescent/less differentiated HSCs (Wilson et al., 2004).

Stem cell factor (SCF) is secreted by osteoblasts and interacts with the SCF receptor (c-Kit; transmembrane tyrosine kinase receptor) activating intracellular transducer molecules that regulate HSC activity in vivo (Ikuta and Weissman, 1992; Miller et al., 1997; Miller et al., 1996) and self-renewal in vitro (Audet et al., 2002; Zandstra et al., 1997). In addition, it has been shown that c-Kit expression increases in hypoxia (Jogi et al., 2002). Mutations in c-Kit and SCF lead to reduced HSC activity and a defective HSC niche, respectively (McCulloch et al., 1964; McCulloch et al., 1965). Mice deficient in Lnk (one of the downstream effectors of SCF) exhibited increased number of HSCs (Ema et al., 2005) and the HSCs from these mice showed higher self-renewal activity in vitro (Seita et al., 2007). Thus, this data suggests that SCF regulates HSC potential in vivo and HSC self-renewal activity in vitro.

Angiopoietin-1 and -2 (Ang-1 and -2) are secreted by osteoblasts and the receptor for these ligands, Tie2, is expressed on HSCs. This signaling axis is thought to prevent HSC exhaustion (Arai et al., 2004). Upon binding of Ang-1, Tie2 is phosphorylated and the downstream phosphatidylinositol 3-kinase (PI3-K)/AKT signaling is activated, resulting in activation of the cell cycle regulators like p21, which induces cell cycle arrest (Li et al., 2002). Tie2+ HSCs in the BM are considered to be quiescent HSCs (Arai et al., 2004). Furthermore, Tie2-deficient and wild type (WT) ES cell chimeric animal studies demonstrated that Tie2 deficiency has no effect on embryonic hematopoiesis, whereas it affects adult BM hematopoiesis. Tie2-/- BM HSCs do not engraft irradiated adult recipients in competition with WT cells in transplantation assays (Puri and Bernstein, 2003).

Thrombopoetin (TPO)-expressing osteoblasts interact with HSCs expressing myeloproliferative leukemia virus oncogen receptor (Mpl) (Yoshihara et al., 2007). Mpl is expressed more in quiescent HSCs as compared to cycling ones (Arai et al., 2009). Studies of mice deficient for Mpl

or TPO have suggested that their interactions play a role in the quiescence of HSCs (Alexander et al., 1996; Kimura et al., 1998). Furthermore, there is some evidence that hypoxia inducible factor (HIF)1α protein is stabilized in normoxia by some receptor-mediated factors such as SCF and TPO (Pedersen et al., 2008; Yoshida et al., 2008); however, this may not have any biological significance in vivo. HIF1α is the master regulator of hypoxia which will be discussed in detail later in this chapter.

Canonical **Wnt** signaling is active in the adult BM. Some studies suggest that Wnt signaling supports the self-renewal activity of HSCs to maintain their reserves (Reya et al., 2003). However, there is controversy between in vitro and in vivo data obtained from different labs. Retroviral introduction of β-catenin in long-term cultures of HSCs increases the HSC number by 103-fold (Reya et al., 2003). Also, in vitro treatment of HSCs with recombinant Wnt3a results in higher in vivo reconstitution activity of HSCs (Willert et al., 2003). However, in vivo studies analyzing mice that stably express β-catenin, show an overall hematopoietic differentiation defect and a loss of HSC repopulating activity (Kirstetter et al., 2006; Scheller et al., 2006). In another study, the conditional knockout of β-catenin in hematopoietic cells shows no defect in HSC self-renewal activity (Cobas et al., 2004). This may suggest that in vivo β-catenin may affect HSC differently because of the presence of other regulatory signals.

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis during development and throughout adult life. It is one of the target genes of the HIF transcription factors that are active during hypoxia (Haase, 2010; Semenza and Wang, 1992; Yoon et al., 2006; Yu et al., 1999). HIF1α and HIF2α regulate EPO production in the fetal liver and kidney to promote erythroid progenitor proliferation and differentiation. Rankin et al. showed that osteoblasts produce EPO through a HIF-dependent mechanism in mouse. Overexpression of HIF signaling factors in osteoprogenitor cells leads to an increase in vascularization, overexpression of VEGF in the BM, and increased red blood cell production in an EPO-dependent manner (Rankin et al., 2012). Their study introduced osteoblasts as a new source of EPO production and presented a mechanism by which hematopoiesis is regulated by osteoblasts.

Bone marrow vascular niche

Endosteal and vascular niches are both required to maintain and regulate HSC function, but each has its own unique function. The association of hematopoietic cells with vasculature and generation of hematopoietic cells from endothelial cells during embryonic development indicate that the vascular endothelium acts as an important part of the niche. Total destruction of endosteal niche by local irradiation leads to the shift of hematopoiesis from bone marrow to the highly vascularized spleen (Klassen et al., 1972). In one study, in vivo transplantation of phenotypically identical HSCs isolated form endosteal niche and central part of the marrow showed that the HSCs from endosteal and endothelial niches possess different engraftment

abilities (Grassinger et al., 2010). Note that the vascular niche is not restricted to the central part of the BM and the endosteal zone is by itself highly vascularized. Thus, the endosteal and vascular niches are not fully separated but they play their independent roles. The molecular mechanisms by which the vascular niche regulates the HSC function are not known completely. Nevertheless, here we briefly discuss some of the proposed mechanisms operating in the vascular niche (reviewed in Coskun and Hirschi, 2010).

Cell-to-cell interactions

Interactions between endothelial cells and HSCs have been studied by coculture experiments of adult hematopoietic cells with endothelial cells derived from both adult and embryonic tissues. Seven-day coculture of adult $CD34^+$ BM cells with brain endothelial cells results in increased number of $CD34^+$ BM cells and increased repopulating activity of HSCs (Chute et al., 2002). The same result was obtained from coculture experiment with porcine microvascular endothelial cells (Brandt et al., 1998). Additionally, coculture of adult BM LSK cells with primary endothelial cells isolated from E9 YS and PSp leads to 9.4- and 11.4-fold increase in the hematopoietic progenitor activity, respectively. Long-term repopulating activity of LSK cells was also increased (Li et al., 2003). Note that endothelial cells derived from different tissues possess different abilities to regulate hematopoietic progenitor and stem cell (HPSC) function.

Endothelial cell adhesion molecules and cytokines

The vasculature in the BM is unique in the array of adhesion molecules it expresses (except in the case of inflammatory reactions). This vasculature allows the trafficking and homing of HPSCs to the BM through the expression E-selectin, P-selectin, VCAM1, and ICAM1 (Mazo et al., 1998). Stromal-derived factor 1 (SDF-1) is one of the most well-known cytokines secreted by BM endothelial cells (also expressed by osteoblasts) (Ponomaryov et al., 2000; Sipkins et al., 2005). SDF-1 mediates the migration of circulating HPSCs to the niche by binding to the CXCR4 receptor expressed on the surface of these cells (Peled et al., 1999a; Peled et al., 1999b). SDF-1 and CXCR4 knockout animals exhibit defects in BM hematopoiesis (Ara et al., 2003; Nagasawa et al., 1996; Zou et al., 1998). Also, it has been shown that the expression of SDF-1 is regulated by HIF1α, which is induced by hypoxic conditions (Ceradini et al., 2004). HIF1α-induced expression of SDF-1 promotes the migration and homing of circulating CXCR4+ progenitor cells to ischemic sites of injury.

Vascular endothelial growth factor (VEGF) is a well-known angiogenic factor regulated by hypoxia and HIF (Peled et al., 1999a; Shweiki et al., 1992). The two major receptors of VEGF are VEGFR1 (Flt1) and VEGFR2 (Flk1 or KDR), which are expressed on endothelial as well as hematopoietic cells (Hattori et al., 2002; Kabrun et al., 1997). VEGFA has been shown

to be important in hematopoiesis. Complete or haploinsufficient VEFGA-deficient animals die early during development because of failure in hematopoietic and vascular development (Carmeliet et al., 1996; Ferrara et al., 1996). Interestingly, VEGFA-deficient HSCs fail to reconstitute the hematopoietic system of irradiated mice despite the presence of VEGFA in the microenvironment, thus demonstrating a cell-intrinsic role for VEGF in HSCs. Blocking the VEGFA receptor activity by neutralizing antibodies has no effect on the in vitro colony forming ability of BM cells while intracellular inhibition of VEGFA signaling leads to apoptosis. This data demonstrate that the effect of VEGFA on HSCs is mediated via an internal autocrine loop (Gerber et al., 2002). In addition, VEGF regulates HSC function via an extrinsic mechanism. Establishment of the osteoblastic niche depends on VEGFA availability (Chan et al., 2009). Irradiated Flk1-deficient BM is not successfully reconstituted by transplanted WT HSCs due to the failure in regeneration of sinusoidal vasculature of BM (Hooper et al., 2009).

Additionally, a hypoxic niche is essential for upregulation of VEGFA in HSCs. Rehn et al. used mouse model Vegfδ/δ in which the hypoxia-responsive element (HRE) in the VEGF locus was conditionally removed in hematopoietic cells. HIF1α could no longer bind to the promoter region of VEGF and thereby inhibited its upregulation in hypoxia. Under such circumstances, the repopulating ability of HSCs was affected in spite of the increase in phenotypic HSC number (Rehn et al., 2011).

Oxygen gradient in adult bone marrow

Several studies indicate that long-term repopulating HSCs (LTR-HSCs) localize mainly in the endosteal zone of the BM, which has a unique vascular organization. Arteries penetrate the cortical bone, enter the medullary canal and then to extend to the metaphyseal region. The arterioles derived from small arteries subdivide into arterial capillaries. The blood in these capillaries flows into sinusoids that are loosely organized, allowing HSCs to move easily across them. Consequently, perfusion rate is limited in this region and the oxygen tension is very low (Draenert and Draenert, 1980). In addition, the BM is densely populated by hematopoietic cells whose oxygen consumption is high. A stimulation study showed the oxygen tension decreases 10 fold at a distance of several cells from the closest capillary (Chow et al., 2001a, b). As a result, the distinguishing characteristic of the BM microenvironment is that it is hypoxic. Using a mathematical modeling of pO2 distribution in the BM, Chow and colleagues suggested that oxygen concentration is less than 1% in the BM and thus, HSCs are located in a very low-oxygen region (almost anoxic), where they are protected from harmful oxygen radicals (Chow et al., 2001a, b). However, this challenging approach is based on an indirect measurement.

Cells low in oxygen (≤2%) can be identified using hypoxyprobes, which are 2-nitromidazole drugs such as pimonidazole (Mahy et al., 2003).Immunohistochemistry for hypoxyprobe on BM sections demonstrates that most of the hypoxic cells localize in sinusoids far from capillaries close to the bone surface. The hypoxic cells show low level of c-Kit expression. C-Kit+ cells and actively proliferating hematopoietic cells were observed close to capillaries,

which are considered as vascular niche. Long-term label-retaining strategy for BrdU showed that label-retaining 'quiescent' hematopoietic cells are most abundant in the sinusoids of the endosteal zone. Hence, quiescent HSCs are located in the sinusoidal hypoxic sites within the BM (Kubota et al., 2008).

Parmer et al. presented direct evidence that there is oxygen gradient in the BM and that the most immature hematopoietic cells reside in the lowest end of the oxygen gradient in the BM niche. These results implied that a hypoxic microenvironment is required for the proper function of HSCs (Parmar et al., 2007). They applied diffusible Hoechst dye, which yields a visualized gradient of intensity based on the dye uptake and perfusion rate of the cells to indicate their oxygenation status. BM cells were separated to six different subsets based on Hoescht efflux/oxygen level. Staining with hypoxyprobe for different BM fractions confirmed that the cells with lowest intensity of Hoechst were the most hypoxic ones. In vivo transplantation assay with the cells from different subsets along the dye perfusion gradient showed that cells with lowest dye fluorescence possessed 10 times more repopulating activity than whole BM cells and the lowest dye-perfused BM cells contain 90-200 times more HSCs compared to the highest dye-perfused cell fraction. This observation clearly shows that HSCs in the BM are concentrated in the most hypoxic region of this tissue.

Takubo and colleagues performed in vivo injection of pimonidazole (hypoxyprobe), followed by flow cytometric analysis to study the oxygenation status of mononuclear cells (MNCs) directly in the BM. Based on the intracellular uptake of the pimonidazole, the BM MNCs were divided to three different fractions: highly, moderately, and weakly positive for pimonidazole. This indicates that the oxygen levels differ in subpopulations of BM hematopoietic cells. Next, they characterized the hematopoietic cells in the three fractions and found that 84.9% of the quiescent HSCs (Tie2+ LSK cells) were located in highly hypoxic cell fraction. The highly hypoxic fraction also contained 68.2% of LT-HSCs (SLAM LSK cells) and 64.2% of LSK cells. In contrast, the least hypoxic fraction contained just 1.8% of LT-HSCs and 1.8% of the LSK cells. These findings strongly indicate the hypoxic nature of HSCs and the BM niche (Takubo et al., 2010) and confirm that the most immature HSCs are enriched in the highly hypoxic fraction of the BM.

Oxygen level and embryo development

About 2.2 billion years ago, oxygen accumulation due to photosynthesis reactions reached a level that was probably toxic to many obligate anaerobic organisms, resulting in a massive extinction of them (Oxygen Catastrophe). The organisms that could protect themselves against oxidative stress and, at the same time use oxygen to provide energy, expanded and prospered. Overtime, oxygen concentration reached to a high level of ~21% in the Earth's atmosphere and a solubility of ~5 ml/L in water. O_2 level depends on atmospheric pressure, temperature, and water salinity (Fisher and Burggren, 2007). As time went on, the requirement for oxygen became critical and animals developed biochemical and metabolic responses to low levels

of oxygen that promote hypoxia tolerance pathways. The most important ones include the energy and nutrient sensor mTOR, the nuclear factor (NF)-κB transcriptional response, and HIF transcription factors as the most important ones (Perkins, 2007; Wouters and Koritzinsky, 2008).

The role of hypoxia and HIFs in embryogenesis and tissue formation has been widely analyzed. We note that the term "hypoxia" in this chapter describes the naturally low oxygen (usually 2%) level present in normal development, which is a physiological hypoxia or *in situ* normoxia, and is distinct from non-physiological and pathological hypoxia experienced by tumor cells and normal cells in ischemic syndromes.

The association between mammalian embryogenesis and O_2 concentration was first suggested in 1970s by experiments showing that successful neural fold development in *ex utero* cultured mouse embryo is dependent on a hypoxic culture condition (Morriss and New, 1979). Since then, the cloning and characterization of the *HIF* complex has revealed discrete molecular mechanisms through which embryonic development is regulated by oxygen levels. (Bruick, 2003; Semenza, 1999). Developmental defects in knockout embryos clarified the importance of HIF function in ontogeny (reviewed in Dunwoodie, 2009; Simon and Keith, 2008). Normal embryonic development occurs in a hypoxic environment with oxygen levels ranging from 1 to 5% in the uterus (Okazaki and Maltepe, 2006) with HIFs responsible for many aspects of development.

To examine what parts of the embryo are hypoxic, Lee and colleagues performed hypoxyprobe immunohistochemistry on mid- and late-gestational stage embryos (Lee et al., 2001). At E8.5-9, the hypoxic regions were detected in folding neural tube and neural mesenchyme cells as well as in YS, allantois, and ectoplacental cone and decidua (Figure 4A and B). At E9.5-11.5, the hypoxic regions spread into neural tube and mesenchymal regions of the head. At E12.5, intersomitic mesenchyme, the internal lining of the cranial flexure, myelencephalon, choroid plexi, the center of maxillary prominence, ventral and perineural regions were highly hypoxic (Figure 4C). A similar pattern was observed at E13.5 as well. Later at E16.5, the hypoxic regions were distributed to the olfactory lobe, some connective tissues of craniofacial region, and cerebral cortex. Liver, kidney, heart, and gastrointestinal tract were partially hypoxic (Figure 4D) (Lee et al., 2001). In another study, in E14.5 embryos, the hypoxic sites are detectable in developing heart, gut, and skeleton (Figure 5) (Ream et al., 2008). This data indicates that despite the presence of cardiovascular system, the cells of the embryo can still experience hypoxia as oxygen level. This is probably due to high oxygen consumption of cells in some tissues, their proximity to blood vessels, and the tissue architecture. It is likely that the developing embryo lacks the capacity to keep pace with the phenomenal growth and the energy demand during the second half of the gestation.

The YS is hypoxic already at early embryonic stages, most likely related to ongoing vasculogenesis in this tissue (Lee et al., 2001). That hypoxia acts as a signal for the angiogenesis and vasculogenesis is supported by the observation that *HIF1α* KO mice show abnormal cephalic vascularization (Maltepe et al., 1997). When the hypoxic response is abolished, hypoxia does not induce angiogenesis, vasodilation or erythropoiesis, and as a result, neural

development is defective even under local low oxygen tension. However, in WT embryos the local hypoxia, which is partly generated by the local cell proliferation, activates the hypoxia response that induces angiogenesis and erythropoiesis to maintain the oxygen homeostasis.

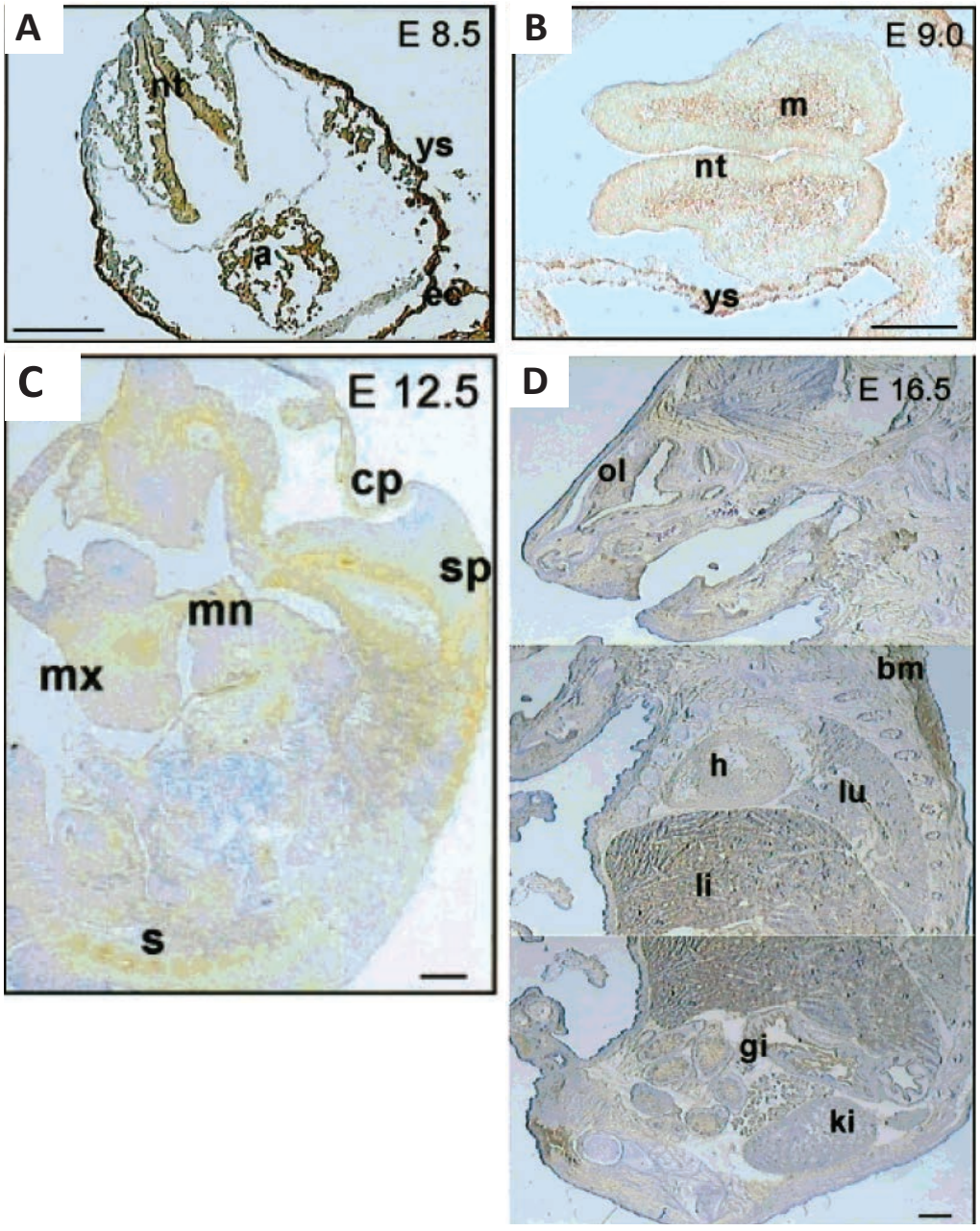

Figure 4. Detection of hypoxic cells by pimonidazole in developing mouse embryo. (A) Section of anterior neural fold at E8.5 indicates that neural tube (nt), head mesenchyme, allaontois, yolk sac (ys), and ectoplacental cone (ec) are hypoxic. (B) In E9 embryos, yolk sac, brain mesenchyme (m), and neural tube are hypoxic. (C) Sagittal section through E12.5 embryos shows that chroid pleux (cp), ependemal layer, marginal layer and mesenchymal cells of brain, maxillary (mx), mandibullar prominences (mn), spinal cord (sp), and intersomitic regions were hypoxic. (D) In E16.5 embryos, cerebral hemisphere, connective tissues of craniofacial region, olfactory lobe (ol), back muscle (bm), and gastrointestinal tracts (gi) were highly hypoxic. Liver (li), heart (h), kidney (ki), and lung (lu) were weakly stained. Scale bar=200μm (adopted from Lee et al., 2001).

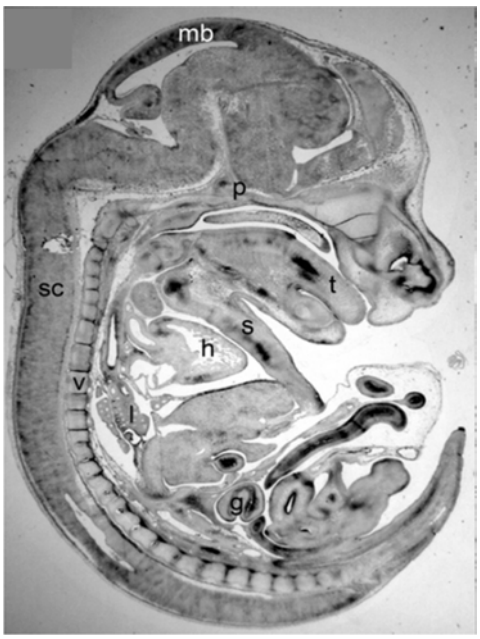

Figure 5. Cellular hypoxia in the mouse embryo. Piminidazole staining of E14.5 embryo showed hypoxic cells in the heart (h), gut (g), lung (l), midbrain (mb), pituitary (p), spinal cord (sc), sternum (s), tongue (t), and vertebra (v) (adopted from Dunwoodie, 2009)

Lee and colleagues checked whether the pattern of hypoxia marker expression matches the HIF1α immunostaining pattern. In E13.5 brain, the marginal layers appeared to be hypoxic by the colocalization of piminidazole and HIF1α, while ependymal regions appeared to be weakly labeled for both piminidazole and HIF1α. The same expression pattern was observed for VEGF. They explained that the marginal layer of the neural tube and mesenchyme in brain is highly hypoxic because the proliferative cells migrate to this area. This data is in agreement with findings that the hypoxic sites in tumors are adjacent to the proliferative cell region (Durand and Raleigh, 1998; Shweiki et al., 1995; Varia et al., 1998; Waleh et al., 1995). They discussed another possibility that perhaps the injected piminidazole does not diffuse to the ependymal regions of the embryo within a certain time, although it has been shown that the dosage of 100mg/kg and time of 3 hours are sufficient for efficient binding. Furthermore, they checked the VEGF expression pattern and found out that VEGF colocalized with hypoxic regions in brain, trunk, heart, and intersomitic vessels. These findings suggest that probably hypoxia acts as a signal for VEGF expression that attracts endothelial cells to supply blood in the developing tissue. However, there is no direct evidence and further investigation of hypoxia-regulated genes in the embryo is required.

When hypoxia occurs, HIF transcription factors activate genes that are involved in erythropoiesis, angiogenesis, energy metabolism, autophagy, etc. depending on the context. The goal of this regulation is to promote tolerance against hypoxia by decreasing the oxygen requirement and increasing the supply of oxygen (Dunwoodie, 2009). The roles of hypoxia and HIFs were elaborately investigated in development of stem cells, placenta, tracheal, cardiovascular system, bone morphogenesis, and adipogenesis (reviewed in Dunwoodie, 2009; Simon and Keith, 2008).

Impact of oxygen level and HIF activity on placenta development

The placenta is a tissue derived from extraembryonic cells of the conceptus and forms at E8/8.5 in mouse embryo (Cross et al., 2003). It provides oxygen, nutrients, and immunoprotection for developing embryo. The placenta is a highly vascularized tissue and it harbors a large pool of hematopoietic progenitors and HSCs (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Placenta formation is such a complex process that at least 85 genes have been identified as playing a role in its development (Watson and Cross, 2005).

The mature placenta consists of three different fetal layers (Dunwoodie, 2009): Proximal to the embryo is the labyrinth, where the fetal vasculature and maternal blood sinuses are brought into proximity to allow the nutrient and oxygen exchange. Next to the labyrinth is the spongiotrophoblast layer that provides structural support for the developing labyrinth layer and is a source for trophoblast cells. The trophoblast giant cell (TGCs) layer borders the decidua. These cells facilitate the embryo implantation and decidua invasion, and produce the pregnancy hormones. The placenta develops in a hypoxic environment and some of placenta cells remain hypoxic until late in the development, despite the flow of maternal blood (Okazaki and Maltepe, 2006; Withington et al., 2006).

Hypoxic cells and the cells expressing HIFs are present in decidua and placenta between E6.5 to E14.5 (Pringle et al., 2007; Schaffer et al., 2006; Withington et al., 2006). Germline deletion of *HIF1α*, *HIF2α*, and *HIFβ* in mouse leads to failure in placenta formation and embryo lethality by E10.5 (Abbott and Buckalew, 2000; Adelman et al., 2000; Cowden Dahl et al., 2005a; Cowden Dahl et al., 2005b; Kozak et al., 1997). The key placenta defects are: incomplete labyrinth development, limited vascularization of placenta, failure in chorion-allantois interactions, trophoblast differentiation, and the higher number of TGCs. It has been shown that the defect in chorion-allantois interaction is the result of the impaired integrins and ligands such as Itga4, Cyr61, and VCAM1 (Watson and Cross, 2005), some of which are induced by hypoxia (Cowden Dahl et al., 2005a; Cowden Dahl et al., 2005b; Kilburn et al., 2000). Also vascularization of placenta depends on HIF activity as some of HIF-target genes like *VEGF, Flk1, Ang1* and *Tie2* are reduced in their expression in the knockout embryos (Abbott and Buckalew, 2000). In addition, HIFs are required for trophoblast proliferation and differentiation (reviewed in Dunwoodie, 2009).

Immunohistochemistry on the E5.5-E7.5 placenta showed that many in different cell types of the placenta are HIF1α-expressing and hypoxic (Pringle et al., 2007).

Maintenance of HPSCs depends on oxygen concentration

Many studies have investigated the role of hypoxia on mouse/human hematopoietic activity, comparing the survival and proliferation, hematopoietic progenitor activity, *in vivo* repopulating activity and cell cycle of cultured hematopoietic cells in normoxia (20% O_2) versus hypoxia (1-5% O_2). The studies differed in the donor tissues examined, the hematopoietic culture system

used (detecting at least 5 different progenitor types), level of oxygen, time of exposure to hypoxia, and different combinations of cytokines granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukine-3 (IL3) and -6 (IL-6), stem cell factor (SCF), thrombopoietin (TPO), and Fms-like tyrosine kinase 3 (FLT-3) ligand in the culture medium. Depending on the culturing conditions, the number of hematopoietic cells decreased or did not change compared to time zero. Hematopoietic progenitor activity decreased, did not change, or increased (although the increase was not as much as the increase in normoxia). For human cells, increases in BFU-E were reported in some studies. Hypoxia improved the repopulating activity of HSCs in most cases since hypoxia is known to maintain HSCs in a low cycling primitive state, which improves their repopulating activity.

Cipolleschi et al. showed that incubation of mouse BM cells in 1% oxygen reversibly blocks their expansion and CFU-GM progenitor activity. CFU-C cultures were supplemented with GM-CSF and IL3. Also, in hypoxia-incubated cultures treated with 5-FU (a cell cycle-specific cytotoxic drug), a considerable number of hematopoietic cells and CFU-GM colonies were generated while few viable hematopoietic cells and no progenitor activity were observed in normoxia cultures, suggesting that hypoxia cultures contained some 5-FU-resistant hematopoietic cells and progenitors (Cipolleschi et al., 2000). Ivanovic et al. improved the culturing condition by using IL3, IL6, GM-CSF, and SCF. The number of BM cells in hypoxia was initially reduced but then reached or passed the time zero level while it increased 3 fold in normoxia. The number of CFU-GM progenitors increased compared to time zero after incubation in hypoxia but not as much as the increase in normoxia cultures. These observations suggest that there are mechanisms that regulate the response of hematopoietic cells to cytokines in hypoxia, and the hypoxic conditions can be optimized by using different combinations of cytokines (Ivanovic et al., 2000a).

In another study, neither committed nor more primitive CFCs could be maintained in 0.9% oxygen culture supplemented with SCF, IL6, GM-CSF with or without IL3. Moreover, CFC generation was completely suppressed in hypoxia when the cells were treated with 5-FU, which is in contradiction with the previous studies. However, hypoxia maintained the CFC activity of $CD34^+$ cells. The percentages of viable mouse BM $CD34^+$ cells that remained undivided were significantly higher after 8 days in hypoxic cultures compared to normoxic cultures. (Ivanovic et al., 2002).

With respect to the effect of hypoxia on mouse HSC function, in one study BM LSK cells were cultured for four days in either hypoxia (1% oxygen) or normoxia in the presence of SCF, TPO, and IL-6. The cell numbers increased in hypoxia only by 28 fold, compared to 168 fold in normoxia. The cell cycle analysis of cultured LT-HSC ($FLT3^-$ $CD34^-$ LSK) showed that HSCs of hypoxic cultures were less dividing, as compared to the normoxic ones. Moreover, the cyclin-dependent kinase inhibitors P21, P27, and P57 were upregulated by 12.7, 12, and 21.2 fold, respectively, in LSK cells cultured for 24 hours in hypoxia. *In vivo* transplantation assay demonstrated the same engraftment level in the recipients of HSCs from both hypoxic and normoxic cultures. However, since a fraction of cultured cells was used for this experiment, it was concluded that *in vivo* repopulating activity was improved in hypoxic cultures due to

the presence of fewer HSCs. The use of HIF1α inhibitors in the culture showed that HIF1α is responsible for upregulation of P21 and P27 in LSK cells (Eliasson et al., 2010).

When human $CD34^+$ cord blood cells were cultured for 14 days in clonogenic medium in severe hypoxia (1% O_2), an increase in erythroid burst-forming unit (BFU-E) and a decrease in CFU-GM colonies was observed (Cipolleschi et al., 1997). However, an increase in both BFU-E and CFU-GM activity in BM $CD34^+$ cells cultured in 1.5% O_2 for six hours reported by another group (Quinlan et al., 1998). In contrast, the culture of peripheral blood (PB) $CD34^+$ cells under hypoxia (1% O_2) for 7 days resulted in a decrease in the total number of CFCs (Ivanovic et al., 2000b).

In another study, the expansion ability of human BM $Lin^- CD34^+$ cells decreased under 1.5% O_2 conditions while that of $Lin^- CD34^+ CD38^-$, a subpopulation enriched in HPSCs, increased. Cell cycle analysis showed that the proliferation rate in hypoxic $Lin^- CD34^+$ cells is less compared to the normoxic ones while they are mostly arrested in G1 phase. Furthermore, the O_2 level had no significant effect on the lineage-committed progenitors with the exception of the CFU-G numbers, which were decreased in hypoxia. However, for the primitive progenitors, the BFU-Es were increased in hypoxia. $Lin^- CD34^+ CD38^-$ cells were cultured in the presence of IL3, IL6, SCF, Flt3 ligand, and G-CSF for 4 days under either hypoxic or normoxic conditions, and were then transplanted into NOD/SCID mice. The hypoxic cultures contained 5.8 fold more SRCs (SCID repopulating cells) than normoxic cultures. Also, HIF1α expression was detected only in the hypoxic $Lin^- CD34^+ CD38^-$ cells (Danet et al., 2003).

Another study analyzed human cord blood $CD34^+$ cells cultured in 0.1% and 20% oxygen levels. IL3 improved the cell survival in both conditions equally. Cell cycle analysis showed a higher number of cells in G0 and a lower number of cells in G1 at 0.1% O_2 compared to 3% and 20% O_2. Moreover, the number of CFU-GM, but not BFU-E and CFU-MIX, colonies was significantly lower in 0.1% O_2 compared to 20% O_2 conditions. The SRC activity of cultured cells was tested after 72 hours of culturing. There was no difference in repopulating activity of hypoxic or normoxic $CD34^+$ cells, but a better myeloid and lymphoid differentiation potential of HSCs was detected in normoxia (Hermitte et al., 2006).

The effect of hypoxia on hematopoietic cells has been also analyzed in a co-culture system. Co-cultures of human PB $CD34^+$ cells and BM stromal cells showed that the proportion of hypoxic cells (detected by hypoxyprobe) in phase-dim cells (the hematopoietic cells that migrate beneath the stromal cells) is much higher compared to phase-bright cells. Also, in hypoxic cultures there are significantly more phase-dim cells compared to normoxic cultures, which suggests that the oxygen level affects hematopoietic cell distribution in the BM niche. Moreover, more VEGFA concentrations were detected in hypoxic mesenchymal cultures and cocultures compared to normoxia. The expression of both HIF1α and HIF2α increased in short-term hypoxic mesenchymal cultures; however, in the longer term HIF1α expression was undetectable. Inhibition of HIF pathway resulted in decreased VEGF production in hypoxic cultures, which suggests its HIF-dependency. This data suggests that hypoxia regulates HSC niche and its interaction with hematopoietic cells *in vitro* (Jing et al., 2012).

Collectively based on the mentioned observations, optimizing the culturing conditions is a

key factor in studying the effect of hypoxia on hematopoietic progenitor and stem cell activity. To this end, understanding the regulatory mechanisms in hematopoietic cells that mediate the responses to hypoxia is crucial.

Effect of hypoxia on BM-derived mesenchymal stem cells

Mesenchymal stem cells (MSCs) have the potential to differentiate to the bone, cartilage, fat, and muscle lineages and of interest in tissue engineering and regenerative medicine approaches. They are an important component of the hematopoietic supportive microenvironment. The effect of hypoxia on MSC behavior including their survival, proliferation, and differentiation capacity has been studied (Das et al., 2010). MSCs can withstand an oxygen level of 1% for at least 48 hours and rely on glycolysis to provide ATP. Moreover, their ATP requirement for survival is low. However, 99% of MSCs transplanted into ischemic heart die after 3-4 days, thus limiting the use of MSCs in therapeutic applications (Reinecke and Murry, 2003; Toma et al., 2002; Zhang et al., 2001). The survival rate of hypoxia-incubated MSCs increases by HIF1α-dependent upregulation of EPO/EPOR and anti-apoptotic factors Bcl-2 and bcl-xL. Hypoxia also increases the expression of VEGF and IL6 in MSCs (Hu et al., 2008; Theus et al., 2008). Understanding apoptotic and survival pathways in MSCs can benefit the design of their culturing conditions in potential clinical settings.

Many studies have investigated the proliferation potential of MSCs under hypoxic condition. Lennon and colleagues showed that rat BM-derived MSCs have a 40% higher proliferation rate in 5% O_2 culture compared to 20% O_2 condition (Lennon et al., 2001). Also, hypoxic human MSC cultures showed a 30-fold increase in cell numbers compared to normoxic cultured cells (Grayson et al., 2007). However, there is contradictory data regarding proliferation of MSCs in hypoxia. While one group showed that duration of MSC cell cycling lengthens in hypoxia (Grayson et al., 2006), another group showed that hypoxia decreases population doubling time in MSCs and increases the number of G2/S/M cells (D'Ippolito et al., 2006). Other studies showed either increase or decrease or no change in MSC proliferation in hypoxia (reviewed in Das et al., 2010). In general, a reduced level of oxygen leads to the increase in the number of MSCs either by increased proliferation capacity or decreased doubling time. Culturing MSCs under hypoxic conditions has been shown to enhance the release of some trophic factors, as well as the factors (such as CXCR4) that play important role in mobilization and homing ability of MSCs into sites of injury. As discussed by Das and colleagues, the discrepancies between these data could be due to differences in culturing conditions including level of oxygen, use of oxygen chelators or sealed chambers, serum conditions, duration of hypoxia, and age of the cells (reviewed in Das et al., 2010). Despite the discrepancies, it is clear that oxygen tension is important in MSC biology.

Hypoxia Inducible Factor (HIF/VHL/PHD) signaling

The heterodimeric transcription factor hypoxia-inducible factor (HIF) is the major regulator of molecular response to hypoxia. HIFs interact via Per-Arnt-Sim (PAS) domains, bind to DNA via N-terminal basic helix-loop-helix (bHLH) domains, and activate transcription with C-terminal transcriptional transactivation domains (TADs) (Figure 6). The HIF complex consists of two proteins: an oxygen-sensitive HIFα subunit and an oxygen-insensitive HIFβ subunit (aryl hydrocarbon receptor nuclear translocator, Arnt). Arnt is expressed constitutively, but the expression and activity of HIFα subunits are regulated by cellular oxygen concentration (Wang et al., 1995). Three HIFα subunits have been identified: HIF1α, HIF2α, and HIF3α. HIF1α is expressed ubiquitously, whereas HIF2α and HIF3α expression seems to be restricted to certain tissues (reviewed in Simon and Keith, 2008). HIF3α has three isoforms: HIF3α, neonatal and embryonic PAS (NEPAS), and inhibitory PAS protein (IPAS).

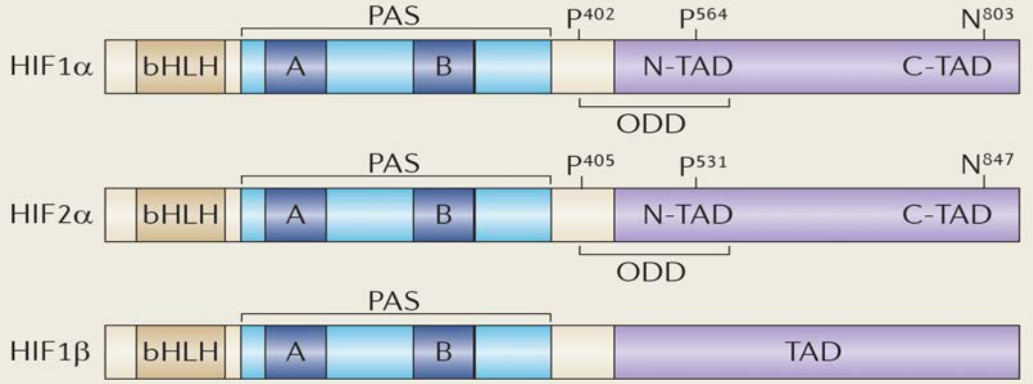

Figure 6. The structural domains of HIF transcription factors. The basic helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains are required in the DNA binding and heterodimerization. The oxygen-dependent degradation domain (ODD) is involved in oxygen-dependent hydroxylation. The N- and C-terminal transactivation domains (N-TAD and C-TAD) are important in transcriptional activation (adopted from Keith et al., 2012).

Regulation of HIF activity is mediated by posttranslational modification of the oxygen-dependent degradation domain (ODD) in the α subunit. In the absence of hypoxia, i.e. at the oxygen level above 5%, prolyl hydroxylase domain protein (PHD1-3) hydroxylates the two prolin residues (402 and 564) in the ODD of HIF1α (in a reaction requiring oxygen, 2-oxoglutarte, ascorbate, and iron as a cofactor) and enables binding of the von Hippel-Lindau tumor suppressor protein, which leads to ubiquitylation and degradation of HIFα by the proteasome (Figure 7) (Jaakkola et al., 2001; Maxwell et al., 1999; Ruas and Poellinger, 2005; Schofield and Ratcliffe, 2005). VHL forms the recognition module of an E3 ubiquitin ligase complex, which consists of elongin C, elongin B, cullin-2, and ring-box 1. Moreover, the factor inhibiting HIF (Fih) hydroxylates an aspargine residue in the TAD of HIFα, preventing binding of the transcriptional coactivator CBP/p300 (Lisy and Peet, 2008). In hypoxic conditions, the rate of prolyl and aspargine hydroxylation is decreased resulting in stabilization and

accumulation of HIFα. Then, HIFα translocates to the nucleus followed by dimerization with HIFβ and binding to the hypoxia responsive element (HRE) sequence in the promoter region of hypoxia-targeted genes (Figure 8) (Maxwell et al., 2001). Of the three HIFα subunits, HIF1α and HIF2α are best studied. HIF1α and HIF2α have 48% amino acid sequence similarity and similar protein structures. They have both common and distinct target genes. HIF3α has high similarity to HIF1α and HIF2α in bHLH and PAS domains. HIF1α and HIF2α have two TADs (Figure 6), HIF3α and NEPAS have one, and IPAS does not have a TAD (Yamashita et al., 2008).

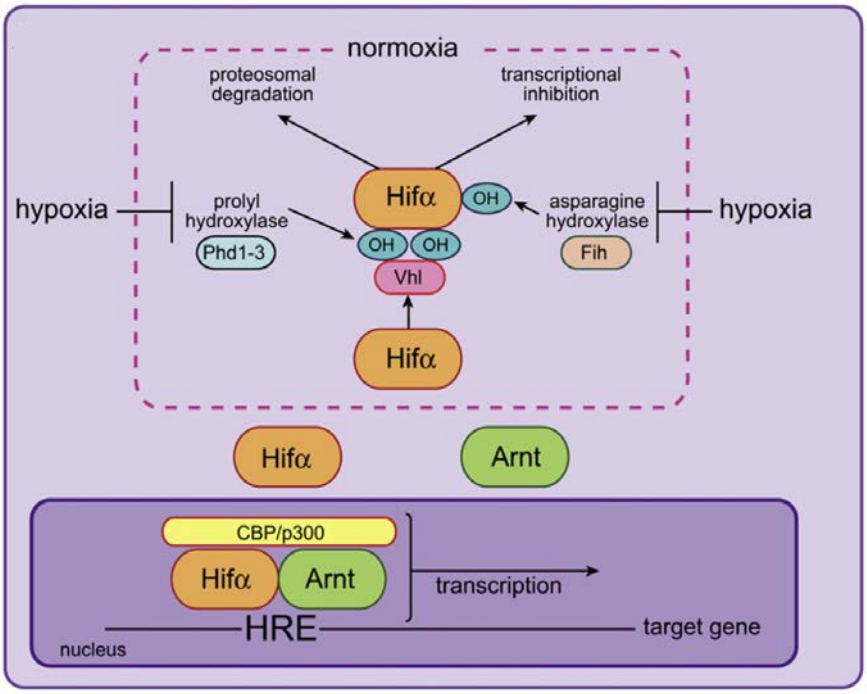

Figure 7. HIF regulation by oxygen level. Under normoxic conditions, the two proline residues in the HIFα subunit are hydroxylated by PHD enzymes using oxygen as a cofactor. The hydroxylated HIFα is recognized by VHL and undergoes ubiquitin-mediated degradation. Also, HIFα is hydroxylated in an aspargine residue in transactivation domain by FIH enzyme, preventing binding of the transcriptional coactivator CBP/p300. In hypoxia, HIFα is not hydroxylated, thereby it is stabilized and translocates to the nucleus, dimerizes with HIFβ, and binds to HRE in the promoter region of hypoxia-targeted genes (adopted from Dunwoodie, 2009).

In addition to VHL, other alternative oxygen-dependent and -independent HIF regulatory pathways have been characterized, some of which are common to both HIF1α and HIF2α, and some are restricted to only one of them. Oxygen-independent regulators include receptor of activated protein kinase C (RACK1), which has a role in HIF1α degradation by competing with heat shock protein (Hsp) 90 for binding to HIF1α, and human double minute 2 (hdm2), through which HIF1α degradation is induced by HIF1α-p53 interaction (Liu and Semenza, 2007; Ravi et al., 2000). Hypoxia associated factor (HAF) promotes HIF1α degradation but induces HIF2α transactivation under long periods of hypoxia. Also, Hsp70/CHIP (carboxyl terminus of Hsp70-interacring protein) degrades HIF1α under long hypoxic or high glucose conditions (reviewed in Koh and Powis, 2012).

The mechanism of oxygen-dependent posttranslational regulation of HIF1α is well understood, but little is known about its transcriptional regulation. Some reports indicate that HIF1α is regulated differentially at the transcriptional level during hypoxia and normoxia (Hirota et al., 2004; Laughner et al., 2001).

Individual and overlapping roles of HIF transcription factors

HIF1α was first described by Semenza and colleagues in 1995 as an oxygen level-dependent transcriptional activator (Wang et al., 1995). HIF2α was identified by other groups who called it endothelial PAS protein 1 (EPAS1) (Tian et al., 1997), HIF-related factor (HRF) (Flamme et al., 1997), HIF1α-like factor (HLF) (Ema et al., 1997), and member of PAS family 2 (MOP2) (Hogenesch et al., 1997). It appears that HIF1α is expressed in all cell types. *HIF2α in situ* hybridization studies in the mouse embryo showed that its expression is more restricted, with high expression in endothelial cells. Also, there is close correlation between mRNA expression patterns of *VEGFA* and *HIF2α* (Tian et al., 1997). These observations led to the hypothesis that the main role of HIF2α is related to endothelial cell function. Later, the evidence of HIF2α expression in tissues such as hypoxic rat kidney, lung, colonic epithelia, hepatocytes, macrophages and muscle cells indicated that HIF1α and HIF2α are co-expressed in many cell types and the regulation of HIF is more complex than thought before (Wiesener et al., 2003). The other subunits, HIF3α and ARNT2, were also identified (Hirose et al., 1996; Makino et al., 2002). However, studies to date suggest that HIF1α and HIF2α are involved in most HIF-regulated phenomena.

HIFα expression has been observed to be expressed in variety of cancer cell types associated with poor prognosis, such as breast, ovarian, colorectal, prostate, pancreatic, and melanoma cancers. The HIFs affect many steps in initiation, progression, and invasion of tumor cells making them potent therapeutic targets. In some cases such as renal cell carcinoma and lung adenocarcinoma, it has been shown that HIF1α and HIF2α function as a tumor suppressor (Keith et al., 2012).

HIF1α and HIF2α regulate some common genes, while each has its own unique targets (Hu et al., 2003; Raval et al., 2005). Recent data showed that HIF1α and HIF2α regulate hypoxia-dependent gene expression through complex and in some cases (such as kidney cancer cells) antagonistic interactions (Raval et al., 2005). Both HIF1α and HIF2α bind to the HRE sequence in the promoter region of target genes. DNA binding does not necessarily imply gene upregulation suggesting the necessity of post-DNA binding mechanisms for their transactivation (Mole et al., 2009).

Generally, HIF1α induces the expression of glycolytic enzyme genes such as phosphofructokinase (*PFK*) and lactate dehydrogenase A (*LDHA*), and anti-apoptotic factors such as BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3)* and BCL2/adenovirus E1B 19kDa interacting protein 3-like (*BNIP3L/NIX*), and PH regulatory factors such as monocarboxylate transporter 4 (*MCT4*) and carbonic anhydrase 9 (*CA-IX*). HIF2α promotes the expression of genes involved in invasion such as matrix metalloproteinases *(MMP)2, MMP13*,

and stem cell factor *OCT-3/4* (Keith et al., 2012; Koh and Powis, 2012). Interestingly, HIF1α has been shown to regulate the *MMPs* and HIF2α has role in regulating glycolysis enzymes in the absence of HIF1α. This indicates that HIF1α and HIF2α can replace each other for some specific functions (Fujiwara et al., 2007; Warnecke et al., 2004). In addition, HIF1α and HIF2α regulate some common genes such as *VEGF* and glucose transporter 1 *(GLUT1*). Therefore, the regulation of specific genes by HIF1α and HIF2α seems to be context-dependent. Moreover, despite the intrinsic ability of HIF1α and HIF2α in regulating particular downstream genes, their different temporal and functional responses in hypoxia are regulated by some upstream or downstream modulators as well (Koh and Powis, 2012).

Isoform-specific, post-translational and other types of regulation of HIF activity

As we discussed previously, regulation of HIFα is mediated by oxygen-dependent hydroxylases. Of the PHDs, PHD2 is more important for HIF1α function and PHD3 for HIF2α regulation (Appelhoff et al., 2004). HIF2α is hydroxylated at a lower efficiency than HIF1α, which causes stabilization and activation of HIF2α in a higher oxygen level compared to HIF1α. In addition to post-translational regulation, HIF1α mRNA stability is repressed under prolonged hypoxia because of the HIF1/2α-dependent expression of HIF1α antisense RNA (Uchida et al., 2004). Moreover, HIF2α translation is dependent on iron metabolism due to the presence of an iron-responsive element (IRE) in the 5' untranslated region (UTR) of *HIF2α* and is suppressed in iron-deficiency condition inhibiting erythropoiesis (Sanchez et al., 2007). Additionally, context-specific regulation is also important as the controller of isoform-specific cofactors and transactivators. The isoform-specific function of HIF1α versus HIF2α has been attributed to their differences in the N-terminal transactivation domains (Hu et al., 2007). Post-translational modifications such as phosphorylation and acetylation control HIFα activity (reviewed in Keith et al., 2012). Altogether, many mechanisms are involved in context-dependent and the specific isoform activation of HIF transcription factors in response to variations in duration and levels of hypoxia.

It has been shown that in some cell lines, HIF1α is active in short periods (2-24 hours) of severe hypoxia or anoxia (<0.1% O_2), while HIF2α is active in mild hypoxia (<5 % O_2) and remains active for long periods (48-72 hours) of hypoxia (Holmquist-Mengelbier et al., 2006). Therefore, it seems that HIF1α drives the initial response to hypoxia, but later in chronic hypoxic condition, HIF2α comes to play and has the main role in regulating the response to hypoxia (Holmquist-Mengelbier et al., 2006; Koh et al., 2011).

HIF deficiency and effects on hematopoiesis

Genetic ablation in the mouse model has been used to examine the role of HIFs. A variety of *HIF*-deficient mice have been generated and studies show broad effects in many tissues during development, including the hematopoietic system. However, due to the overwhelming

effect of HIF transcription factors during embryogenesis, it is not clear whether impaired hematopoiesis is a direct effect of HIF deficiency. Here we briefly address some of the related studies.

HIFβ (Arnt)

Maltepe and colleagues (Maltepe et al., 1997) analyzed the role of Arnt in embryonic stem cells (ES). Under hypoxic conditions, it is known that cells shift from aerobic to anaerobic respiration by activation of glycolytic pathways, increase their glucose uptake to compensate for the low level of ATP production by glycolysis, and also increase the tissue vascularization by inducing the angiogenesis factors. *Arnt*$^{-/-}$ ES cells were examined for the induction of hypoxia-induced genes in low-oxygen and low-glucose cultures. In contrast to WT ES cells, mutant ES cells did not show the upregulation of hypoxia-induced genes involved in glycolysis, i.e. phosphoglycerate kinase (*PGK-1*), aldolase A (*ALDA*), glocuse transporter (*GLUT-1)*, and *VEGF*. In the glucose-deprived condition, the *Arnt*$^{-/-}$ ES cells, in contrast to *Arnt*$^{+/+}$ ES cells, did not grow well and failed to upregulate genes involved in hypoglycemia such *as GLUT-1, VEGF, PGK-1*, and *ALDA*. *Arnt*$^{-/-}$ embryos died by E10. At E9-10, most mutant embryos were distinguishable from WT littermates by defective YS vascularization, small vitelline arteries, enlarged capillaries, fewer vessels, and smaller size. But the heart, dorsal aorta, and intersomitic vessels appeared normal. Moreover, the mutant embryos showed lower expression of *VEGF* in yolk sac and other tissues, as analyzed by *in situ* hybridization. Since Arnt2 is expressed in the *Arnt*$^{-/-}$ embryos, it partially compensates for the absence of Arnt.

Another group (Kozak et al., 1997) analyzed *Arnt*$^{-/-}$ mice and observed most of the same phenotypes. They also detected impaired neural development in 40% of the mutant embryos. Furthermore, in E9-9.5 mutant embryos the fetal part of the placenta was smaller, with defects in vascularization, a smaller chorio-allantoic plate and fewer labyrinth cavities. In contrast to the other study, no abnormalities were observed in the YS.

In a later study, Adelman and colleagues (Adelman et al., 1999) differentiated *Arnt*$^{-/-}$ ES cells to embryoid bodies (EB) and tested these cells in the CFU-C hematopoietic progenitor assay. CFU-C activity (all hematopoietic colony types) was significantly reduced in mutant EB cells. In contrast to WT cells, no hypoxic induction of progenitor activity was observed in KO EB cells. Thus, it appears that hypoxia induces the expansion of hematopoietic progenitors in developing EB through HIF activity. Studies of mutant embryos showed a lack of blood-filled vitelline vessels, suggesting an *in vivo* defect in hematopoiesis. Mutant YS showed considerably less hematopoietic progenitor activity at both E8.5 and E9.5, demonstrating that Arnt is required for production of hematopoietic progenitor in the YS. However, CFU-C analysis of YS and adult BM cells from *Arnt*$^{-/-}$ ES chimeric animals showed that defect in hematopoietic progenitor activity is not cell-intrinsic and is due to the defect in other cell types. Mutant EB cells express less VEGF and EPO, which are critical cytokines for hematopoiesis. Since the CFU-C activity of mutant EB cells could be rescued by adding the VEGF to cultures, it was concluded that hypoxia-induced

VEGF production is responsible for the survival, and expansion of hematopoietic progenitors in developing embryo. The defect in hematopoietic progenitors is likely the result of an early defect in the hemangioblast, HSCs, or lineage committed progenitors (Adelman et al., 1999).

Interestingly, the vessels in E9.5 *Arnt*$^{-/-}$ embryos were found to be disorganized, especially in the PSp/AGM region where the first definitive hematopoietic cells arise. The vessels exhibited less PECAM (CD31) staining and fewer CD34^{+} cells in the dorsal aorta and throughout the embryo, suggesting a role for hypoxic response in vessel formation. Other phenotypes related to a general developmental delay were also observed in the KO embryos. In the KO PSp explant cultures, in contrast to WT ones, few if any vascular beds were formed and much lower frequency of CD45^{+} hematopoietic cells and decreased CFU-C activity were detected, demonstrating that the defect extends into the hematopoietic system. Surprisingly, the addition of WT Sca-1^{+} BM cells to KO PSp cultures rescued the vessel and hematopoietic defect, despite the fact that DiI-Ac-LDL labeling of PSps showed no contribution of WT Sca-1^{+} cells into vessel development. Immunostaining showed HIF1α expression in KO embryos, but with less nuclear localization. Also, the number of TUNEL^{+} hematopoietic cells was increased in KO embryos, suggesting that Arnt affects the survival of hematopoietic cells. VEGF treatment could rescue the vessel and hematopoietic defect in KO PSp cultures showing that the decreased level of paracrine VEGF is partially responsible for impaired vasculogenesis and hematopoiesis in *Arnt*-deficient embryos (Ramirez-Bergeron et al., 2006). This data indicates that HIF activity is essential for the early development of the hematopoietic system through mechanisms that include the production of growth factors.

HIF2α

Depending on the genetic background, *HIF2α* KO mice die either prenatally by E12.5 because of vascular defects (Peng et al., 2011), bradycardia (Tian et al., 1998) and placenta deficiency (Cowden Dahl et al., 2005a), or in neonatal stages due to impaired lung maturation (Compernolle et al., 2002), or months after birth due to ROS-mediated multi-organ failure (Scortegagna et al., 2003a).

In a study in which 20% of *HIF2α* KO embryos survived to adulthood, hypocellularity was observed in the BM and the number of cells in all hematopoietic lineages was decreased. HIF2α expression is normally observed in BM stroma, vascular, and bone lining cells. To examine whether HSC deficiency is the source of the hematopoietic defect, BM cells from WT and KO embryos were injected into irradiated recipients and analyzed for multipotent progenitors (CFU-S_8; colony forming unit-spleen day 8) and repopulating HSCs (2 months post-injection) by *in vivo* transplantation assay. There was no difference in hematopoietic activity between the two groups. However, in reverse transplantation assay (transplantation of WT cells into KO recipients), differences in hematocrit values and peripheral blood cell counts of WT and KO recipients were detected. Also, the mRNA level of some genes involved in hematopoiesis and hematopoietic cell migration, such as vascular cell adhesion molecule (*VCAM*) and

fibronectin, was decreased in KO BM cells. However, *VEGF* was not decreased, suggesting that HIF2α is not involved in regulation of VEGF in BM. Hence, the pancytopenia in *HIF2α* KO animals is a secondary effect of dysregulated receptors, extracellular matrix proteins, and altered microenvironment of hematopoietic cells (Scortegagna et al., 2003b).

HIF1α

Iyer and colleagues (Iyer et al., 1998) focused their research on the role of HIF1α in ES cell and mouse development. *HIF1α*$^{+/+}$ and *HIF1α*$^{-/-}$ ES cells were cultured under hypoxic and normoxic conditions and analyzed for 15 different hypoxia-induced glucose transporter genes and glycolytic enzymes by Northern blotting of total RNA. Most of these genes were upregulated in WT ES cells in hypoxia, while they were downregulated in KO ES cells cultured under both hypoxic and normoxic conditions. Also, *VEGF* mRNA expression was induced in hypoxic WT ES cells but not in KO ones. However, VEGF expression increased in both WT and KO ES cells under glucose deprivation conditions showing that this regulation can be HIF1α-independent. This data shows that HIF1α is essential for oxygen level-dependent activation of VEGF and genes involved in the glycolytic pathway.

HIF1α expression is very high in E8.5 WT embryos and increases 7 to 8-fold, 12to 18-fold, and 10-fold in E9.5-10, E11-12, and E18, respectively, indicating developmental regulation of *HIF1α*. *HIF1α*$^{-/-}$ deficiency led to embryonic lethality by E10.5. At E8.5-8.75, the majority of KO embryos looked normal but by E9.75-10 they are developmental delayed, as compared to WT littermates. Other developmental abnormalities include pericardial effusion, failure of neural tube closure with cystic degeneration and prolapse of the neural folds, cystic enlargement of hindbrain, and hypoplasia of the branchial arches. In E9.75-10 KO embryos, extensive mesenchymal cell death was detected compared to stage-matched WT embryos, leading to an open neural tube. Hyperplasia of the myocardium and large vascular structures were observed in cephalic region of KO embryos. Whole-mount staining for endothelial cell markers demonstrated normal vasculature in E8 KO embryos, but E9.25 KO embryos had significant vasculature defects, especially in the cephalic region and dorsal aorta. Others confirmed these same phenotypes in *HIF1α*-deficient ES cells and embryos (Ryan et al., 1998). This data shows that in *HIF1α*-deficient embryos, vascularization initiates and progresses normally for a short while but later fails, resulting in embryonic lethality (Iyer et al., 1998).

Assays to detect the level of oxygenation in KO embryos showed that there is a significant 10-fold increase in hypoxyprobe staining in KO embryos as compared to WTs (Ryan et al., 1998). The increased level of hypoxia was associated with increased apoptotic cells and lack of vascularization. Also E9.5 KO YSs showed defective vascular structure, implying that oxygen level is a key factor in regulating vascular development of the YS. Furthermore, loss of *HIF1α* led to the delay in solid tumor growth and reduced vessels density in tumors probably because of the reduced level of VEGF.

While these studies focused on the role of HIF1a in ES cells and embryos, HIF transcription factors play fundamental roles in placenta morphogenesis (described earlier in this chapter). Cowden Dahl and colleagues focused on placental development in *Arnt*$^{-/-}$, *HIF1α*$^{-/-}$, and *HIF2α*$^{-/-}$ embryos and found extensive vascular deficiencies in this tissue. Also found were failures in chorioallantoic interactions and defects in spongiotrophoblast and trophoblast giant cell formation. Defects in *HIF1α*$^{-/-}$ or *HIF2α*$^{-/-}$ placentas were less severe compared to double KO and *Arnt*$^{-/-}$ placentas. These results show that hypoxia regulates placenta development through both HIF1α/ARNT and HIF2α/ARNT pathways (Cowden Dahl et al., 2005a). However, these studies did not examine the hematopoietic compartment of the placenta and it is as yet unknown whether blood production is affected. Understanding the molecular mechanisms by which HIFs regulate the placenta formation will provide new insights to design therapeutic agents for medical conditions such as preeclampsia.

Specific examination of erythroid lineage development in E9.5 HIF1α$^{-/-}$ YS was performed by Yoon and colleagues. Erythropoiesis in the YS is normally induced through EPO/EPOR- and VEGF/VEGFR-signaling pathways. HIF1α$^{-/-}$ embryos showed a moderate developmental delay, with smaller YSs. Measure of erythroid progenitors showed in decrease in BFU-E colonies and the erythroid cells were not fully hemoglobinized. *EPO, EPOR* and *VEGFR1* mRNA levels were found to be significantly decreased in HIF1α$^{-/-}$ embryos and YS as compared to WTs. Furthermore, the transferrin receptor (Tfr) protein levels (provides the iron required for YS erythropoiesis) were lower in KO embryos and YSs. The authors concluded that HIF1α is not necessary for the formation of hematopoietic progenitors, but has a role in their expansion and differentiation at least to the erythroid lineage (Yoon et al., 2006).

To more specificially study the role of Hif1α in angiogenesis, the *HIF1α* gene was removed from Tie2 expressing endothelial cells (Tie2 is a receptor tyrosine kinase specifically expressed by vascular endothelial cells) by a conditional knockout strategy. Endothelial cells, from the lung of a mouse strain contained a lox-flanked (fl) *HIF1α* allele, were cultured under hypoxic or normoxic conditions and infected with Cre-recombinase expressing adenovirus to delete the *HIF1α* gene in these cells. The expression levels of three HIF1α target genes, i.e. phosphoglycerate kinase (*PGK), GLUT-1,* and *VEGF*, were induced in hypoxia in WT endothelial cells, whereas *HIF1α* deficient endothelial cells showed no upregulated expression. Also, the 1.6-fold increase of intracellular ATP production observed in hypoxic WT endothelial cells was lost in conditionally deleted endothelial cells (Tang et al., 2004).

Tie2-Cre/HIF1α$^{fl/fl}$ mice were generated and the murine matrigel plug assay was performed to investigate the role of HIF1α in angiogenesis *in vivo*. The mutant embryos showed a 50% reduction in vessel density compared to WT embryos, but the vessels contained erythrocytes showing that they were functionally intact. Hence, VEGF-dependent angiogenic signaling is impaired in the *Tie2-Cre/HIF1α*$^{fl/fl}$ mice. Moreover, mutant embryos exhibited delayed wound healing and in tumorogenesis assays, tumors in mutant mice were smaller, necrotic, and less vascularised. Both WT and mutant endothelial cells had the same growth rate in normoxic cultures, whereas in hypoxic cultures mutant cells grew slower during the exponential growth phase. Also, the elongation of mutant endothelial cells was affected in hypoxia. As this process

depends on paracrine or autocrine VEGF, the VEGF protein level was dramatically reduced in the medium of mutant cell hypoxic cultures. The induced expression of *VEGFR1* and *VEGFR2* was blocked in hypoxic mutant endothelial cells compared to WT cells (Tang et al., 2004). This study demonstrated that the loss of HIF1α activity in endothelial cells leads to a decrease in ATP levels during hypoxia, which results in defective proliferation and invasion capacity of endothelial cells.

Conditional deletion of *HIF1α* specifically in other cell types demonstrates its role in chondrogenesis (Schipani et al., 2001), adipogenesis (Yun et al., 2002), B-cell development (Kojima et al., 2002), T-cell differentiation (Dang et al., 2011), HSC regulation (Takubo et al., 2010), and osteogenesis (Wang et al., 2007).

HIF1α in HSC regulation

We previously described in this chapter that LTR-HSCs are located in the most hypoxic region of the BM, where they are kept in a quiescent state. Also, in hypoxic *ex vivo* cultures of murine/human BM cells and human cord blood cells, HIF1α is stabilized and seems to have an effect on the HSC behavior. This raised the question whether hypoxia and HIF1α contribute to the *in vivo* regulation of HSC maintenance and function in the BM.

Takuba et al. evaluated the mRNA levels of *HIF1α, HIF2α*, and *HIF3α* in different subsets of hematopoietic cells in the adult mouse BM including LTR-HSCs ($CD34^-$ LSK), STR-HSCs and multipotent progenitors ($CD34^+$ LSK), Lin^-, and Lin^+ cells. *HIF1α* expression was highest in LTR-HSCs and lowest in Lin^+ cells. The same expression pattern was observed for *HIF3α*, which itself is a direct target of HIF1α, although its role is not clear yet. The highest expression of *HIF2α* was in Lin^- cells. They checked *PHD*s, the HIF degradation machinery, and found that *PHD2* (most important in HIF1α degradation) is expressed in $CD34^+$ LSK fraction more than other fractions (Takubo et al., 2010).

Immunohistochemistry on BM sections for HIF1α, pimonidazole, and FOXO3a (a transcription factor required for quiescence of HSCs (Miyamoto et al., 2007; Tothova et al., 2007)) revealed that most of the pimonidazole$^+$ c-Kit^+ cells had nuclear staining of HIF1α and FOXO3a. Thus, c-Kit^+HIF1α^+FOXO3a^+ cells are hypoxic LTR-HSCs in the BM. In addition, SLAM HSCs localized in the endosteal zone express HIF1α. HIF1α is highly stabilized in $CD34^-$ LSK cells cultured in 1% O_2 but not in other subpopulations. Together, these data indicate that HIF1α is a regulator of LTR-HSC function in hypoxia.

To investigate the role of HIF1α in adult BM HSCs, *MX-Cre: HIF1α$^{fl/fl}$* (inducible conditional KO) mice were analyzed. Following induction of Cre with polyIpolyC, the frequency of phenotypic HSCs was found to be comparable between KO and WT BM cells. Normal hematopoietic progenitor activity was observed in KO animals. However, *in vivo* transplantation of BM LSK cells showed significantly higher peripheral blood cell donor chimerism in recipients receiving KO cells (at 1-4 months post-injection) as compared to WT cells. Recipients of KO HSCs possessed fewer donor-derived LSK cells at 4 months after transplantation, indicating that HSC

content was decreased. The expression level of *Ink4a* gene product (marker of senescent stem cells) increased in the *HIF1α*-KO LSK cells of primary recipients. Suppression of *Ink4a* products in KO LSK cells resulted in the significant rescue of their reconstitution activity. Secondary transplantation of the BM LSK cells showed failure of long-term reconstitution ability of KO HSCs. Based on these interesting results, it was concluded that HIF1α plays a protective role against senescence and exhaustion of HSCs mediated by *Ink4a* gene products (Figure 8).

Cell cycle analysis of conditional-KO animals revealed a decrease in the fraction of *HIF1α*-KO $CD34^-$ LSK cells in G_0 phase, whereas the fraction in the G_1 phase increased. This was not observed for $CD34^+$ LSK cells. Moreover, increased production of ROS (the inducer of senescence in HSCs) was observed in *HIF1α*-KO LTR-HSCs, suggesting that ROS-associated over-cycling of KO HSCs is responsible for their defective function. This data clearly showed that HIF1α acts as a cell cycle regulator of LTR-HSCs, and its absence leads to their over-proliferation and exhaustion in stress conditions of serial transplantations.

Takubo and colleagues next showed that in aged *HIF1α*-deficient mice the reduction in the frequency of long-term and quiescent HSCs is much more severe compared to WT animals. The distribution of long-term and quiescent HSCs in different BM oxygen level-based subpopulations was disturbed. They were mostly found in the weakly hypoxic fraction, instead of the highly hypoxic one observed in young animals. This data suggests that HIF1α plays a role in the resistance of HSCs to aging, which is a physiological stress condition, via modulating the oxygenation status of HSCs.

To verify the data obtained by *MX-Cre:HIF1α*$^{fl/fl}$ mice, *MX-Cre:VHL*$^{fl/fl}$ and *MX-Cre:VHL*$^{fl/+}$ mice were analyzed. By this model, Takubo et al. were able to analyze the effect of HIF1α stabilization in a dose-dependent manner. $CD34^-$ LSK from *MX-Cre:VHL*$^{fl/+}$ animals showed an increase in G_0 and G_1 and a decrease in $S/G_2/M$ phase. In the *VHL*$^{fl/fl}$ BM model, accumulation of $CD34^-$ LSK cells and significant reduction in Ki-67-labeled LSK cells were detected. In *VHL*$^{fl/+}$ aged animals, the frequency of LTR-HSCs was significantly higher than that in WT animals. PB chimerism was identical after *in vivo* transplantation of *VHL*$^{fl/+}$ and *VHL*$^{+/+}$ LSK cells. However, the donor-derived HSC content was considerably increased in the recipients of KO cells. Thus, stabilization of HIF1α leads to improved maintenance of HSCs in conditions of stress, (in this context, transplantation and aging) (Figure 8). However, *in vivo* transplantation assay showed that repopulating activity of *VHL*$^{fl/fl}$ LSK cells is completely destroyed. Moreover, the *VHL*$^{fl/fl}$ HSCs showed increase in apoptosis rate and their homing capacity was defected. Hence, over-stabilization of HIF1α results in defective HSC cycling and function. Collectively, these findings show that hypoxia is a hallmark of HSCs in the BM niche and HIF1α regulates the cell cycle and function of HSCs in a dose-dependent manner (Takubo et al., 2010). Yet molecular mechanisms and the targeted gene through which this regulating occurs are not clear.

VEGFA is the best-known and perhaps most important HIF1α target gene. It has been shown to play a role in the maintenance of HSCs (Gerber et al., 2002). To investigate the role of VEGFA regulation on HSCs in the hypoxic BM niche, Rehn and colleagues analyzed the mouse model *Vegfa*$^{\delta/\delta}$, in which the HRE in *Vegfa* promoter region is mutated, preventing HIF binding and subsequent regulation of *Vegf* expression by HIF and hypoxia (Rehn et al., 2011). The *Vegfa*

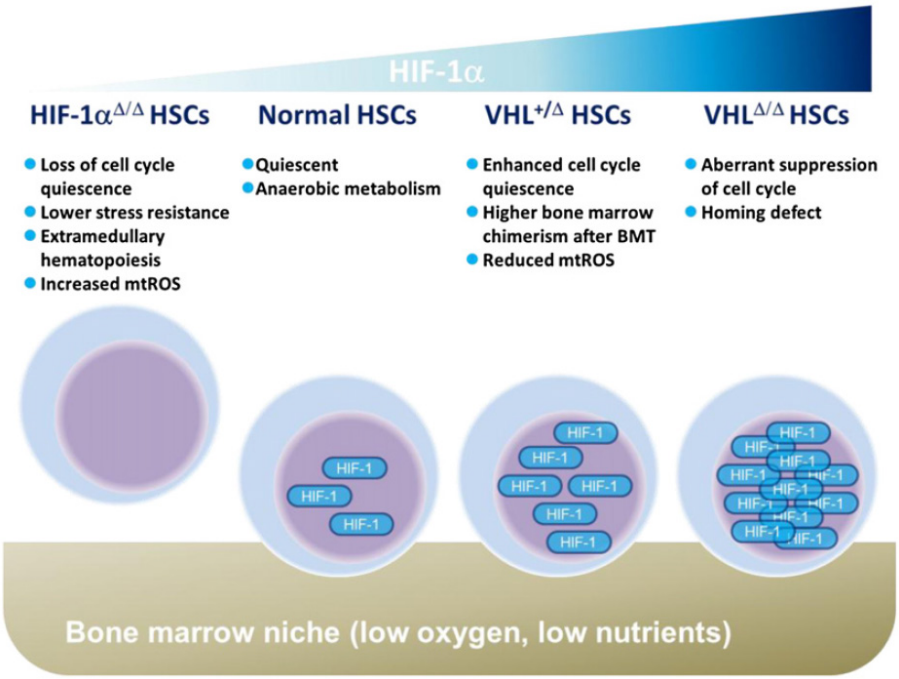

Figure 8. Schematic representation of results of HIF1α or VHL deficiency in HSC regulation. HIF1α affects HSC behavior in a dose-dependent manner in BM of adult mice (adopted from Suda et al., 2011)

expression level remained unchanged in BM c-Kit$^+$ cells of the KO animals cultured in hypoxia while it increased for the WT cells. Also, *Vegfa* expression was highest in CD34$^-$ LSK cells compared to other fractions, which altogether suggests that it is the hypoxic nature of HSCs in the BM that induces Vegfa expression. Total BM cellularity was decreased in the KO animals, but there was no significant change in CFU-C activity and the percentages of different HSC fractions except for CD34$^-$ LSK, which slightly increased. However, both PB and BM chimerism was notably decreased in the recipients of KO BM cells compared to WT cells 15 weeks after *in vivo* transplantation, showing that HSCs lacking hypoxia-regulated *Vegfa* expression are impaired in repopulating activity. In conclusion, Vegfa is one of the targets of HIF and hypoxia in the BM through which HSC function is regulated (Rehn et al., 2011).

Cripto is another molecule regulated by HIF1α. It is the ligand of GRP78, a heat shock protein that plays role in development and cell growth. Cripto is highly expressed in LTR-HSCs compared to STR-HSCs and in ex vivo cultures Cripto increases HSC maintenance. GRP78R$^+$ LT-HSCs are located in endosteal region and are more hypoxic compared to *GRP78*$^-$ LTR-HSCs. They are also quiescent with high glycolysis activity. Furthermore, *GRP78*$^+$ and *GRP78*$^-$ HSCs show different behaviors in transplantation assays. Blocking of GRP78 led to the migration of HSCs from endosteal zone to the central zone of the BM. *HIF1α*-deficient mice showed fewer GRP78$^+$ HSCs and less Cripto expression by endosteal cells. Collectively, GRP78 and Cripto regulate HSC quiescence and maintenance under hypoxic conditions as an intermediary of HIF1α (Miharada et al., 2011).

HIF1α and HIF2α are responsible for hypoxic induction of Erythropoietin (EPO) and regulate erythropoiesis (Haase, 2010; Semenza and Wang, 1992; Yoon et al., 2006). Rankin and colleagues introduced osteoblasts as a new source of EPO and showed that EPO is a regulator of HSCs and erythropoiesis in osteoblasts. Both HIF1α and HIF2α are expressed in the osteoblast niche of BM. They showed that the *in vivo* increased level of HIF expression in osteoprogenitors (by inhibition of PHDs) leads to a dramatic increase in trabecular bone and expansion of functional HSCs and erythroid lineages (the latter happening in an EPO-dependent manner). Collectively, manipulation of HIF/VHL/PHD signaling in order to activate HIFs in osteoblasts can be an approach to expand HSCs or erythrocytes in hematological diseases such as anemia (Rankin et al., 2012).

Metabolic profile of HSCs reflects their hypoxic niche

It has been recently proposed that HSCs possess specific metabolic properties that meet the requirements for their self-renewal and multi-potent differentiation activity (Macarthur et al., 2009). The quiescent versus the cycling nature of stem cells is perhaps a strategy to avoid damage accumulation from physiological stress. Quiescent stem cells are more resistance to cytotoxic agents compared to cycling ones because these agents affect DNA in the S or M phase of the cell cycle (Blanpain et al., 2011; Branzei and Foiani, 2008; reviewed in Suda et al., 2011). Additionally, HSCs have the characteristic of drug efflux by ATP-dependent transporters, preventing the accumulation of toxic agents (Challen and Little, 2006). Nevertheless, HSCs accumulate some damage, as they remain in the body for a long time.

HIF1α is the master regulator of metabolism and regulates the switch from oxidative respiration to anaerobic glycolysis (Hagg and Wennstrom, 2005; Kim et al., 2006; Marin-Hernandez et al., 2009; Maxwell et al., 2007; Papandreou et al., 2006; Wang et al., 1995; Zhang et al., 2007). Simsek and colleagues analyzed the metabolic profile of HSCs. They showed that LTR-HSCs have low rate of mitochondrial respiration and ATP level, while having a high rate of glycolysis compared to the whole BM (Simsek et al., 2010). In terms of metabolic profile, more than 80% of LTR-HSCs were localized in low mitochondrial potential (MP) fraction, which comprise 6-9% of the whole BM cell population. Oxygen consumption and ATP content is significantly greater in the high MP population compared to the low MP population. The low MP population utilized glycolysis. Moreover, they showed that in low MP cells the hypoxia-inducible genes are upregulated in normoxic condition and can survive in hypoxic and anoxic conditions better than high MP cells. HIF1α is expressed significantly higher in low MP cells compared to high MP cells at both the mRNA and protein level. All this data shows that the BM low MP cells, which mostly contain LSK and LTR-HSCs, are primed to get adapted to hypoxic environment and that they rely on glycolysis rather than oxidative respiration to meet their energy demands.

To know if it is possible to isolate different BM cells based on their metabolism, *in vitro* and *in vivo* assays were performed on low and high MP cells. Low MP cells showed considerably

higher progenitor activity compared to high MP cells. *In vivo* transplantation assays showed significantly more repopulating activity by low MP cells, indicating the enrichment of LTR-HSCs in this fraction. The majority of LTR-HSCs express HIF1α while only a small percentage of whole BM cells do so. Collectively, the metabolic properties of HSCs support their adaptation and survival in hypoxic niche (Simsek et al., 2010).

Scope of this thesis

In this introduction an overview of hematopoietic progenitor and stem cells generation and regulation during murine development was described. HSC niches in the adult BM and their various hematopoiesis regulatory factors were addressed. One of the hallmarks of BM HSC niche is its hypoxic nature which plays a crucial role in HSC regulation. The master transcriptional regulator of hypoxia is HIF1α, which has been shown to be essential for HSC function in the adult BM. Hypoxia and HIF also play physiological roles during fetal organogenesis and placenta morphogenesis. Yet, little is known about the role of hypoxia and HIF1α on embryonic HPSCs. The mouse placenta is an embryonic hematopoietic tissue that harbors a large pool of HSCs making it an attractive tissue to study HSC niche.

In Chapter 2, analysis of the human placenta is performed to see whether it (like the mouse placenta) harbors any HSCs. In this study we show that the human placenta contains hematopoietic progenitor and stem cells already at gestational week 6 and onward. Furthermore, mesenchymal cells derived from human placenta have the potential to differentiate along osteogenic, adipogenic, and enotheilial lineages and support the expansion of cord blood $CD34^+$ cells and immature hematopoietic progenitors.

In Chapter 3, to acquire more insight into HPSC regulation, the hematopoietic niche in mouse placenta and the role of hypoxia as a niche factor in HPSC maintenance/expansion is analyzed. We demonstrate that the stromal cells from E12 placenta have the osteogenic and adipogenic differentiation potential and support expansion and progenitor activity of mouse BM hematopoietic cells. Faster proliferation of placenta stromal cells and the induction of hypoxia-sensitive genes in them under hypoxic conditions show the placenta HSPC niche is ready to respond to oxygen-decreased conditions.

More importantly, in Chapter 4, to understand the role of HIF1α in HPSC development, we analyze the affects of HIF1α-deficiency in the endothelial compartment and the emerging hematopoietic cells in the mouse embryo. We show that HIF1α is essential in HPSC generation and/or expansion in the mouse embryo, as the hematopoietic progenitor activity and HSC repopulating potential of HIF1α-deficient embryonic tissues are diminished. Moreover, phenotypic HPSCs and the number of aortic hematopoietic clusters/c-Kit^+ cells are reduced in KO embryos. In adult mice, HIF1α is required to maintain the normal number of HSCs and keeps them in a quiescent state under stress conditions.

We conclude the thesis in Chapter 5 by a general discussion of the aforementioned findings and the future avenues for research in this area.

References

Abbott, B.D., and Buckalew, A.R. (2000). Placental defects in ARNT-knockout conceptus correlate with localized decreases in VEGF-R2, Ang-1, and Tie-2. Developmental dynamics : an official publication of the American Association of Anatomists *219*, 526-538.

Adelman, D.M., Gertsenstein, M., Nagy, A., Simon, M.C., and Maltepe, E. (2000). Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes & development *14*, 3191-3203.

Adelman, D.M., Maltepe, E., and Simon, M.C. (1999). Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes & development *13*, 2478-2483.

Alexander, W.S., Roberts, A.W., Maurer, A.B., Nicola, N.A., Dunn, A.R., and Metcalf, D. (1996). Studies of the c-Mpl thrombopoietin receptor through gene disruption and activation. Stem Cells *14 Suppl 1*, 124-132.

Appelhoff, R.J., Tian, Y.M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., and Gleadle, J.M. (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. The Journal of biological chemistry *279*, 38458-38465.

Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. (2003). Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity *19*, 257-267.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Arai, F., Yoshihara, H., Hosokawa, K., Nakamura, Y., Gomei, Y., Iwasaki, H., and Suda, T. (2009). Niche regulation of hematopoietic stem cells in the endosteum. Annals of the New York Academy of Sciences *1176*, 36-46.

Audet, J., Miller, C.L., Eaves, C.J., and Piret, J.M. (2002). Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. Biotechnology and bioengineering *80*, 393-404.

Blanpain, C., Mohrin, M., Sotiropoulou, P.A., and Passegue, E. (2011). DNA-damage response in tissue-specific and cancer stem cells. Cell stem cell *8*, 16-29.

Boggs, D.R., Boggs, S.S., Saxe, D.F., Gress, L.A., and Canfield, D.R. (1982). Hematopoietic stem cells with high proliferative potential. Assay of their concentration in marrow by the frequency and duration of cure of W/Wv mice. The Journal of clinical investigation *70*, 242-253.

Boisset, J.C., and Robin, C. (2012). On the origin of hematopoietic stem cells: progress and controversy. Stem cell research *8*, 1-13.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature *464*, 116-120.

Brandt, J.E., Galy, A.H., Luens, K.M., Travis, M., Young, J., Tong, J., Chen, S., Davis, T.A., Lee, K.P., Chen, B.P*., et al.* (1998). Bone marrow repopulation by human marrow stem cells after long-term expansion culture on a porcine endothelial cell line. Experimental hematology *26*, 950-961.

Branzei, D., and Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nature reviews Molecular cell biology *9*, 297-308.

Bruick, R.K. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes & development *17*, 2614-2623.

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*, 841-846.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C*., et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nature medicine *10*, 858-864.

Challen, G.A., and Little, M.H. (2006). A side order of stem cells: the SP phenotype. Stem Cells *24*, 3-12.

Chan, C.K., Chen, C.C., Luppen, C.A., Kim, J.B., DeBoer, A.T., Wei, K., Helms, J.A., Kuo, C.J., Kraft, D.L., and Weissman, I.L. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. Nature *457*, 490-494.

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. Proceedings of the National Academy of Sciences of the United States of America *96*, 3120-3125.

Chow, D.C., Wenning, L.A., Miller, W.M., and Papoutsakis, E.T. (2001a). Modeling pO(2) distributions in the bone marrow hematopoietic compartment. I. Krogh's model. Biophysical journal *81*, 675-684.

Chow, D.C., Wenning, L.A., Miller, W.M., and Papoutsakis, E.T. (2001b). Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. Biophysical journal *81*, 685-696.

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

Chute, J.P., Saini, A.A., Chute, D.J., Wells, M.R., Clark, W.B., Harlan, D.M., Park, J., Stull, M.K., Civin, C., and Davis, T.A. (2002). Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow. Blood *100*, 4433-4439.

Cipolleschi, M.G., D'Ippolito, G., Bernabei, P.A., Caporale, R., Nannini, R., Mariani, M., Fabbiani, M., Rossi-Ferrini, P., Olivotto, M., and Dello Sbarba, P. (1997). Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E in vitro. Experimental hematology *25*, 1187-1194.

Cipolleschi, M.G., Rovida, E., Ivanovic, Z., Praloran, V., Olivotto, M., and Dello Sbarba, P. (2000). The expansion of murine bone marrow cells preincubated in hypoxia as an in vitro indicator of their marrow-repopulating ability. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *14*, 735-739.

Cobas, M., Wilson, A., Ernst, B., Mancini, S.J., MacDonald, H.R., Kemler, R., and Radtke, F. (2004). Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. The Journal of experimental medicine *199*, 221-229.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F*., et al.* (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas

treatment with VEGF prevents fatal respiratory distress in premature mice. Nature medicine *8*, 702-710.

Coskun, S., and Hirschi, K.K. (2010). Establishment and regulation of the HSC niche: Roles of osteoblastic and vascular compartments. Birth defects research Part C, Embryo today : reviews *90*, 229-242.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P., and Simon, M.C. (2005a). Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Molecular and cellular biology *25*, 10479-10491.

Cowden Dahl, K.D., Robertson, S.E., Weaver, V.M., and Simon, M.C. (2005b). Hypoxia-inducible factor regulates alphavbeta3 integrin cell surface expression. Molecular biology of the cell *16*, 1901-1912.

Cross, J.C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D.G., Yamamoto, H., and Kingdom, J.C. (2003). Genes, development and evolution of the placenta. Placenta *24*, 123-130.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907-916.

D'Ippolito, G., Diabira, S., Howard, G.A., Roos, B.A., and Schiller, P.C. (2006). Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone *39*, 513-522.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A., and Simon, M.C. (2003). Expansion of human SCID-repopulating cells under hypoxic conditions. The Journal of clinical investigation *112*, 126-135.

Dang, E.V., Barbi, J., Yang, H.Y., Jinasena, D., Yu, H., Zheng, Y., Bordman, Z., Fu, J., Kim, Y., Yen, H.R*., et al.* (2011). Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. Cell *146*, 772-784.

Das, R., Jahr, H., van Osch, G.J., and Farrell, E. (2010). The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue engineering Part B, Reviews *16*, 159-168.

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. The EMBO journal *19*, 2465-2474.

Delassus, S., and Cumano, A. (1996). Circulation of hematopoietic progenitors in the mouse embryo. Immunity *4*, 97-106.

Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457-462.

Draenert, K., and Draenert, Y. (1980). The vascular system of bone marrow. Scanning electron microscopy, 113-122.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N*., et al.* (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nature immunology *6*, 314-322.

Dunwoodie, S.L. (2009). The role of hypoxia in development of the Mammalian embryo. Developmental cell *17*, 755-773.

Durand, R.E., and Raleigh, J.A. (1998). Identification of nonproliferating but viable hypoxic tumor cells in vivo. Cancer research *58*, 3547-3550.

Dzierzak, E., and Speck, N.A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nature immunology *9*, 129-136.

Eliasson, P., and Jonsson, J.I. (2010). The hematopoietic stem cell niche: low in oxygen but a nice place to be. Journal of cellular physiology *222*, 17-22.

Eliasson, P., Rehn, M., Hammar, P., Larsson, P., Sirenko, O., Flippin, L.A., Cammenga, J., and Jonsson, J.I. (2010). Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. Experimental hematology *38*, 301-310 e302.

Ema, H., Sudo, K., Seita, J., Matsubara, A., Morita, Y., Osawa, M., Takatsu, K., Takaki, S., and Nakauchi, H. (2005). Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. Developmental cell *8*, 907-914.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. Proceedings of the National Academy of Sciences of the United States of America *94*, 4273-4278.

Ferkowicz, M.J., and Yoder, M.C. (2005). Blood island formation: longstanding observations and modern interpretations. Experimental hematology *33*, 1041-1047.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Fisher, S.A., and Burggren, W.W. (2007). Role of hypoxia in the evolution and development of the cardiovascular system. Antioxidants & redox signaling *9*, 1339-1352.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mechanisms of development *63*, 51-60.

Fujiwara, S., Nakagawa, K., Harada, H., Nagato, S., Furukawa, K., Teraoka, M., Seno, T., Oka, K., Iwata, S., and Ohnishi, T. (2007). Silencing hypoxia-inducible factor-1alpha inhibits cell migration and invasion under hypoxic environment in malignant gliomas. International journal of oncology *30*, 793-802.

Gekas, C., Dieterlen-Lievre, F., Orkin, S.H., and Mikkola, H.K. (2005). The placenta is a niche for hematopoietic stem cells. Developmental cell *8*, 365-375.

Gerber, H.P., Malik, A.K., Solar, G.P., Sherman, D., Liang, X.H., Meng, G., Hong, K., Marsters, J.C., and Ferrara, N. (2002). VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature *417*, 954-958.

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. Proceedings of the National Academy of Sciences of the United States of America *92*, 773-777.

Grassinger, J., Haylock, D.N., Williams, B., Olsen, G.H., and Nilsson, S.K. (2010). Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. Blood *116*, 3185-3196.

Grayson, W.L., Zhao, F., Bunnell, B., and Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochemical and biophysical research communications *358*, 948-953.

Grayson, W.L., Zhao, F., Izadpanah, R., Bunnell, B., and Ma, T. (2006). Effects of hypoxia on human

mesenchymal stem cell expansion and plasticity in 3D constructs. Journal of cellular physiology *207*, 331-339.

Gustafsson, M.V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J.L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. Developmental cell *9*, 617-628.

Haase, V.H. (2010). Hypoxic regulation of erythropoiesis and iron metabolism. American journal of physiology Renal physiology *299*, F1-13.

Hagg, M., and Wennstrom, S. (2005). Activation of hypoxia-induced transcription in normoxia. Experimental cell research *306*, 180-191.

Harrison, D.E., Astle, C.M., and Lerner, C. (1988). Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. Proceedings of the National Academy of Sciences of the United States of America *85*, 822-826.

Harrison, D.E., Jordan, C.T., Zhong, R.K., and Astle, C.M. (1993). Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. Experimental hematology *21*, 206-219.

Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., Hicklin, D.J., Zhu, Z., Bohlen, P., Witte, L.*, et al.* (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nature medicine *8*, 841-849.

Haug, J.S., He, X.C., Grindley, J.C., Wunderlich, J.P., Gaudenz, K., Ross, J.T., Paulson, A., Wagner, K.P., Xie, Y., Zhu, R.*, et al.* (2008). N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell stem cell *2*, 367-379.

Hermitte, F., Brunet de la Grange, P., Belloc, F., Praloran, V., and Ivanovic, Z. (2006). Very low O2 concentration (0.1%) favors G0 return of dividing CD34+ cells. Stem Cells *24*, 65-73.

Hidalgo, A., Weiss, L.A., and Frenette, P.S. (2002). Functional selectin ligands mediating human CD34(+) cell interactions with bone marrow endothelium are enhanced postnatally. The Journal of clinical investigation *110*, 559-569.

Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996). cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). Molecular and cellular biology *16*, 1706-1713.

Hirota, K., Fukuda, R., Takabuchi, S., Kizaka-Kondoh, S., Adachi, T., Fukuda, K., and Semenza, G.L. (2004). Induction of hypoxia-inducible factor 1 activity by muscarinic acetylcholine receptor signaling. The Journal of biological chemistry *279*, 41521-41528.

Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H., and Bradfield, C.A. (1997). Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. The Journal of biological chemistry *272*, 8581-8593.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K.*, et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer cell *10*, 413-423.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K.*, et al.* (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell stem cell *4*, 263-274.

Hu, C.J., Sataur, A., Wang, L., Chen, H., and Simon, M.C. (2007). The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Molecular biology of the cell *18*, 4528-4542.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., and Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Molecular and cellular biology *23*, 9361-9374.

Hu, X., Yu, S.P., Fraser, J.L., Lu, Z., Ogle, M.E., Wang, J.A., and Wei, L. (2008). Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. The Journal of thoracic and cardiovascular surgery *135*, 799-808.

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*, 625-630.

Ikuta, K., and Weissman, I.L. (1992). Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proceedings of the National Academy of Sciences of the United States of America *89*, 1502-1506.

Ivanovic, Z., Bartolozzi, B., Bernabei, P.A., Cipolleschi, M.G., Rovida, E., Milenkovic, P., Praloran, V., and Dello Sbarba, P. (2000a). Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. British journal of haematology *108*, 424-429.

Ivanovic, Z., Belloc, F., Faucher, J.L., Cipolleschi, M.G., Praloran, V., and Dello Sbarba, P. (2002). Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells. Experimental hematology *30*, 67-73.

Ivanovic, Z., Dello Sbarba, P., Trimoreau, F., Faucher, J.L., and Praloran, V. (2000b). Primitive human HPCs are better maintained and expanded in vitro at 1 percent oxygen than at 20 percent. Transfusion *40*, 1482-1488.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y.*, et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes & development *12*, 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J.*, et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Jing, D., Wobus, M., Poitz, D.M., Bornhauser, M., Ehninger, G., and Ordemann, R. (2012). Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. Haematologica *97*, 331-339.

Jogi, A., Ora, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., Axelson, H., and Pahlman, S. (2002). Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proceedings of the National Academy of Sciences of the United States of America *99*, 7021-7026.

Kabrun, N., Buhring, H.J., Choi, K., Ullrich, A., Risau, W., and Keller, G. (1997). Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development *124*, 2039-2048.

Kaimakis, P., Crisan, M., and Dzierzak, E. (2012). The biochemistry of hematopoietic stem cell development. Biochimica et biophysica acta.

Katayama, Y., Hidalgo, A., Furie, B.C., Vestweber, D., Furie, B., and Frenette, P.S. (2003). PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. Blood *102*, 2060-2067.

Keith, B., Johnson, R.S., and Simon, M.C. (2012). HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nature reviews Cancer *12*, 9-22.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell stem cell *1*, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kilburn, B.A., Wang, J., Duniec-Dmuchowski, Z.M., Leach, R.E., Romero, R., and Armant, D.R. (2000). Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. Biology of reproduction *62*, 739-747.

Kim, C.G., Lee, J.J., Jung, D.Y., Jeon, J., Heo, H.S., Kang, H.C., Shin, J.H., Cho, Y.S., Cha, K.J., Do, B.R*., et al.* (2006). Profiling of differentially expressed genes in human stem cells by cDNA microarray. Molecules and cells *21*, 343-355.

Kimura, S., Roberts, A.W., Metcalf, D., and Alexander, W.S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proceedings of the National Academy of Sciences of the United States of America *95*, 1195-1200.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nature immunology *7*, 1048-1056.

Klassen, L.W., Birks, J., Allen, E., and Gurney, C.W. (1972). Experimental medullary aplasia. The Journal of laboratory and clinical medicine *80*, 8-17.

Koh, M.Y., Lemos, R., Jr., Liu, X., and Powis, G. (2011). The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. Cancer research *71*, 4015-4027.

Koh, M.Y., and Powis, G. (2012). Passing the baton: the HIF switch. Trends in biochemical sciences *37*, 364-372.

Kojima, H., Gu, H., Nomura, S., Caldwell, C.C., Kobata, T., Carmeliet, P., Semenza, G.L., and Sitkovsky, M.V. (2002). Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1alpha -deficient chimeric mice. Proceedings of the National Academy of Sciences of the United States of America *99*, 2170-2174.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., and Rafii, S. (2005). The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) *20*, 349-356.

Kozak, K.R., Abbott, B., and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation.

Developmental biology *191*, 297-305.

Kubota, Y., Takubo, K., and Suda, T. (2008). Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. Biochemical and biophysical research communications *366*, 335-339.

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*, 4891-4899.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C., and Semenza, G.L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Molecular and cellular biology *21*, 3995-4004.

Lee, Y.M., Jeong, C.H., Koo, S.Y., Son, M.J., Song, H.S., Bae, S.K., Raleigh, J.A., Chung, H.Y., Yoo, M.A., and Kim, K.W. (2001). Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. Developmental dynamics : an official publication of the American Association of Anatomists *220*, 175-186.

Lemischka, I.R. (1992). The haematopoietic stem cell and its clonal progeny: mechanisms regulating the hierarchy of primitive haematopoietic cells. Cancer surveys *15*, 3-18.

Lennon, D.P., Edmison, J.M., and Caplan, A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. Journal of cellular physiology *187*, 345-355.

Li, W., Johnson, S.A., Shelley, W.C., Ferkowicz, M., Morrison, P., Li, Y., and Yoder, M.C. (2003). Primary endothelial cells isolated from the yolk sac and para-aortic splanchnopleura support the expansion of adult marrow stem cells in vitro. Blood *102*, 4345-4353.

Li, Y., Dowbenko, D., and Lasky, L.A. (2002). AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. The Journal of biological chemistry *277*, 11352-11361.

Li, Z., and Li, L. (2006). Understanding hematopoietic stem-cell microenvironments. Trends in biochemical sciences *31*, 589-595.

Lisy, K., and Peet, D.J. (2008). Turn me on: regulating HIF transcriptional activity. Cell death and differentiation *15*, 642-649.

Liu, Y.V., and Semenza, G.L. (2007). RACK1 vs. HSP90: competition for HIF-1 alpha degradation vs. stabilization. Cell Cycle *6*, 656-659.

Lo Celso, C., Fleming, H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Cote, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature *457*, 92-96.

Lord, B.I., and Hendry, J.H. (1972). The distribution of haemopoietic colony-forming units in the mouse femur, and its modification by x rays. The British journal of radiology *45*, 110-115.

Lux, C.T., Yoshimoto, M., McGrath, K., Conway, S.J., Palis, J., and Yoder, M.C. (2008). All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. Blood *111*, 3435-3438.

Macarthur, B.D., Ma'ayan, A., and Lemischka, I.R. (2009). Systems biology of stem cell fate and cellular

reprogramming. Nature reviews Molecular cell biology *10*, 672-681.

Mahy, P., De Bast, M., Gallez, B., Gueulette, J., Koch, C.J., Scalliet, P., and Gregoire, V. (2003). In vivo colocalization of 2-nitroimidazole EF5 fluorescence intensity and electron paramagnetic resonance oximetry in mouse tumors. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology *67*, 53-61.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. The Journal of biological chemistry *277*, 32405-32408.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403-407.

Marin-Hernandez, A., Gallardo-Perez, J.C., Ralph, S.J., Rodriguez-Enriquez, S., and Moreno-Sanchez, R. (2009). HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. Mini reviews in medicinal chemistry *9*, 1084-1101.

Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Activation of the HIF pathway in cancer. Current opinion in genetics & development *11*, 293-299.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Maxwell, P.J., Gallagher, R., Seaton, A., Wilson, C., Scullin, P., Pettigrew, J., Stratford, I.J., Williams, K.J., Johnston, P.G., and Waugh, D.J. (2007). HIF-1 and NF-kappaB-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells. Oncogene *26*, 7333-7345.

Mazo, I.B., Gutierrez-Ramos, J.C., Frenette, P.S., Hynes, R.O., Wagner, D.D., and von Andrian, U.H. (1998). Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. The Journal of experimental medicine *188*, 465-474.

McCulloch, E.A., Siminovitch, L., and Till, J.E. (1964). Spleen-Colony Formation in Anemic Mice of Genotype Ww. Science *144*, 844-846.

McCulloch, E.A., Siminovitch, L., Till, J.E., Russell, E.S., and Bernstein, S.E. (1965). The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl-Sld. Blood *26*, 399-410.

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

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

Medvinsky, A.L., Samoylina, N.L., Muller, A.M., and Dzierzak, E.A. (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. Nature *364*, 64-67.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature *466*, 829-834.

Miharada, K., Karlsson, G., Rehn, M., Rorby, E., Siva, K., Cammenga, J., and Karlsson, S. (2011). Cripto regulates hematopoietic stem cells as a hypoxic-niche-related factor through cell surface receptor

GRP78. Cell stem cell *9*, 330-344.

Miller, C.L., Rebel, V.I., Helgason, C.D., Lansdorp, P.M., and Eaves, C.J. (1997). Impaired steel factor responsiveness differentially affects the detection and long-term maintenance of fetal liver hematopoietic stem cells in vivo. Blood *89*, 1214-1223.

Miller, C.L., Rebel, V.I., Lemieux, M.E., Helgason, C.D., Lansdorp, P.M., and Eaves, C.J. (1996). Studies of W mutant mice provide evidence for alternate mechanisms capable of activating hematopoietic stem cells. Experimental hematology *24*, 185-194.

Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M.*, et al.* (2007). Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell stem cell *1*, 101-112.

Mole, D.R., Blancher, C., Copley, R.R., Pollard, P.J., Gleadle, J.M., Ragoussis, J., and Ratcliffe, P.J. (2009). Genome-wide association of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha DNA binding with expression profiling of hypoxia-inducible transcripts. The Journal of biological chemistry *284*, 16767-16775.

Moore, M.A., and Metcalf, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. British journal of haematology *18*, 279-296.

Morriss, G.M., and New, D.A. (1979). Effect of oxygen concentration on morphogenesis of cranial neural folds and neural crest in cultured rat embryos. Journal of embryology and experimental morphology *54*, 17-35.

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.

Murray, P. (1932). The Development in vitro of the Blood of the Early Chick Embryo. Proceedings of the Royal Society of London *111*, 497-521.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635-638.

Okazaki, K., and Maltepe, E. (2006). Oxygen, epigenetics and stem cell fate. Regenerative medicine *1*, 71-83.

Orkin, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. Nature reviews Genetics *1*, 57-64.

Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Developmental cell *8*, 377-387.

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*, 5073-5084.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., and Denko, N.C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell metabolism *3*, 187-197.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proceedings of the National Academy of Sciences of the United States of America *104*, 5431-5436.

Patt, H.M., and Maloney, M.A. (1972). Bone formation and resorption as a requirement for marrow development. Proc Soc Exp Biol Med *140*, 205-207.

Paul, W.E. (2008). Fundamental immunology, 6th edn (Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins).

Pedersen, M., Lofstedt, T., Sun, J., Holmquist-Mengelbier, L., Pahlman, S., and Ronnstrand, L. (2008). Stem cell factor induces HIF-1alpha at normoxia in hematopoietic cells. Biochemical and biophysical research communications *377*, 98-103.

Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. (1999a). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. The Journal of clinical investigation *104*, 1199-1211.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L.*, et al.* (1999b). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science *283*, 845-848.

Peng, Y.J., Nanduri, J., Khan, S.A., Yuan, G., Wang, N., Kinsman, B., Vaddi, D.R., Kumar, G.K., Garcia, J.A., Semenza, G.L.*, et al.* (2011). Hypoxia-inducible factor 2alpha (HIF-2alpha) heterozygous-null mice exhibit exaggerated carotid body sensitivity to hypoxia, breathing instability, and hypertension. Proceedings of the National Academy of Sciences of the United States of America *108*, 3065-3070.

Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. Nature reviews Molecular cell biology *8*, 49-62.

Ponomaryov, T., Peled, A., Petit, I., Taichman, R.S., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Magerus, A., Caruz, A., Fujii, N.*, et al.* (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. The Journal of clinical investigation *106*, 1331-1339.

Pringle, K.G., Kind, K.L., Thompson, J.G., and Roberts, C.T. (2007). Complex interactions between hypoxia inducible factors, insulin-like growth factor-II and oxygen in early murine trophoblasts. Placenta *28*, 1147-1157.

Puri, M.C., and Bernstein, A. (2003). Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. Proceedings of the National Academy of Sciences of the United States of America *100*, 12753-12758.

Quinlan, D.P., Jr., Rameshwar, P., Qian, J., Maloof, P.B., Mohr, A.M., Hauser, C.J., and Livingston, D.H. (1998). Effect of hypoxia on the hematopoietic and immune modulator preprotachykinin-I. Arch Surg *133*, 1328-1334.

Ramirez-Bergeron, D.L., Runge, A., Adelman, D.M., Gohil, M., and Simon, M.C. (2006). HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. Developmental cell *11*, 81-92.

Rankin, E.B., Wu, C., Khatri, R., Wilson, T.L., Andersen, R., Araldi, E., Rankin, A.L., Yuan, J., Kuo, C.J., Schipani, E.*, et al.* (2012). The HIF signaling pathway in osteoblasts directly modulates erythropoiesis through the production of EPO. Cell *149*, 63-74.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Molecular and cellular biology *25*, 5675-5686.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes & development *14*, 34-44.

Ream, M., Ray, A.M., Chandra, R., and Chikaraishi, D.M. (2008). Early fetal hypoxia leads to growth restriction and myocardial thinning. American journal of physiology Regulatory, integrative and comparative physiology *295*, R583-595.

Rehn, M., Olsson, A., Reckzeh, K., Diffner, E., Carmeliet, P., Landberg, G., and Cammenga, J. (2011). Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. Blood *118*, 1534-1543.

Reinecke, H., and Murry, C.E. (2003). Cell grafting for cardiac repair. Methods Mol Biol *219*, 97-112.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

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*, 252-263.

Ruas, J.L., and Poellinger, L. (2005). Hypoxia-dependent activation of HIF into a transcriptional regulator. Seminars in cell & developmental biology *16*, 514-522.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. The EMBO journal *17*, 3005-3015.

Sabin, F. (1920). Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during the second day of incubation. Contributions to Embryology *9*, 213-262.

Sanchez, M., Galy, B., Muckenthaler, M.U., and Hentze, M.W. (2007). Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. Nature structural & molecular biology *14*, 420-426.

Schaffer, L., Vogel, J., Breymann, C., Gassmann, M., and Marti, H.H. (2006). Preserved placental oxygenation and development during severe systemic hypoxia. American journal of physiology Regulatory, integrative and comparative physiology *290*, R844-851.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nature immunology *7*, 1037-1047.

Schipani, E., Ryan, H.E., Didrickson, S., Kobayashi, T., Knight, M., and Johnson, R.S. (2001). Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes & development *15*, 2865-2876.

Schofield, C.J., and Ratcliffe, P.J. (2005). Signalling hypoxia by HIF hydroxylases. Biochemical and biophysical research communications *338*, 617-626.

Schweitzer, K.M., Drager, A.M., van der Valk, P., Thijsen, S.F., Zevenbergen, A., Theijsmeijer, A.P., van der Schoot, C.E., and Langenhuijsen, M.M. (1996). Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. The American journal of pathology *148*, 165-175.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A*., et al.* (2003a). Multiple organ pathology, metabolic abnormalities and

impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nature genetics *35*, 331-340.

Scortegagna, M., Morris, M.A., Oktay, Y., Bennett, M., and Garcia, J.A. (2003b). The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood *102*, 1634-1640.

Seita, J., Ema, H., Ooehara, J., Yamazaki, S., Tadokoro, Y., Yamasaki, A., Eto, K., Takaki, S., Takatsu, K., and Nakauchi, H. (2007). Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. Proceedings of the National Academy of Sciences of the United States of America *104*, 2349-2354.

Semenza, G.L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annual review of cell and developmental biology *15*, 551-578.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and cellular biology *12*, 5447-5454.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Shweiki, D., Neeman, M., Itin, A., and Keshet, E. (1995). Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proceedings of the National Academy of Sciences of the United States of America *92*, 768-772.

Simon, M.C., and Keith, B. (2008). The role of oxygen availability in embryonic development and stem cell function. Nature reviews Molecular cell biology *9*, 285-296.

Simsek, T., Kocabas, F., Zheng, J., Deberardinis, R.J., Mahmoud, A.I., Olson, E.N., Schneider, J.W., Zhang, C.C., and Sadek, H.A. (2010). The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell stem cell *7*, 380-390.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Cote, D., Means, T.K., Luster, A.D., Scadden, D.T., and Lin, C.P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature *435*, 969-973.

Suda, T., Takubo, K., and Semenza, G.L. (2011). Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell stem cell *9*, 298-310.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell stem cell *7*, 391-402.

Tang, N., Wang, L., Esko, J., Giordano, F.J., Huang, Y., Gerber, H.P., Ferrara, N., and Johnson, R.S. (2004). Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer cell *6*, 485-495.

Theus, M.H., Wei, L., Cui, L., Francis, K., Hu, X., Keogh, C., and Yu, S.P. (2008). In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. Experimental neurology *210*, 656-670.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes & development *12*, 3320-3324.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a

transcription factor selectively expressed in endothelial cells. Genes & development *11*, 72-82.

Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J., and Kessler, P.D. (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation *105*, 93-98.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C.*, et al.* (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Uchida, T., Rossignol, F., Matthay, M.A., Mounier, R., Couette, S., Clottes, E., and Clerici, C. (2004). Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. The Journal of biological chemistry *279*, 14871-14878.

Ueno, H., and Weissman, I.L. (2006). Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. Developmental cell *11*, 519-533.

Varia, M.A., Calkins-Adams, D.P., Rinker, L.H., Kennedy, A.S., Novotny, D.B., Fowler, W.C., Jr., and Raleigh, J.A. (1998). Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. Gynecologic oncology *71*, 270-277.

Waleh, N.S., Brody, M.D., Knapp, M.A., Mendonca, H.L., Lord, E.M., Koch, C.J., Laderoute, K.R., and Sutherland, R.M. (1995). Mapping of the vascular endothelial growth factor-producing hypoxic cells in multicellular tumor spheroids using a hypoxia-specific marker. Cancer research *55*, 6222-6226.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America *92*, 5510-5514.

Wang, Y., Wan, C., Deng, L., Liu, X., Cao, X., Gilbert, S.R., Bouxsein, M.L., Faugere, M.C., Guldberg, R.E., Gerstenfeld, L.C.*, et al.* (2007). The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. The Journal of clinical investigation *117*, 1616-1626.

Warnecke, C., Zaborowska, Z., Kurreck, J., Erdmann, V.A., Frei, U., Wiesener, M., and Eckardt, K.U. (2004). Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 1462-1464.

Watson, E.D., and Cross, J.C. (2005). Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) *20*, 180-193.

Weber, J.M., and Calvi, L.M. (2010). Notch signaling and the bone marrow hematopoietic stem cell niche. Bone *46*, 281-285.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W.*, et al.* (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *17*, 271-273.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant,

C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes & development *18*, 2747-2763.

Withington, S.L., Scott, A.N., Saunders, D.N., Lopes Floro, K., Preis, J.I., Michalicek, J., Maclean, K., Sparrow, D.B., Barbera, J.P., and Dunwoodie, S.L. (2006). Loss of Cited2 affects trophoblast formation and vascularization of the mouse placenta. Developmental biology *294*, 67-82.

Wouters, B.G., and Koritzinsky, M. (2008). Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nature reviews Cancer *8*, 851-864.

Wu, J.Y., Purton, L.E., Rodda, S.J., Chen, M., Weinstein, L.S., McMahon, A.P., Scadden, D.T., and Kronenberg, H.M. (2008). Osteoblastic regulation of B lymphopoiesis is mediated by Gs{alpha}-dependent signaling pathways. Proceedings of the National Academy of Sciences of the United States of America *105*, 16976-16981.

Xie, Y., Yin, T., Wiegraebe, W., He, X.C., Miller, D., Stark, D., Perko, K., Alexander, R., Schwartz, J., Grindley, J.C.*, et al.* (2009). Detection of functional haematopoietic stem cell niche using real-time imaging. Nature *457*, 97-101.

Xu, M.J., Matsuoka, S., Yang, F.C., Ebihara, Y., Manabe, A., Tanaka, R., Eguchi, M., Asano, S., Nakahata, T., and Tsuji, K. (2001). Evidence for the presence of murine primitive megakaryocytopoiesis in the early yolk sac. Blood *97*, 2016-2022.

Yamashita, T., Ohneda, O., Nagano, M., Iemitsu, M., Makino, Y., Tanaka, H., Miyauchi, T., Goto, K., Ohneda, K., Fujii-Kuriyama, Y.*, et al.* (2008). Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. Molecular and cellular biology *28*, 1285-1297.

Yoon, D., Pastore, Y.D., Divoky, V., Liu, E., Mlodnicka, A.E., Rainey, K., Ponka, P., Semenza, G.L., Schumacher, A., and Prchal, J.T. (2006). Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. The Journal of biological chemistry *281*, 25703-25711.

Yoshida, K., Kirito, K., Yongzhen, H., Ozawa, K., Kaushansky, K., and Komatsu, N. (2008). Thrombopoietin (TPO) regulates HIF-1alpha levels through generation of mitochondrial reactive oxygen species. International journal of hematology *88*, 43-51.

Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., Miyamoto, K.*, et al.* (2007). Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell stem cell *1*, 685-697.

Yoshimoto, M., Montecino-Rodriguez, E., Ferkowicz, M.J., Porayette, P., Shelley, W.C., Conway, S.J., Dorshkind, K., and Yoder, M.C. (2011). Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. Proceedings of the National Academy of Sciences of the United States of America *108*, 1468-1473.

Yoshimoto, M., Porayette, P., Glosson, N.L., Conway, S.J., Carlesso, N., Cardoso, A.A., Kaplan, M.H., and Yoder, M.C. (2012). Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. Blood *119*, 5706-5714.

Yu, A.Y., Shimoda, L.A., Iyer, N.V., Huso, D.L., Sun, X., McWilliams, R., Beaty, T., Sham, J.S., Wiener,

C.M., Sylvester, J.T*., et al.* (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. The Journal of clinical investigation *103*, 691-696.

Yun, Z., Maecker, H.L., Johnson, R.S., and Giaccia, A.J. (2002). Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Developmental cell *2*, 331-341.

Zandstra, P.W., Conneally, E., Petzer, A.L., Piret, J.M., and Eaves, C.J. (1997). Cytokine manipulation of primitive human hematopoietic cell self-renewal. Proceedings of the National Academy of Sciences of the United States of America *94*, 4698-4703.

Zhang, H., Gao, P., Fukuda, R., Kumar, G., Krishnamachary, B., Zeller, K.I., Dang, C.V., and Semenza, G.L. (2007). HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer cell *11*, 407-420.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q*., et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhang, M., Methot, D., Poppa, V., Fujio, Y., Walsh, K., and Murry, C.E. (2001). Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. Journal of molecular and cellular cardiology *33*, 907-921.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595-599.

Chapter 2

Human Placenta Is a Potent Hematopoietic Niche Containing Hematopoietic Stem and Progenitor Cells throughout Development

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Human Placenta Is a Potent Hematopoietic Niche Containing Hematopoietic Stem and Progenitor Cells throughout Development

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SUMMARY

Hematopoietic stem cells (HSCs) are responsible for the life-long production of the blood system and are pivotal cells in hematologic transplantation therapies. During mouse and human development, the first HSCs are produced in the aorta-gonad-mesonephros region. Subsequent to this emergence, HSCs are found in other anatomical sites of the mouse conceptus. While the mouse placenta contains abundant HSCs at midgestation, little is known concerning whether HSCs or hematopoietic progenitors are present and supported in the human placenta during development. In this study we show, over a range of developmental times including term, that the human placenta contains hematopoietic progenitors and HSCs. Moreover, stromal cell lines generated from human placenta at several developmental time points are pericyte-like cells and support human hematopoiesis. Immunostaining of placenta sections during development localizes hematopoietic cells in close contact with pericytes/perivascular cells. Thus, the human placenta is a potent hematopoietic niche throughout development.

INTRODUCTION

Hematopoiesis in the human conceptus progresses in a wave-like manner in several different embryonic sites: the yolk sac (YS), the splanchnopleura/aorta-gonad-mesonephros (AGM) region, the liver, and the bone marrow (BM) (Tavian and Peault, 2005; Zambidis et al., 2006). Blood generation begins at day 16 of development in the YS with the production of primitive erythroid cells. At day 19, the intraembryonic splanchnopleura becomes hematopoietic. The emergence of multipotent progenitors and HSCs, organized in clusters of cells closely adherent to the ventral wall of the dorsal aorta, starts at day 27 in the developing splanchnopleura/AGM region (Tavian et al., 1996, 1999, 2001). Starting at day 30, the first erythroid progenitors (BFU-E, burst forming unit erythroid) are found in the liver, with multilineage hematopoietic progenitors (CFU-Mix or -GEMM; colony forming unit granulocyte, erythroid, macrophage, megakaryocyte) appearing in this tissue at week 13 (Hann et al., 1983). Hematopoietic progenitors and long-term culture-initiating cells have been found in the human placenta at 8–17 weeks in gestation (Barcena et al., 2009; Zhang et al., 2004). Thereafter, the BM becomes hematopoietic. This sequence of hematopoietic events closely parallels that found in the mouse conceptus, in which the spatial/temporal appearance and the quantitative/qualitative characteristics of hematopoietic progenitor and stem cells have been carefully mapped (Ferkowicz et al., 2003; Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Palis et al., 1999). Importantly, the developing hematopoietic cells in the conceptus are increasing in their complexity (multilineage and higher proliferative potentials) and culminate with the generation of adult-type HSCs that sustain hematopoiesis throughout adult life (Dzierzak and Speck, 2008). While the YS generates the transient embryonic erythroid cells, the AGM is the first tissue to generate more complex hematopoietic progenitors and stem cells (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). The liver and the BM are thought to be colonized by these cells and provide a potent supportive microenvironment for the growth of the fetal and life-long blood system.

In addition to the AGM (Cumano et al., 1996; de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996), the chorioallantoic placenta of the mouse conceptus generates and supports hematopoietic cells at early developmental stages (Alvarez-Silva et al., 2003; Corbel et al., 2007; Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Rhodes et al., 2008; Zeigler et al., 2006). Quantitatively, the midgestation mouse placenta contains more hematopoietic progenitors and HSCs than the AGM region and the YS, indicating that the placenta provides a potent supportive

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microenvironment for HSC amplification and may be, with the liver, a predominant source of adult BM HSCs (Alvarez-Silva et al., 2003; Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). In contrast to the mouse, there is little information concerning the hematopoietic potential of the human placenta (Bailo et al., 2004; Barcena et al., 2009; Challier et al., 2005; Zhang et al., 2004). Human studies have focused on umbilical cord blood (UCB), revealing that it is an important and easily accessible source of potent hematopoietic progenitors and HSCs for clinical transplantation procedures (Tse et al., 2008). However, the HSC dose limitation in UCB samples and the increasing transplantation needs for treating hematologic disorders has stimulated the search for additional sources of potent HSCs and/or improved methods of ex vivo amplification of HSCs prior to transplantation.

Generally, the human placenta has been thought to function as a facilitator of nutrient and waste exchange between the mother and fetus, a provider of immunoprotection for the fetus, and a producer of important factors and hormones for fetal growth (Gude et al., 2004). In this report, we present data showing that the human placenta beginning from gestation week 6 onward contains fetal-derived immature hematopoietic progenitors and stem cells, differentially expressing CD34 through ontogeny. Furthermore, mesenchymal stromal cells, isolated from human placenta throughout development that we identify as pericyte-like cells, can support the in vitro maintenance of human cord blood hematopoietic progenitors. Together, our results show that the human placenta is a potent hematopoietic niche and a potentially useful source of cells at term for regenerative medicine.

RESULTS

Human Placenta Contains Hematopoietic Progenitor Cells throughout Gestation

The human term placenta is comprised of the highly vascular fetal-derived chorionic plate and villi and maternally-derived blood components that circulate in the intervillous space. We examined whether the human placenta obtained at the time of delivery contains hematopoietic progenitors. Blood from inside the placenta was collected (placenta blood). The remaining cells inside the vasculature were collected in wash steps (vessels PBS) and following collagenase treatment (vessels collagenase). Finally, the placenta was dissociated after enzymatic treatment (placenta collagenase) (Figure 1A).

Flow cytometric analysis for CD34 and CD38 markers was performed on human placenta cell populations and UCB (Figure 1B). $CD34^+CD38^+$ cells (mature hematopoietic progenitors) and $CD34^+CD38^-$ (immature hematopoietic progenitors/HSCs) were found in the vessel PBS wash, vessel collagenase, and placenta collagenase preparations. Compared to UCB and placenta blood, the percentages of $CD34^+CD38^-$ cells were increased (about 6- to 10-fold), and an extra population of cells, $CD34^{++}CD38^-$, was found in the vessel collagenase and placenta collagenase cell preparations. Some of these cells coexpress CD31, but not CD45, and represent a population of endothelial cells (Figure S1 available online).

Hematopoietic progenitor activity in term placental cell preparations was tested in the colony forming unit (CFU) assay. Colonies with typical morphology representing all hematopoietic lineages were found in both the vessel and placenta preparations—BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Mix (Figure S2). The combined number of CFU-Cs in the placenta vessels and tissue obtained at the time of delivery (38 weeks) was found to be 8000 per 10^5 $CD34^+$ cells (Figure 1C) and is a lower frequency than that found in UCB (23,000 per 10^5 $CD34^+$ cells) or placental blood. This is a slight underestimate of placenta progenitor frequency since the $CD34^{++}CD38^-$ population contains a proportion of endothelial cells: 19% for placenta vessels and 37% for tissue (Figure S1).

Clonogenic hematopoietic assays were also performed on placentas obtained from the first and second gestational trimesters. Colonies of all erythromyeloid lineages were found beginning at gestational week 6, the earliest stage placenta tested (Figure 1D), and were in both the $CD34^+$ and $CD34^-$ cell fractions. While the frequency of BFU-E remained similar between placentas obtained at gestational weeks 6, 9, and 15, abundant increases (up to 10-fold) of CFU-GM and CFU-Mix were found beginning at week 9. Until week 9, CFU-GM and CFU-Mix are mainly in the $CD34^-$ placenta fraction. Genotyping of CFU-Mix colonies from $CD34^+$ and $CD34^-$ placenta cells (gestation week 9) revealed that the hematopoietic cells were fetal-derived (data not shown). By week 15 (and 38; term), these progenitors are in the $CD34^+$ fraction, suggesting a developmental regulation in the appearance, phenotype, and frequency of more complex hematopoietic progenitors in the developing placenta. $CD34^+$ $CD45^+$ hematopoietic cells are localized in the placenta villi (Figure 1E) and vasculature (Figure 1F) as shown by immunostaining of week 16 placenta sections.

Early-Stage Human Placenta Contains Hematopoietic Stem Cells

Hematopoietic engraftment of NOD-SCID (or *Rag* $\gamma C^{-/-}$) immunodeficient mice is considered the gold-standard functional assay for detection of human HSCs (hereafter called hu-SRC, human SCID repopulating cells) (Coulombel, 2004). Published results from developmental studies show that the mouse placenta contains a high number of HSCs during midgestation (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Since the analogous developmental period in the human begins at approximately week 6 in gestation, first and second trimester human placentas (total of 17) were examined for hu-SRCs. Placenta cells were injected into 47 NOD-SCID recipients (Table 1), and multilineage engraftment was measured by flow cytometry and PCR.

Figure 2A shows PCR results of hematopoietic tissue DNA from recipients receiving male 6, 9, and 19 week placenta cells. The male Y-chromosome-specific *AMELY* fragment was found in the blood, spleen, and BM, demonstrating that engraftment was due to placenta cells from the fetal part of the placenta. Flow cytometric analysis of the recipient injected with week 9 placenta (TCB) cells shows multilineage hematopoietic engraftment (Figure 2B). Human (h)$CD45^+$ cells were found in the blood, BM, and spleen and were of the myeloid ($CD15^+$) and B lymphoid ($CD19^+$) lineages. A small population of $hCD34^+CD38^-$ cells, indicative of immature hematopoietic progenitors, was found in the recipient BM. BM cells from this repopulated mouse produced colonies of all myeloerythroid lineages (Figure 2C),

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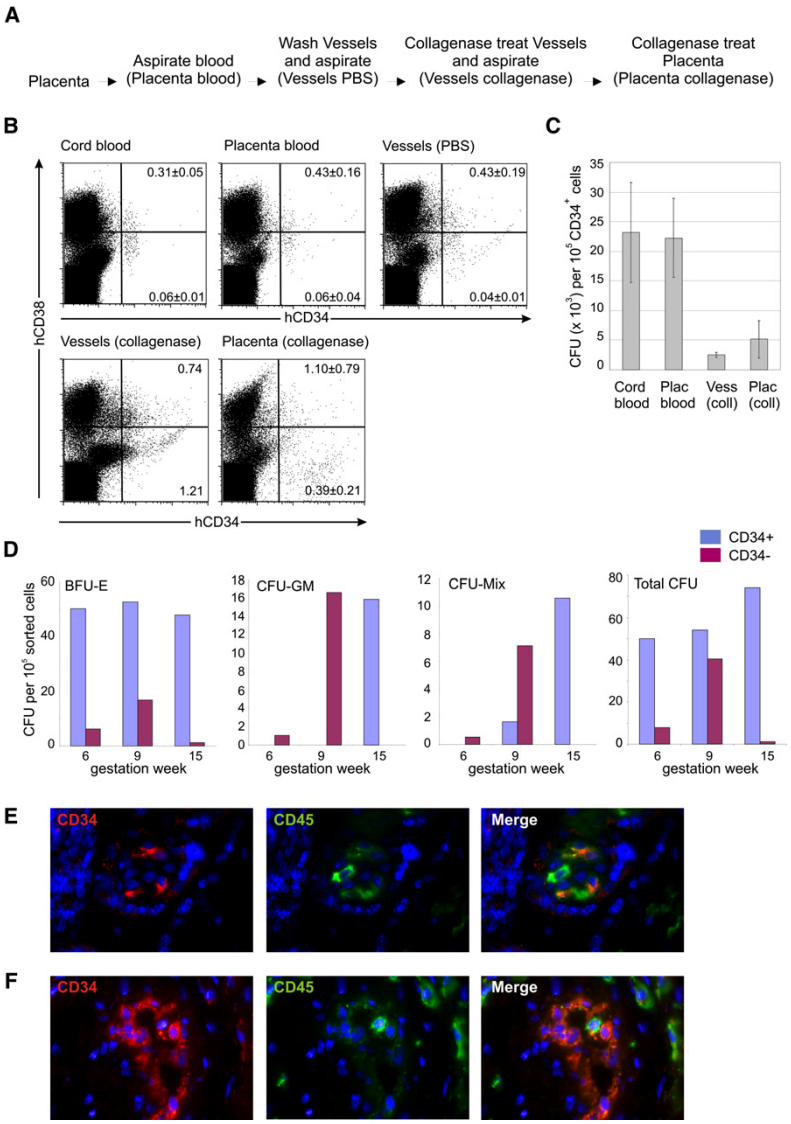

Figure 1. Human Placenta Contains Hematopoietic Progenitors throughout Development

(A) Procedure for the isolation of cell populations from the human placenta.

(B) Flow cytometric analyses of term blood and placenta. Cord blood cells, placental blood cells, and cells recovered after extensive washes of the placental vasculature (Vessels PBS), from collagenase treatment of the placental vessels, and subsequent collagenase treatment of the remaining placenta tissue were stained with anti-human (h)CD34 and CD38 antibodies, and viable cells were analyzed. Mean percentage ± SD (n = 1–4) of relevant populations is indicated.

(C) Clonogenic progenitors in term placenta were analyzed in methylcellulose cultures. Frequency of total hematopoietic progenitors (CFU, colony forming unit) in the $CD34^+$ cell fraction sorted from the different tissues. Sort purity for cord blood > 96%, placenta blood > 98%, vessel collagenase > 93%, and placenta collagenase > 81%. Error bars display SEM (n = 5).

(D) Clonogenic progenitors in the sorted $CD34^+$ (92%–94% purity) and $CD34^-$ (98%–100% purity) cell fractions of early stage placentas were analyzed in methylcellulose cultures. Frequencies of the different hematopoietic progenitor types (BFU-E, CFU-GM, CFU-Mix, and the sum of these, total CFU) in both $CD34^+$ and $CD34^-$ cell fractions sorted from placentas of gestational weeks 6, 9, and 15 are displayed.

(E and F) Villus (E) and vasculature (F) from 16 human placenta cryosections; CD34 (red), CD45 (green), and merged fluorescence are shown.

including the most immature multilineage colonies, CFU-Mix. PCR analysis of DNA prepared from individual CFU and pooled CFU verified that these human progenitors were fetal-derived (Figure 2D). Thus, early gestational stage human placenta cells home to the BM and provide multilineage hematopoietic repopulation of NOD-SCID mice.

Engraftment of NOD-SCID mice with placenta cells from female conceptuses was tested by PCR for human chromosome 17 (*hChr 17*) alpha-satellite DNA (Figure 2A, TC69A), followed by a forensic PCR to discriminate fetal- from maternal-derived engraftment. Fifteen highly polymorphic short tandem repeat (*STR*) loci normally used for human identity testing were measured, and the STR profiles of recipient NOD-SCID hematopoietic tissue DNAs were compared to embryo STR profiles. As shown for the TC69A placenta (Figure 2E), the STR profiles of the spleen and BM cells of the NOD-SCID recipient were identical to the profile of the embryo, thus demonstrating exclusive engraftment from fetal-derived female placenta cells. In summary, of the 17 placentas transplanted into a total of 47 recipients, 14 recipients (30%) were repopulated by hu-SRCs of fetal placenta origin (Table 1).

To further characterize the placenta-derived hu-SRCs, transplantation experiments into NOD-SCID and *Rag* $\gamma C^{-/-}$ mice were performed with placenta cells sorted on the basis of expression of the CD34 marker. As shown in Table 2, HSCs are both in $CD34^+$ and $CD34^-$ fractions in 6-week-old placenta, the earliest time point tested. By 16 to 18 weeks, the HSC population appears to be enriched in the $CD34^+$ fraction. An example of human multilineage analyses in a *Rag* $\gamma C^{-/-}$ recipient is shown (Figure S3).

Human Full-Term Placenta Contains NOD-SCID Hematopoietic Repopulating Cells

Previously, it was shown that HSCs were almost undetectable in the mouse placenta at term (E18) (Gekas et al., 2005). To examine if this was also the case for the human placenta, cells from term human placenta vessels and tissues were prepared and injected into NOD-SCID mice. Recipients were analyzed at

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Table 1. Summary of NOD-SCID Recipient Repopulation with Fetal-Derived Cells from First and Second Trimester Human Placenta Cells

Gestation Week	Number of Placentas: Male	Female	Total	Cell Number Injected	Number Repopulated / Number Injected
19	1	1	2	1–3 × 10^6	3[a]/4
18	2		2	1.5–2.1 × 10^6	0/2
17	3		3	1–3 × 10^6	0/7
13	1		1	0.8–3 × 10^6	0/2
11	1		1	1.3 × 10^6	1/1
9	3		3	1–6 × 10^6	6/16
8	1	1	2	3 × 10^6	3[a]/5
7	2		2	1–3 × 10^6	0/9
6	1		1	1.5 × 10^6	1/1
Total	15	2	17		14/47 (30%)

First and second trimester human placenta cells were prepared, and various cell doses were injected into NOD-SCID recipients. All 47 recipients were tested for donor cell engraftment with *AMEL* PCR for placental cells from a male conceptus and *STR* PCR for placenta cells from a female conceptus. Recipients were considered positive for repopulation if at least one hematopoietic tissue at the time of sacrifice (5–10 weeks post-injection) showed *AMELY* signal or an STR profile that matched that of the embryo. All PCR results were verified two to three times.

[a] Samples for which STR profiles were established. One recipient injected with 19 week female placenta and three recipients injected with 8 week female placenta were profiled.

5 months posttransplantation for human hematopoietic cell engraftment by flow cytometric analysis and PCR.

Three experiments (3 male placentas) resulted in human hematopoietic repopulation of NOD-SCID mice (Figure 3 and Table 3). hCD45$^+$ cells (Figure 3A) were detected in the blood of a NOD-SCID recipient receiving 20 × 10^6 placenta tissue cells from male term placenta (tP2). The BM and spleen contained high percentages of hCD45$^+$cells (51% and 22%, respectively) of which many were B lymphoid cells, with a few myeloid cells. Control transplantation of 20 × 10^6 human UCB cells showed similar levels of NOD-SCID BM engraftment (66% hCD45$^+$, 49% hCD19$^+$, and 6% hCD15$^+$ cells) that were comparable to published cord blood NOD-SCID transplantation data (Bhatia et al., 1997). Collagenase-treated placenta vessel cells (7 × 10^6 cells injected) from tP2 (Figure 3B) also resulted in similar engraftment, with high percentages of hCD45$^+$ cells in BM and spleen, including B lymphoid and myeloid cells. Interestingly, injection of 7 × 10^6 vessel cells was sufficient to highly repopulate a NOD-SCID recipient, while injections of 5–6 × 10^6 tissue cells from tP3 and tP1 were not (Table 3). These data suggest that placental hu-SRCs are concentrated inside the placental labyrinth, possibly attached to the vascular endothelium.

A combination of three enzymes (collagenase, dispase, and pancreatin) was used to further optimize placenta cell preparations. The improved digestion conditions resulted in a higher viable cell recovery (13-fold) and increased percentages of CD34$^+$CD38$^+$ and CD34$^+$CD38$^-$ cells (Figure S4), as compared to single collagenase treatment (Figure 1B). After injection of 10 × 10^6 cells from male tP3 (prepared using this method) into a NOD-SCID recipient, high percentages of hCD45$^+$ cells were found in the blood, spleen, and BM (Figure 3C), and cells were of the B lymphoid and myeloid lineages. Moreover, the recipient mouse BM contained immature human CD34$^+$CD38$^-$ cells, strongly suggesting that the term placenta contains bona fide hematopoietic progenitors/stem cells.

The human hematopoietic cells detected in the flow cytometric analysis of NOD-SCID recipients (Figures 3A, 3B, and 3C) transplanted with term placenta cells were derived from the fetal (male) part of the placenta, as shown by *AMEL* PCR analysis (Figure 3D) of BM, blood, spleen, lymph node, and thymus DNA. Thus, term human placenta contains fetal-derived hu-SRCs that home to the BM and provide robust long-term multilineage hematopoietic engraftment of recipients.

Human-Placenta-Derived Cell Lines Support Human Hematopoietic Progenitors and Possess Characteristics of Pericytes/Perivascular Placenta Cells

To examine whether the human placenta contains cells typical of a hematopoietic supportive microenvironment (i.e., mesenchymal stromal cells), cell lines were established at various developmental stages—3, 6, 16, 18, and 38 weeks of gestation. All the cell lines showed a fibroblastic morphology, and 2 cell lines from each developmental time point were analyzed.

The growth rates of the placenta cell lines varied. Early stage (maternally derived) and term placenta cell lines showed slower growth than cell lines from the first and second trimester tissues (Table 4). In agreement with the previously described cell surface phenotype of first trimester and term placenta stromal cells (Bhatia et al., 1997; Fukuchi et al., 2004; Li et al., 2005; Yen et al., 2005; Zhang et al., 2004, 2006), our lines are CD13$^+$, CD29$^+$, CD44$^+$, CD105$^+$, HLA-DR$^-$, CD14$^-$, CD34$^-$, CD45$^-$, CD19$^-$, CD2$^-$, CD3$^-$, CD4$^{lo/-}$, CD8$^-$, and CD11b$^{lo/-}$ (Figure S5A; Table 4). Also, in cultures allowing for osteogenic differentiation, our placenta lines (second trimester and term) were positive for alkaline phosphatase (Figure S5B) and mineralization, and most cell lines also could be differentiated into adipocytes (Figure S5C). Interestingly, when three of these cell lines were tested (H93-6, H92-1, and H91-2) in matrigel, they formed tubules indicative of endothelial potential (Figure S5D; Table 4). Thus, the human placental cell lines have the same mesenchymal potential as reported previously in hematopoietic supportive AGM (Durand et al., 2006).

Since a recent publication has highlighted pericytes/perivascular cells as the in vivo correlate/precursors to mesenchymal stromal/stem cells (Crisan et al., 2008), we examined our cell lines for pericyte characteristics. Flow cytometric analyses showed that cell line H92-1 expressed pericyte markers NG2 and CD146 (Figure 4A), as did H93-6 and H91-1 (data not shown). To localize these cells in vivo, cryosections from week 16 human placenta were immunostained with three pericyte markers CD146, NG2, and α-SMA (smooth muscle actin) (Figures 4B and 4C). As previously shown (Crisan et al., 2008), the only cells coexpressing CD146, NG2, and α-SMA in situ are pericytes/perivascular cells closely associated to endothelial cells in microvessels (MV), capillaries (C), and large vessels (LV). These data demonstrate that placenta stromal cell lines at week

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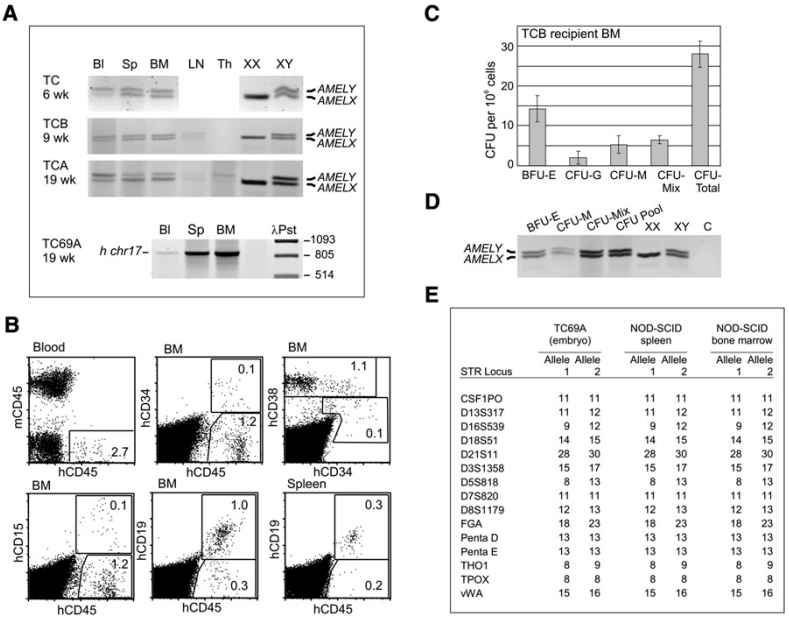

STR Locus	TC69A (embryo) Allele 1	TC69A (embryo) Allele 2	NOD-SCID spleen Allele 1	NOD-SCID spleen Allele 2	NOD-SCID bone marrow Allele 1	NOD-SCID bone marrow Allele 2
CSF1PO	11	11	11	11	11	11
D13S317	11	12	11	12	11	12
D16S539	9	12	9	12	9	12
D18S51	14	15	14	15	14	15
D21S11	28	30	28	30	28	30
D3S1358	15	17	15	17	15	17
D5S818	8	13	8	13	8	13
D7S820	11	11	11	11	11	11
D8S1179	12	13	12	13	12	13
FGA	18	23	18	23	18	23
Penta D	13	13	13	13	13	13
Penta E	13	13	13	13	13	13
THO1	8	9	8	9	8	9
TPOX	8	8	8	8	8	8
vWA	15	16	15	16	15	16

Figure 2. Long-Term Multilineage NOD-SCID Hematopoietic Repopulating Cells Are Present in Placenta throughout Gestation

(A) Human placenta cell engraftment was examined by (A) PCR for the human amelogenin gene (*AMEL*) or for the human chromosome 17 alpha-satellite sequence (*h chr17*) in blood (Bl), spleen (Sp), bone marrow (BM), and/or thymus (Th) and lymph node (LN) DNA isolated from cells of NOD-SCID mice transplanted with collagenase-/dispase-/pancreatin-treated placenta tissue cells from the 6, 9, and 19 week (wk) gestation stages. 1.5 × 10^6 of TC, 3 × 10^6 of TCB, 3 × 10^6 of TCA, and 3 × 10^6 of TC69A placenta cells were injected per mouse. TC, TCB, and TCA placentas were from male conceptuses, and TC69A was from a female conceptus. TC, TCB, TCA, and TC69A recipients were analyzed respectively at 6, 10, 11, and 7 weeks post-transplantation.

(B) Flow cytometric multilineage analyses of blood, bone marrow (BM), and spleen cells isolated from NOD-SCID mice 10 weeks after injection of 3 × 10^6 cells from collagenase-/dispase-/pancreatin-treated TCB placenta tissue. Cells were stained with anti-mouse (m) CD45 and anti-human (h) CD34, CD38, CD45, CD19, and CD15 antibodies and analyzed in the viable population. Number of events analyzed were 3 × 10^5 for blood and 9 × 10^4 for BM and spleen. Percentages of gated populations are indicated.

(C) Frequencies of the different hematopoietic progenitor types (BFU-E, CFU-G, CFU-M, and CFU-Mix) present in the total BM isolated from the TCB reconstituted NOD-SCID recipient shown in (A) and (B). Error bars display SEM (triplicate).

(D) PCR analysis for the amelogenin gene was performed on each colony type and on a pool of colonies (CFU pool) harvested from the culture experiments in (C). The presence of *AMELY* fragment reveals their fetal origin.

(E) STR profiling of DNA from the spleen and BM of the NOD-SCID recipient transplanted with TC69A (female) placenta tissue cells. TC69A embryo DNA (female) served as the control for fetal-derived cells. STR alleles are designated as numbers of polymorphic repeats.

16 of gestation are pericyte-like cells, and together with data in Figures 1E and 1F, suggest that the perivascular/vascular microenvironment and the hematopoietic system develop in parallel in the placenta.

The hematopoietic supportive properties of placenta stromal cell lines were tested in cocultures. Confluent monolayers of stromal cells (3, 16, and 18 week stages) were overlayed with 5000 $CD34^+$ UCB cells and cultured in factor-supplemented medium. After 12 days, the number of $CD34^+$ cells was increased 2- to 8-fold (Table 4). Clonogenic activity was also tested. As compared to the input number of CFU (in freshly sorted CB $CD34^+$ cells), the placenta cell lines supported a 65- to 370-fold expansion of CFU-GM and an up to 8-fold expansion of CFU-Mix (Table 4 and Figure S6). Thus, based on the results of the cell lines, the human placenta contains hematopoietic supportive pericytes/perivascular stromal cells.

DISCUSSION

Prior to this study, only the presence of progenitors in the human placenta has been reported (Barcena et al., 2009). Here, we confirm that the human placenta contains all types of hematopoietic progenitors but more importantly, we show that the human placenta also contains hu-SRCs. hu-SRCs are detected

Table 2. Summary of NOD-SCID and *Rag* $\gamma C^{-/-}$ recipient repopulation with $CD34^+$ and $CD34^-$ sorted cells from first and second trimester human placenta

Recipients	Gestation Week	Cell Number Injected		Number Repopulated/Number Injected	
		$CD34^+$	$CD34^-$	$CD34^+$	$CD34^-$
NOD-SCID	6	0.6 × 10^6	1.9 × 10^6	2/2	1/1
Rag $\gamma C^{-/-}$	16–17	0.6–1.0 × 10^6	2 × 10^6	1/3	0/4
NOD-SCID	16–18	0.06–0.6 × 10^6	0.75–1.6 × 10^6	1/2	1/2
NOD-SCID	19–20	0.5 × 10^6	5–10 × 10^6	1/1	0/2

Tissue DNA from all recipients injected with human placenta cells was tested for human engraftment by *AMEL* PCR. NOD-SCID adult recipients were irradiated with 3 Gy, injected intravenously with the indicated numbers of sorted placenta cells (and 1 × 10^5 NOD-SCID spleen cells), and analyzed for human cell engraftment 5 weeks postinjection. *Rag* $\gamma C^{-/-}$ 5-day-old recipients were irradiated with 3 Gy, injected in the liver with the indicated numbers of sorted placenta cells (10 μl volume), and, 11 weeks postinjection, analyzed for human cell engraftment.

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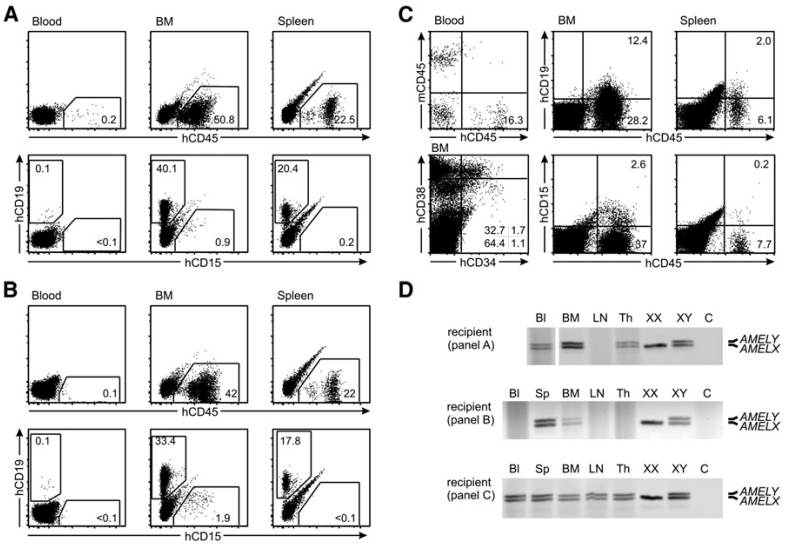

Figure 3. Long-Term Multilineage NOD-SCID Hematopoietic Repopulating Potential of Full-Term Placenta Cells

(A–C) Flow cytometric multilineage analyses of blood, bone marrow (BM), and spleen cells isolated from NOD-SCID mice repopulated 5 months after injection of term placenta cells. (A) 20 × 10^6 cells from collagenase-treated placenta tissue (tP2); (B) 7 × 10^6 cells from collagenase-treated placental vessels (tP2); and (C) 10 × 10^6 cells from collagenase-/dispase-/pancreatin-treated placenta tissue (tP3). Cells are stained with anti-mouse (m) CD45 and/or anti-human (h) CD45, CD19, CD15, CD34, and CD38 antibodies and analyzed in the viable population. Number of events analyzed was 3 × 10^4 for all tissues in (A) and (B), and 2 × 10^5 for blood and 1 × 10^5 for BM and spleen in (C). Percentages of gated and quadrant populations are indicated.

(D) To verify the fetal (male) origin of the engraftment, PCR for the amelogenin gene was performed on blood (Bl), spleen (Sp), bone marrow (BM), thymus (Th), and lymph node (LN) DNA isolated from cells of the reconstituted recipients described in (A), (B), and (C). Control female (XX) cell DNA produces a single product (*AMELX* at 106 bp), whereas control male (XY) DNA produces two products (*AMELY* at 112 bp and *AMELX*).

in the human placenta as early as week 6 in gestation, throughout fetal development, and most surprisingly, at term. This is an unexpected result since previous data in the mouse term placenta show almost no adult repopulating HSCs (Gekas et al., 2005). Given that human placenta cells throughout ontogeny provide long-term repopulation of the NOD-SCID hematopoietic system to the same levels and in the same hematopoietic lineages (B lymphoid and myeloid) as UCB cells, they are bona fide hu-SRCs (Cashman et al., 1997; Coulombel, 2004; Larochelle et al., 1996; Pflumio et al., 1996). Thus, the human placenta can now be acknowledged as a new territory of hu-SRCs, and this routinely discarded tissue can now be used to provide further insight into cell-cell interactions and molecules relevant to human hematopoietic progenitor/stem cell growth.

Our data demonstrate that the temporal sequence of hematopoietic cell appearance in the human placenta is generally conserved as compared to the mouse placenta. In the mouse, adult repopulating HSCs appear in the AGM region, vitelline, and

Table 3. Summary of NOD-SCID Recipient Repopulation by Fetal-Derived Cells from Term Placenta

Term Placenta (Male)	Cell Preparation	Number of Mice Injected	Cell Number injected	Bleed I (2 Months Postinjection)	Bleed II (4.5–5 Months Postinjection)
tP2	vessel	1[a]	7 × 10^6	+++	+++
	placenta fresh (c)	1[b]	20 × 10^6	+++	+++
tP3	placenta fresh (cdp)	2	1 × 10^6	negative	negative
		2	5 × 10^6	negative	negative
		1[c]	10 × 10^6	+++	+++
tP1	vessel	1	21 × 10^6	+++	dead
	placenta fresh (c)	1	6 × 10^6	+/−	negative
	placenta frozen (c)	1[d]	1.3 × 10^6	+++	+++

Term (male) placentas were treated and made into cell suspensions as indicated and injected at various cell doses into NOD-SCID recipients. Out of ten injected mice, six were positive (by flow cytometry) at 2 months postinjection. Multilineage flow cytometric analysis performed (at 2 months postinjection) on the blood of the recipient repopulated with tP1 vessel cells (20 × 10^6). 67% hCD45$^+$, 0.2% hCD15$^+$, 49% CD19$^+$, 67% hCD38$^+$, 2.1% hCD34$^+$, and 0.07% hCD34$^+$38$^-$ cells were found and were similar to percentages obtained from a control recipient transplanted (in the same experiment) with 20 × 10^6 cord blood cells. The tP1 recipient transplanted with 6 × 10^6 fresh placenta cells was considered +/− at 2 months postinjection because only 0.35% hCD45$^+$, 0.79% hCD38$^+$, and 0.52% hCD19$^+$ cells were found. cdp, collagenase, dispase, and pancreatin treatment; c, collagenase treatment.

[a] Flow cytometric analysis of recipient shown in Figure 3B.
[b] Flow cytometric analysis of recipient shown in Figure 3A.
[c] Flow cytometric analysis of recipient shown in Figure 3C.
[d] Flow cytometric analysis of recipient shown in Figure S7.

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Table 4. Phenotypic Characteristics and Functional Properties of Human Placental Stromal Cell Lines through Development

Line	Age (Weeks)	Doubling Time (hrs)	Origin	Mesenchymal Markers	Osteogenic Potential	Adipogenic Potential	Endothelial Potential	Coculture Fold Increase in CB CD34$^+$	Coculture Fold Increase in CB CFU-GM	Coculture Fold Increase in CB CFU-Mix
R19-a	3	59	M	+	–	–	ND	3.9 ± 1.6	80.4	7.9
R19-3	3	50	M	+	–	–	ND	6.6 ± 1.9	69.3	3.0
R17-2	6	32	F or M	ND	+++	ND	ND	ND	ND	ND
R17-3	6	34	F and M	ND	–	ND	ND	ND	ND	ND
H93-6	16	29	F	+	+++	+/–	+	3.6 ± 1.3	65.0	0
H92-1	16	29	F	+	+++	++	+	7.7 ± 2.9	370.6	6.1
H91-1	18	35	F	+	+++	++	+	2.2 ± 0.5	80.4	3.1
H91-2	18	35	F	+	+++	+++	ND	3.8 ± 0.3	102.7	0
L13-1	term	41	ND	ND	+++	ND	ND	ND	ND	ND
L13-5	term	41	ND	ND	+++	ND	ND	ND	ND	ND

Cell line origin was determined by STR profiling. The profile of R17-2 yielded only two alleles for each gene, while R17-3 gave a mix of alleles for many genes. ND, not done; M, maternal; F, fetal.

umbilical arteries first and are thereafter found in the YS and placenta (de Bruijn et al., 2000; Dzierzak and Speck, 2008; Gekas et al., 2005; Ottersbach and Dzierzak, 2005). In the human conceptus, hematopoietic progenitor/stem cells are found at day 27 in the aorta, concomitant to the appearance of clusters of cells closely adherent to the aortic lumenal wall (Tavian et al., 1996, 1999). Hematopoietic progenitors are found in the human YS, but with a less robust hematopoietic potential (Tavian et al., 2001). Our results indicate that fetal-derived hu-SRCs are present in the human placenta already at gestational week 6. The presence of HSCs at earlier stages, particularly between gestational weeks 3–6, is still undetermined. Most placentas we analyzed at these stages were of variable quality. Considering that, in the mouse placenta, limiting numbers of fetal-derived HSCs are found at E11 and rapidly increase to the highest numbers at E12 to E13 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), our future analyses on the possible earlier appearance of HSCs in human placenta will depend on improved placenta isolation and more sensitive maternal/fetal genotyping.

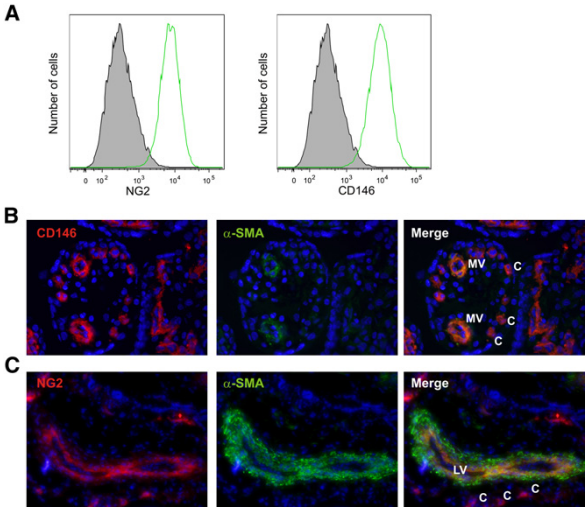

Figure 4. Pericyte Marker Expression on Human Placenta Stromal Cell Lines and Human Placenta Tissue

(A) Histogram of flow cytometric analysis for NG2 and CD146 expression on H92.1 placenta stromal cell line is shown. Immunostained cryosections from 16 week human placenta costained for (B) CD146 (red) and α-SMA (green) (20× lens) or (C) NG2 (red) and α -SMA (green) (10× lens) are shown. Single and merged fluorescence are shown. MV, microvessels; C, capillaries; LV, large vessel.

Another important and timely result comes from our panel of placental stromal cell lines. These cells are identified as CD146- and NG2-expressing pericyte-like cells. These stromal cell lines support the expansion of cord blood CD34$^+$ cells and immature hematopoietic progenitors in cocultures. Interestingly, such pericyte-like cells were found in situ in the developing human placenta, suggesting an in vivo role in hematopoietic support. At all gestational stages, the placenta stromal cells express classical mesenchymal markers and, after gestation week 6, possess mesenchymal lineage potentials (osteo- and adipogenic), in agreement with other reported placenta cell lines (Fukuchi et al., 2004; Igura et al., 2004; Li et al., 2005; Miao et al., 2006; Parolini et al., 2008; Portmann-Lanz et al., 2006; Wulf et al., 2004; Yen et al., 2005; Zhang et al., 2006). Since some mesenchymal cell lines constitute a suitable feeder layer for in vitro maintenance and/or expansion of primate and human ESCs (Kim et al., 2007; Miyamoto et al., 2004) and long-term culture-initiating cells (Zhang et al., 2004), it will be interesting to determine whether they are pericyte-like and are of maternal or fetal origin (In't Anker et al., 2004). Our cell lines from gestation week 3 placenta were found to be maternally derived (by STR profiling) and exhibited slow growth, as compared to week 6, 16, and 18 placenta cell lines. Nonetheless, these cells effectively support the growth of CD34$^+$ cells in cocultures, yielding an 8-fold increase in CFU-Mix. Maternal stromal cells, therefore, may contribute at early stages to hematopoietic support, and later in gestation, the more rapidly doubling fetal stromal cells predominate in the growth of the placenta as a highly vascular and hematopoietic territory.

At early developmental time points (week 6 and 9), hematopoietic progenitors are in both the CD34$^+$ and CD34$^-$ fractions. CFU-GM and CFU-Mix are restricted initially to the

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$CD34^-$ fraction of week 6 placenta and switch to the $CD34^+$ fraction by week 15, suggesting developmental regulation of this marker on progenitors. Similarly, hu-SRCs are found in both $CD34^+$ and $CD34^-$ fractions at week 6. Later at week 16–20, hu-SRCs are in both fractions but appear to be more enriched in the $CD34^+$ fraction. This is in agreement with the published data showing a subset of hu-SRCs in the $CD34^-$ fraction of UCB (Bhatia et al., 1998; Wang et al., 2003). Interestingly, immunostainings of week 16 placenta sections show $CD34^+CD45^+$ hematopoietic cells within placental villi stroma and $CD45^{high}$ expressing cells that appear to be budding from the vasculature. Moreover, high percentages of $CD34^+CD38^-$ cells and hu-SRCs were found in the collagenase-treated vessel cell preparations (after extensive prewashing of the placenta to remove circulating blood) and in collagenase-treated placenta tissue. Since previous studies demonstrated the hematopoietic potential of human YS, embryonic liver, and fetal BM vascular endothelium (Oberlin et al., 2002) and also was suggested in the early mouse placenta (Corbel et al., 2007; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006; Gekas et al., 2005), our data support the notion that hu-SRCs are generated, harbored, and/or amplified in vascular labyrinth placenta niche.

Finally, in addition to revealing the fundamental aspects of human placenta HSC development, our results have implications for the human placenta as source of HSCs alongside UCB for banking and potential clinical use. From our data, a conservative estimate of the HSC content of a human placenta (using the three enzyme treatment) is about 10% of the published number of HSCs contained in one unit of UCB (Bhatia et al., 1997; Wang et al., 1997). As a 13-fold increase was already achieved through the implementation of three enzymes versus collagenase only, further increases in placenta hu-SRC harvest are expected. Importantly, if placental cells are to be a source of clinically useful HSCs, they must withstand storage procedures. In preliminary experiments, we found that the percentage of $CD34^+$ placenta cells increased 1.4-fold and that hu-SRC potential was retained and enriched after storage in liquid nitrogen. Remarkably, only 1.3×10^6 thawed unsorted cells from placenta tP1 were required for robust NOD-SCID multilineage hematopoietic engraftment (Figure S7) as compared to the low engraftment yielded with 6×10^6 freshly prepared collagenase-treated tP1 cells (Table 3). Taken together, the human placenta is a highly hematopoietic tissue throughout development, containing potent hu-SRCs. As a rest tissue normally discarded in the birthing process, the human placenta can be considered as potential source for additional hematopoietic progenitor/stem cells useful for hematologic clinical applications and human regenerative medicine.

EXPERIMENTAL PROCEDURES

Tissues and Cell Preparation

Human fetal tissues were obtained from elective abortions (CASA Clinics, Leiden and Rotterdam) and were contingent on informed consent. Umbilical cord blood and term placentas were obtained from vaginal deliveries or by Cesarean section. The use of fetal tissues was approved by the Medical Ethical Committee of the Erasmus Medical Center (MEC-2006-202). Gestational age was determined by ultrasonic fetal measurements. Placenta cells were isolated directly or after overnight storage at 4°C. The umbilical cord was cut and removed, along with the amniotic sac and deciduas, from the placenta under sterile conditions. The outside of the placenta was washed with cold PBS/EDTA/PS (phosphate-buffered saline added with EDTA, penicillin [100 U/ml] and streptomycin [100 μg/ml]). The blood remaining inside the placenta was aspirated and collected (placenta blood), and the placental vasculature was flushed extensively with PBS/EDTA (up to ten times) via the umbilical vein and arteries to eliminate residual blood within the placental vascular labyrinth.

Collagenase (0.125% w/v type I collagenase [Sigma] in PBS/10% fetal calf serum [FCS]/PS) was injected inside the placental vascular labyrinth. After 1 hr of incubation at 37°C, intravascular cells detached by the collagenase treatment were aspirated and collected (vessels collagenase).

Placenta tissue was minced and washed thoroughly in cold PBS/FCS/PS and treated with 0.125% w/v type I collagenase in PBS/FCS/PS for 1 to 1.5 hr under agitation. Five grams of placenta tissue per 200 ml of buffer was found to be optimal for enzymatic digestion. The placenta was treated, in some cases, with Collagenase, Pancreatin (Sigma, 0.3%), and Dispase I (neutral protease grade I, Roche, 0.33 mg/ml) (three enzyme treatment). All enzymatic treatments were performed in presence of DNase (Sigma). Tissue was dissociated by repeated pipetting and passed through cotton gauze to eliminate nondigested tissue clumps, and the filtered cell suspension was washed twice. Mononuclear cells were recovered by Ficoll density gradient fractionation (Density 1.077 g/ml, Lymphoprep, Axis-Shield PoC AS), washed twice, and filtered through a 40 μm nylon cell strainer. Umbilical cord blood was diluted (1/2) into PBS/FCS/PS, and mononuclear cells were collected after Ficoll. Cells were washed, counted, and kept at 4°C for further utilization.

Enzyme Stock Solution Preparation

Pancreatin (2.5%) was prepared with pancreatin powder from porcine pancreas dissolved in 0.5% PVP solution (polyvinylpyrolidone K30, Fluka). DNase and dispase I (5 mg/ml) were prepared in sterile MilliQ water. Collagenase (2.5%) was prepared with collagenase powder dissolved in sterile PBS.

Mouse Transplantations and Posttransplantation Tissue Collection

Cells from placenta preparations or placenta cells sorted on the basis of CD34 expression (along with 1–2 × 10^5 helper spleen cells) were intravenously injected into irradiated (3 to 3.5 Gy) NOD-SCID (adult) or intrahepatically into *Rag* $\gamma C^{-/-}$ (5-day-old) mice. Four weeks to 5 months later, hematopoietic tissues were collected. Peripheral blood was diluted and mononuclear cells were isolated using Ficoll or lysing solution. Spleen, lymph nodes, and thymus were crushed separately on a 40 μm nylon cell strainer. BM cells were flushed from the femurs and tibias of recipient mice. For all tissues collected, cells were kept for further DNA analyses.

Flow Cytometry Analysis

Placenta cells were stained with the following antibodies: purified CD16/CD32 (preblock), fluorescein isothiocyanate (FITC) or APC-human (h)CD34, FITC-mouse (m)CD45, phycoerythrin (PE) or PE Cy7-hCD38, FITC-hCD31, and PE or PerCP Cy5.5-hCD45. Cells from tissues of NOD-SCID or *Rag* $\gamma C^{-/-}$ recipient mice were stained with the following antibodies: purified CD16/CD32, FITC or PE or PerCP-Cy5.5-hCD45, FITC or PE-mCD45, FITC-hCD15, PE-hCD19, FITC-hCD34, PE-hCD38, PE Cy7-hCD14, and APC Cy7-HLA-DR. After 30 min of staining, cells were washed and stained with 7AAD (Molecular Probes, Leiden, NL) or Hoechst 33258 (1 μg/ml, Molecular Probes) for dead cell exclusion. Blood samples from a noninjected mouse and from informed consent individuals were used as controls. Stromal cell lines were stained with primary antibodies NG2 and CD146, followed by goat anti-mouse Alexa 488. All antibodies were from BD PharMingen, Immunotech or Invitrogen, and analyses were performed on a FACScan or Aria (Becton Dickinson).

Immunohistochemistry

Cryosections (7 μm) of human placenta (1 to 2 cm pieces) were prepared and immunostained as described previously (Crisan et al., 2008). Primary antibodies used are as follows: CD146 (BD PharMingen), NG2 (BD PharMingen), CD45 (eBioscience), α-SMA-FITC (Sigma), and biotinylated anti-CD34 (NOVUS Biologicals). Secondary goat anti-mouse antibody was biotinylated (Dako) or coupled to Alexa 488 (Invitrogen). Streptavidin-Cy3 (Sigma) was used with biotinylated antibodies. Sections were mounted with medium for fluorescence (Vector)-containing DAPI. An isotype-matched negative control

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was performed for each immunostaining. Sections were observed on an epifluorescence microscope (Zeiss).

Hematopoietic Colony Assay

$CD34^+$ and $CD34^-$ cells from human placentas at different stages of development or BM from NOD-SCID recipients tranplanted with human placenta cells were cultured in methylcellulose medium (Methocult H4434; Stem Cell Technologies, Inc.) at 37°C. CFU-GM and Mix and BFU-E colonies were scored with an inverted microscope after day 21 and 28 of culture.

Generation of Human Placenta-Stromal Cell Lines

Placenta tissues were dissected into small pieces and explant cultured on 0.1% gelatin coated 6-well plates at the air-medium interface in hu-LTCSM medium (50% H5100, Stem Cell Technologies; 15% heat-inactivated FCS, GIBCO; 35% α-MEM, GIBCO; 1% Pen/Strep, GIBCO; 1% Glutamax-I (100×), GIBCO; 10 μM β-mercaptoethanol, Merck) at 37°C, 5% CO_2. After several days, cells were harvested using trypsin-EDTA and were seeded on new precoated dishes supplemented with 20% filtered supernatant from the previous passage. Six lines per placenta at 3, 6, 16, and 18 weeks of gestation and 11 lines from term placentas were established. Lines were checked daily and split when subconfluency was reached. Growth curves were established from passage 3 onward.

Mesenchymal Differentiation

Osteogenic differentiation was performed in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 15% heat-inactivated FCS (GIBCO), 1% PS (GIBCO), 200 μM ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma), and 10^{-7} M dexamethasone (Sigma). Cells were seeded in uncoated 6-well plates (500, 1000, and 2000 cells/cm^2) and incubated at 37°C. After 11 and 14 days, alkaline phosphatase activity was determined (Sigma) and at 28 days, Alazarin Red staining was performed (Sigma). Adipocyte differentiation of subconfluent cells was performed in DMEM (10% FCS), dexamethasone (1 μM), IBMX (500 μM), indomethacin (60 μM), and 5 μg/ml insulin for 7 days. Cells were stained with oil red. Endothelial differentiation was performed as previously described (Chen et al., 2009) on Matrigel Matrix (BD Matrigel Basement Membrane Matrix, 354234) precoated 96-well dishes (50 μl/well) and incubated at 37°C for 40 min. Stromal cells (1×10^4) were seeded on top of matrigel, incubated at 37°C, and observed up to 6 hr.

Hematopoietic Supportive Stromal Cocultures

Mononuclear cord blood cells were sorted on a FACSAria (Becton Dickinson) based on CD34 expression and Hoechst 33258 exclusion (Molecular Probes). 5000 $CD34^+$ CB cells were cocultured with a preestablished confluent irradiated layer (12 Gy) of human stromal cell lines in a 24-well plate using h-LTCSM medium, supplemented with hFLT3 (50 ng/ml), hSCF (100 ng/ml), and hTPO (20 ng/ml). After 12 days of coculture, cells were harvested, counted, analyzed by flow cytometry, and plated in hematopoietic colony assays.

Conventional PCR Analysis

Embryo gender was determined by PCR amplification of the amelogenin locus (*AMEL*) (Sullivan et al., 1993) that differs in size on the X (106 bp) and Y (112 bp) chromosomes. PCR mixture contained AmpliTaq DNA polymerase PCR Buffer (15 mM $MgCl_2$; Roche, Applied Biosystems), 200 μM of each dNTP, 400 nM of each primer, 0.01 U/μl of SuperTaq DNA polymerase (HT Biotechnology, Applied Biosystems), and products were separated on a 4% agarose gel. Human chromosome 17 α-satellite PCR (*Chr 17*) (Becker et al., 2002) mixture contained AmpliTaq DNA polymerase PCR Buffer (15 mM $MgCl_2$), 200 μM of each dNTP, 250 nM of each primer, $MgCl_2$ 2 mM, and 0.01 U/μl of SuperTaq DNA polymerase, and yields a PCR product of 850 bp. Conditions for all PCRs are in Table S1. 0.5–3 micrograms of DNA from hematopoietic tissues of recipient mice or in vitro cultures was used for *AMEL* PCR, and 0.25–0.5 μg DNA was used for human *Chr 17* PCR. Limits of sensitivity of human *AMEL* PCR and *Chr 17* PCR were both 1 human cell in 10^5 mouse cells.

STR Typing

Human embryo samples and recipient mouse tissue samples were profiled using the PowerPlex 16 System Kit (Promega) as usually applied to human identity testing in forensic DNA analysis. PCR mixtures contained 1.3 μl Gold Star 10× Buffer, 1.3 μl PowerPlex16 10× Primer Pair Mix, 2U of AmpliTaq Gold DNA polymerase (Roche, Applied Biosystems), and 1 ng of human genomic DNA or 50–200 ng of DNA from mouse samples. PCR was done as described in Table S1 using the PTC-200 Thermal Cycler from MJ Research (Bio-Rad). One microliter of PCR product was mixed with 9.6 μl of Hi-Di Formamide (Applied Biosystems) and 0.4 μl of internal lane standard ILS 600 and denatured at 95°C for 3 min. Amplified fragments were detected using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and data were analyzed with the GeneMapperID v3.2 software (Applied Biosystems). The statistical certainty of the profiles obtained from each reconstituted mouse was established by calculating the random match probability with correction for potential allele sharing with the mother (Weir, 2003). For the 19 week placenta recipient (Figure 2E; Table 1) displaying a complete profile, the probability of a match with a profile among a selection of unrelated and related individuals (i.e., not from the embryonic source) was estimated at 8.65×10^{-12}. For the three 8 week placenta recipients showing a partial profile (Table 1), this probability was estimated at 6.87×10^{-11}, 1.89×10^{-8}, and 3.73×10^{-7} respectively. Estimations are based on the European population and may slightly vary if non-European populations are taken in account. The limit of sensitivity of STR profiling in which a complete profile could be obtained was 1 human cell in 1–2 × 10^3 mouse cells.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and one table and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00444-5.

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REFERENCES

Alvarez-Silva, M., Belo-Diabangouaya, P., Salaun, J., and Dieterlen-Lievre, F. (2003). Mouse placenta is a major hematopoietic organ. Development *130*, 5437–5444.

Bailo, M., Soncini, M., Vertua, E., Signoroni, P.B., Sanzone, S., Lombardi, G., Arienti, D., Calamani, F., Zatti, D., Paul, P., et al. (2004). Engraftment potential of human amnion and chorion cells derived from term placenta. Transplantation *78*, 1439–1448.

Barcena, A., Kapidzic, M., Muench, M.O., Gormley, M., Scott, M.A., Weier, J.F., Ferlatte, C., and Fisher, S.J. (2009). The human placenta is a hematopoietic organ during the embryonic and fetal periods of development. Dev. Biol. *327*, 24–33.

Becker, M., Nitsche, A., Neumann, C., Aumann, J., Junghahn, I., and Fichtner, I. (2002). Sensitive PCR method for the detection and real-time quantification of human cells in xenotransplantation systems. Br. J. Cancer *87*, 1328–1335.

Bhatia, M., Bonnet, D., Murdoch, B., Gan, O.I., and Dick, J.E. (1998). A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat. Med. *4*, 1038–1045.

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Human Placenta Is a Potent Hematopoietic Niche

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc. Natl. Acad. Sci. USA *94*, 5320–5325.

Cashman, J., Bockhold, K., Hogge, D.E., Eaves, A.C., and Eaves, C.J. (1997). Sustained proliferation, multi-lineage differentiation and maintenance of primitive human haemopoietic cells in NOD/SCID mice transplanted with human cord blood. Br. J. Haematol. *98*, 1026–1036.

Challier, J.C., Galtier, M., Cortez, A., Bintein, T., Rabreau, M., and Uzan, S. (2005). Immunocytological evidence for hematopoiesis in the early human placenta. Placenta *26*, 282–288.

Chen, Y., Zhang, H., Gou, X., Horikawa, Y., Xing, J., and Chen, Z. (2009). Up-regulation of HAb18G/CD147 in activated human umbilical vein endothelial cells enhances the angiogenesis. Cancer Lett. *278*, 113–121.

Corbel, C., Salaun, J., Belo-Diabangouaya, P., and Dieterlen-Lievre, F. (2007). Hematopoietic potential of the pre-fusion allantois. Dev. Biol. *301*, 478–488.

Coulombel, L. (2004). Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene *23*, 7210–7222.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell *3*, 301–313.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907–916.

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*, 2465–2474.

Durand, C., Robin, C., and Dzierzak, E. (2006). Mesenchymal lineage potentials of aorta-gonad-mesonephros stromal clones. Haematologica *91*, 1172–1179.

Dzierzak, E., and Speck, N.A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat. Immunol. *9*, 129–136.

Ferkowicz, M.J., Starr, M., Xie, X., Li, W., Johnson, S.A., Shelley, W.C., Morrison, P.R., and Yoder, M.C. (2003). CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. Development *130*, 4393–4403.

Fukuchi, Y., Nakajima, H., Sugiyama, D., Hirose, I., Kitamura, T., and Tsuji, K. (2004). Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells *22*, 649–658.

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*, 365–375.

Gude, N.M., Roberts, C.T., Kalionis, B., and King, R.G. (2004). Growth and function of the normal human placenta. Thromb. Res. *114*, 397–407.

Hann, I.M., Bodger, M.P., and Hoffbrand, A.V. (1983). Development of pluripotent hematopoietic progenitor cells in the human fetus. Blood *62*, 118–123.

Igura, K., Zhang, X., Takahashi, K., Mitsuru, A., Yamaguchi, S., and Takashi, T.A. (2004). Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. Cytotherapy *6*, 543–553.

In't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., de Groot-Swings, G.M., Claas, F.H., Fibbe, W.E., and Kanhai, H.H. (2004). Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells *22*, 1338–1345.

Kim, S.J., Song, C.H., Sung, H.J., Yoo, Y.D., Geum, D.H., Park, S.H., Yoo, J.H., Oh, J.H., Shin, H.J., Kim, S.H., et al. (2007). Human placenta-derived feeders support prolonged undifferentiated propagation of a human embryonic stem cell line, SNUhES3: comparison with human bone marrow-derived feeders. Stem Cells Dev. *16*, 421–428.

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*, 4891–4899.

Larochelle, A., Vormoor, J., Hanenberg, H., Wang, J.C., Bhatia, M., Lapidot, T., Moritz, T., Murdoch, B., Xiao, X.L., Kato, I., et al. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nat. Med. *2*, 1329–1337.

Li, C.D., Zhang, W.Y., Li, H.L., Jiang, X.X., Zhang, Y., Tang, P., and Mao, N. (2005). Isolation and identification of a multilineage potential mesenchymal cell from human placenta. Placenta. in press. Published online September 17, 2005.

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

Miao, Z., Jin, J., Chen, L., Zhu, J., Huang, W., Zhao, J., Qian, H., and Zhang, X. (2006). Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. Cell Biol. Int. *30*, 681–687.

Miyamoto, K., Hayashi, K., Suzuki, T., Ichihara, S., Yamada, T., Kano, Y., Yamabe, T., and Ito, Y. (2004). Human placenta feeder layers support undifferentiated growth of primate embryonic stem cells. Stem Cells *22*, 433–440.

Oberlin, E., Tavian, M., Blazsek, I., and Peault, B. (2002). Blood-forming potential of vascular endothelium in the human embryo. Development *129*, 4147–4157.

Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Dev. Cell *8*, 377–387.

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*, 5073–5084.

Parolini, O., Alviano, F., Bagnara, G.P., Bilic, G., Buhring, H.J., Evangelista, M., Hennerbichler, S., Liu, B., Magatti, M., Mao, N., et al. (2008). Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop on placenta derived stem cells. Stem Cells *26*, 300–311.

Pflumio, F., Izac, B., Katz, A., Shultz, L.D., Vainchenker, W., and Coulombel, L. (1996). Phenotype and function of human hematopoietic cells engrafting immune-deficient CB17-severe combined immunodeficiency mice and non-obese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells. Blood *88*, 3731–3740.

Portmann-Lanz, C.B., Schoeberlein, A., Huber, A., Sager, R., Malek, A., Holzgreve, W., and Surbek, D.V. (2006). Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. Am. J. Obstet. Gynecol. *194*, 664–673.

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*, 252–263.

Sullivan, K.M., Mannucci, A., Kimpton, C.P., and Gill, P. (1993). A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. BioTechniques *15*, 636–638, 640–641.

Tavian, M., Coulombel, L., Luton, D., Clemente, H.S., Dieterlen-Lievre, F., and Peault, B. (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. Blood *87*, 67–72.

Tavian, M., Hallais, M.F., and Peault, B. (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. Development *126*, 793–803.

Tavian, M., and Peault, B. (2005). Embryonic development of the human hematopoietic system. Int. J. Dev. Biol. *49*, 243–250.

Tavian, M., Robin, C., Coulombel, L., and Peault, B. (2001). The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. Immunity *15*, 487–495.

Tse, W., Bunting, K.D., and Laughlin, M.J. (2008). New insights into cord blood stem cell transplantation. Curr. Opin. Hematol. *15*, 279–284.

Wang, J., Kimura, T., Asada, R., Harada, S., Yokota, S., Kawamoto, Y., Fujimura, Y., Tsuji, T., Ikehara, S., and Sonoda, Y. (2003). SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood *101*, 2924–2931.

Wang, J.C., Doedens, M., and Dick, J.E. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or

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mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood *89*, 3919–3924.

Weir, B.S. (2003). Forensics. In Handbook of Statistical Genetics. D.J. Balding, M. Bishop, and C. Cannings, eds. (Hoboken, NJ: Wiley and Sons), pp. 830–850.

Wulf, G.G., Viereck, V., Hemmerlein, B., Haase, D., Vehmeyer, K., Pukrop, T., Glass, B., Emons, G., and Trumper, L. (2004). Mesengenic progenitor cells derived from human placenta. Tissue Eng. *10*, 1136–1147.

Yen, B.L., Huang, H.I., Chien, C.C., Jui, H.Y., Ko, B.S., Yao, M., Shun, C.T., Yen, M.L., Lee, M.C., and Chen, Y.C. (2005). Isolation of multipotent cells from human term placenta. Stem Cells *23*, 3–9.

Zambidis, E.T., Oberlin, E., Tavian, M., and Peault, B. (2006). Blood-forming endothelium in human ontogeny: lessons from in utero development and embryonic stem cell culture. Trends Cardiovasc. Med. *16*, 95–101.

Zeigler, B.M., Sugiyama, D., Chen, M., Guo, Y., Downs, K.M., and Speck, N.A. (2006). The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. Development *133*, 4183–4192.

Zhang, X., Mitsuru, A., Igura, K., Takahashi, K., Ichinose, S., Yamaguchi, S., and Takahashi, T.A. (2006). Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering. Biochem. Biophys. Res. Commun. *340*, 944–952.

Zhang, Y., Li, C., Jiang, X., Zhang, S., Wu, Y., Liu, B., Tang, P., and Mao, N. (2004). Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Exp. Hematol. *32*, 657–664.

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Supplemental Data

Human Placenta Is a Potent Hematopoietic Niche Containing Hematopoietic Stem and Progenitor Cells throughout Development

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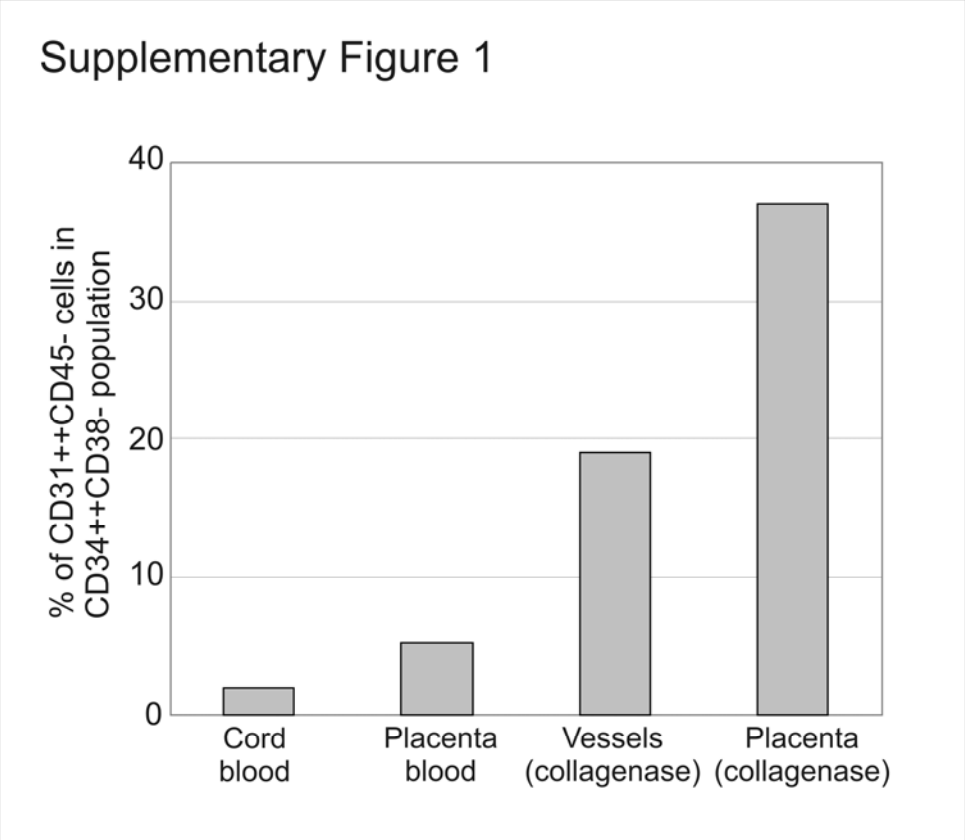
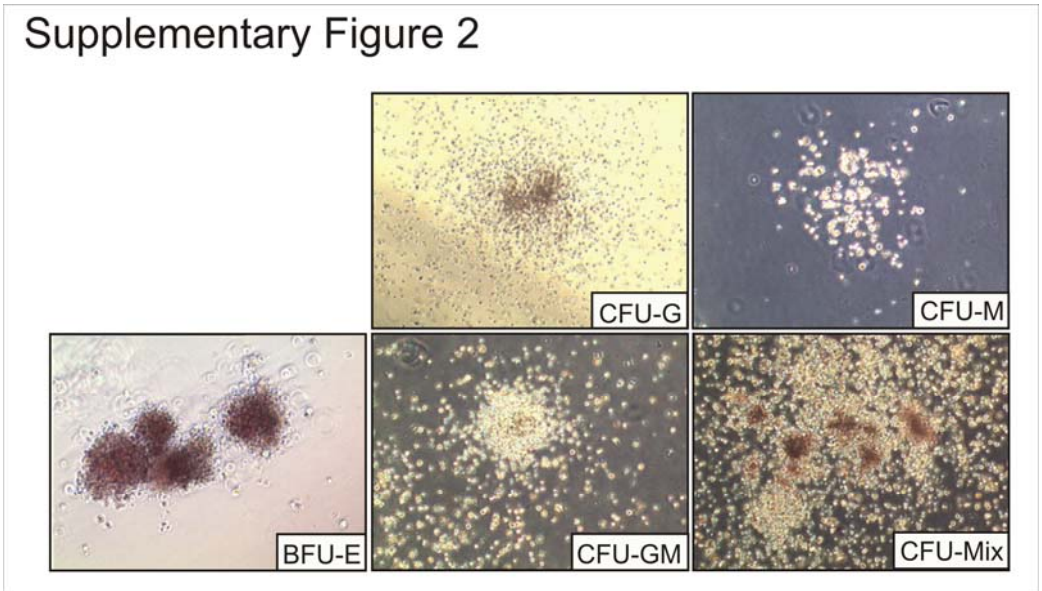
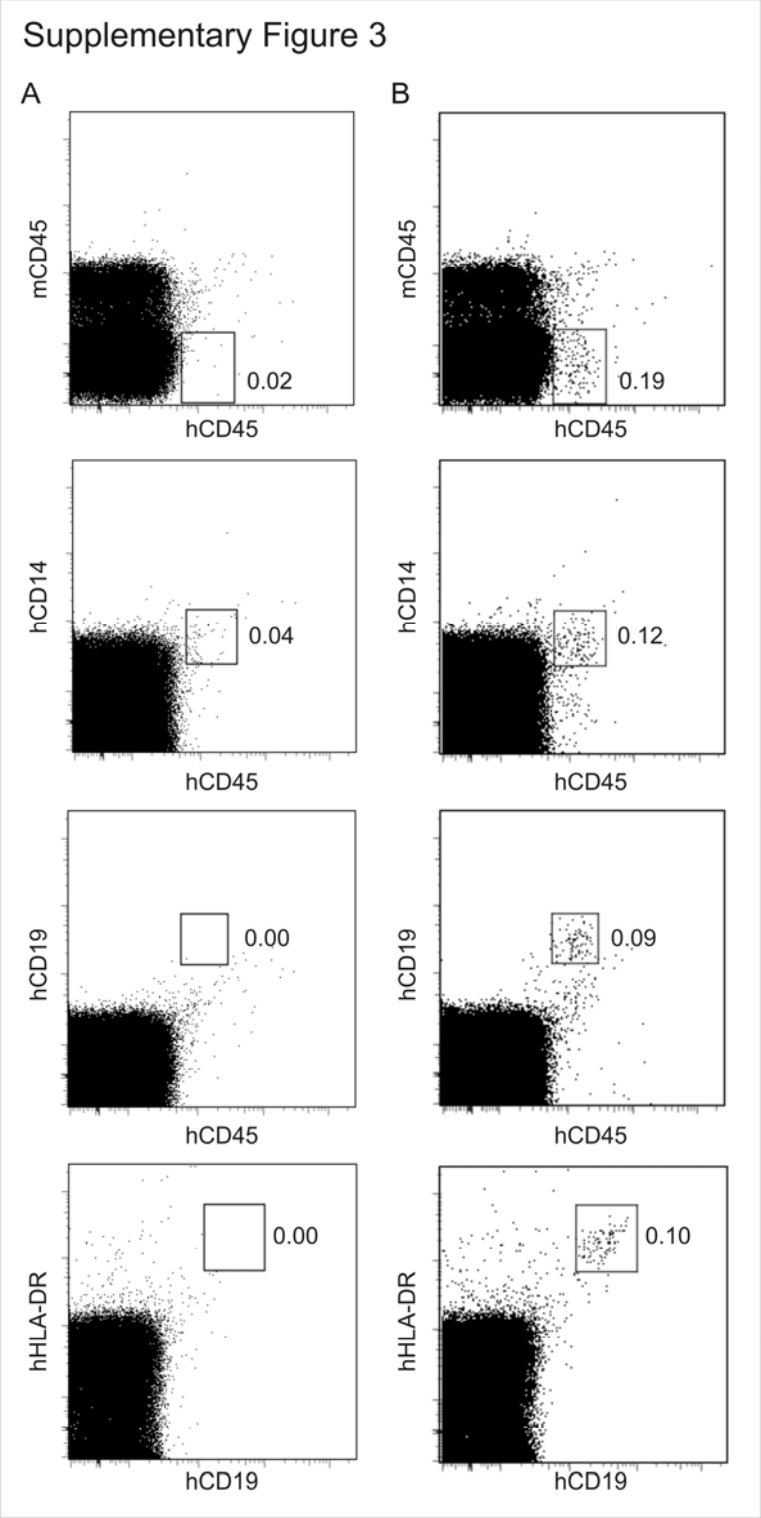


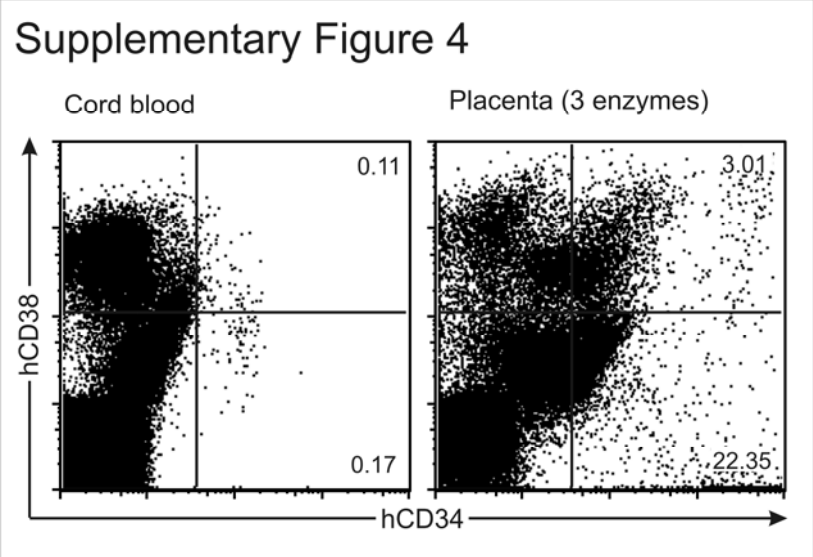
Percentages of $CD31^{++}CD45^{-}$ endothelial cells in term human cord and placenta blood, and placental $CD34^{++}CD38^{-}$ cells.



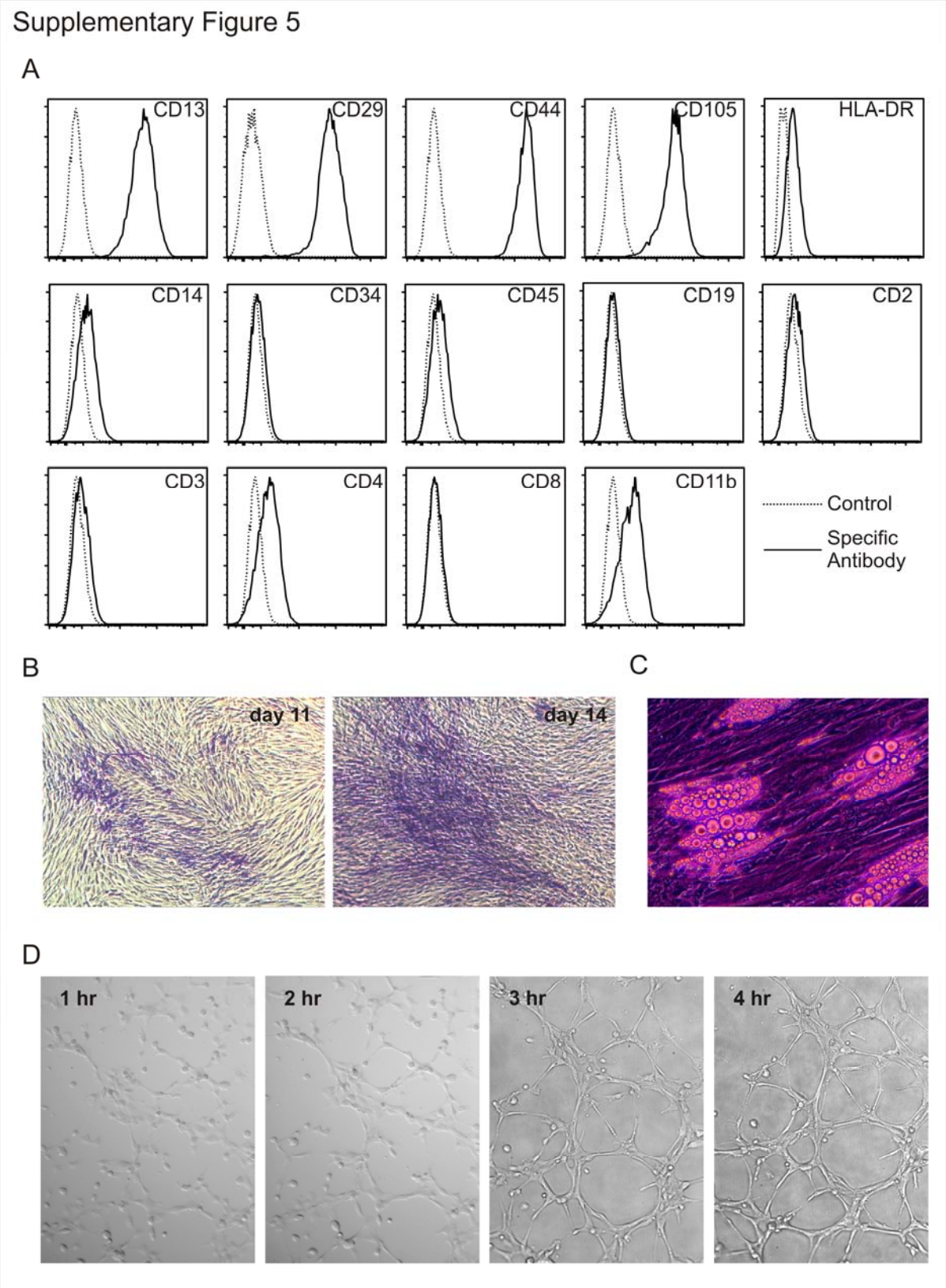
The human placenta contains all types of hematopoietic progenitors (with typical morphology) throughout development. Representative differentiated colonies from human term placenta cells: BFU-E (burst forming unit-erythroid), CFU-G (granulocyte), CFU-M (macrophage), CFU-GM (granulocyte-macrophage) and CFU-Mix (granulocyte, erythroid, macrophage, megakaryocyte).



Multilineage flow cytometric analysis of bone marrow from **(A)** *Rag* $\gamma C^{-/-}$ negative control (not reconstituted) and **(B)** *Rag* $\gamma C^{-/-}$ recipient reconstituted with human week 16-17 placenta $CD34^{+}$ cells. Percentages of relevant hematopoietic populations are indicated.

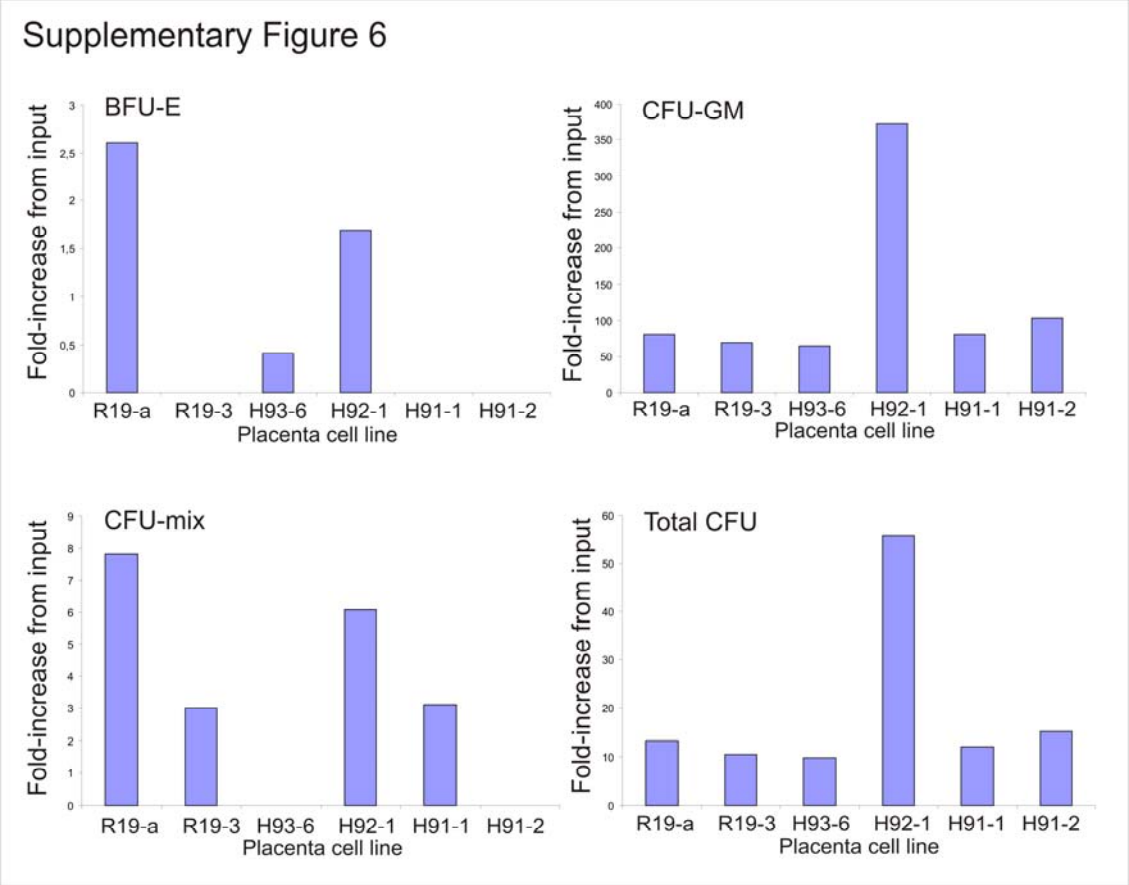


Phenotypic analysis of full term placenta digested with multiple enzymes. Flow cytometry analyses of cord blood and collagenase/dispase/pancreatin treated placenta tissue cells stained with anti-human (h)CD34 and CD38 antibodies. Dot plots are generated from the gated viable population. 10^5 events analysed for cord blood and $5x10^4$ events for placenta (3 enzyme treatment – collagenase, pancreatin, dispase). Percentages of relevant populations are indicated.

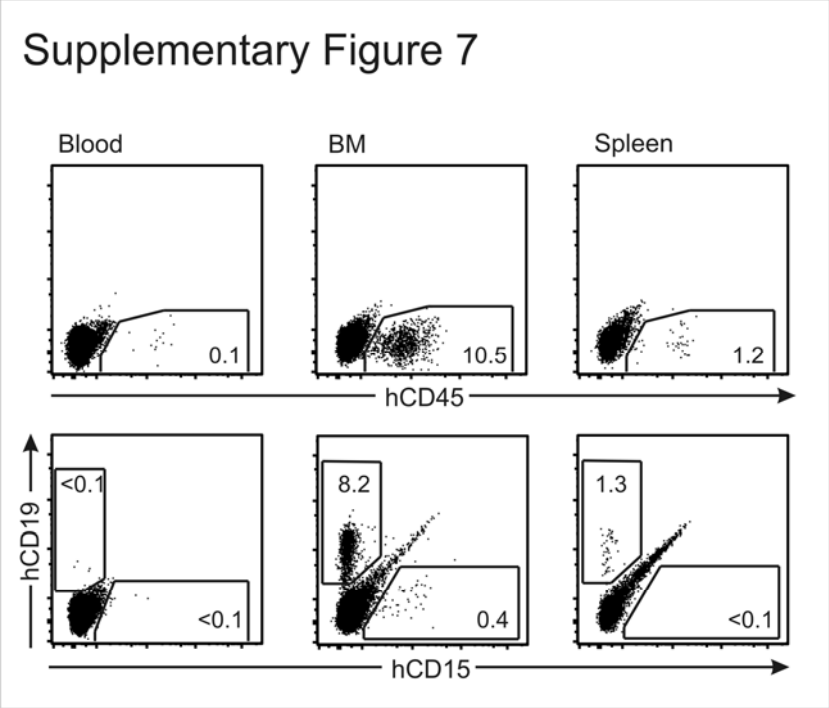


Differentiation potentials and phenotypic analysis of human placenta derived stromal cell lines. **(A)** Flow cytometry analyses of the stromal cell line H92-1 (16 week gestation placenta). After trypsinisation, cells are stained with anti-human (h) CD13, CD29, CD44, CD105, HLA-DR, CD14, CD34, CD45, CD19, CD2, CD3,

CD4, CD8 and CD11b antibodies and analysed in the viable population. **(B)** Osteogenic differentiation of H93.6 cells is demonstrated by staining for alkaline hosphatase (ALP) activity at day 11 and 14 post-induction. **(C)** Adipogenic potential is demonstrated by the accumulation of fat droplets as shown after Oil red staining. **(D)** Endothelial potential is demonstrated by the temporal development of tubules at 1, 2, 3 and 4 hours after seeding of the H93.6 cell line in matrigel culture. Cell lines H92.1 and H92.2 also formed tubules (not shown). 4x lens.



Hematopoietic activity of cord blood cells is maintained/amplified upon co-culture with placental stromal cell lines. Graphic representation of the fold increase (compared to the input) of the different hematopoietic progenitor types (BFU-E, CFU-GM, CFU-Mix) obtained 12 days after co-culture on different placenta stromal cell lines. 3000 $CD34^+$ cord blood sorted cells were plated in triplicate immediately (input) or following co-culture for 12 days on a confluent irradiated placental stromal cell line in presence of TPO, SCF and Flt3L. Stromal cell lines from gestation week 3, 16 and 18 placentas were used (see **Table 4** for stromal cell line details).



Freeze-thawing of placenta cells does not alter their long-term multilineage hematopoietic repopulating potential. Flow cytometry multilineage analyses of blood, bone marrow (BM) and spleen cells isolated from NOD-SCID mouse repopulated 5 months after injection of $1.3x10^6$ collagenase treated placenta tissue (tP1), obtained after frozen-thawing. Cells are stained with anti-human (h) CD45, CD19 and CD15 antibodies and analysed in the viable population. $3x10^4$ events were analysed for each tissue and percentages of relevant populations are indicated.

Table S1. Amplification thermal cycling used for conventional and STR PCRs

Amelogenin PCR	***Ch17 α-satellite PCR***	***Powerplex 16 STR PCR***
94°C for 3min	92°C for 5min	95°C for 11min 96°C for 1min
94°C for 30s 60°C for 30s 72°C for 30s For 35 cycles	92°C for 1min 60°C for 1min 72°C for 1min For 35 cycles	94°C for 30sec ramp 68sec to 60°C (hold for 30sec) ramp 50sec to 70°C (hold for 45sec) For 10 cycles
72°C for 10min	72°C for 10min	90°C for 30sec ramp 60sec to 60°C (hold for 30sec) ramp 50sec to 70°C (hold for 45sec) For 20 cycles
		60°C for 30min

Chapter 3

The Effects of Hypoxia on Hematopoietic Cells and Their Niche in the Developing Mouse Placenta

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In preparation for submission

Abstract

Hypoxia plays a physiological role during fetal organogenesis and in the adult it is involved in bone marrow HSC maintenance. The placenta harbors hematopoietic stem cells (HSCs), yet little is known about hypoxia and its effects on placental HSCs and hematopoietic progenitors. To identify the hypoxia-sensitive placenta cell types (hematopoietic and/or microenvironment), we investigated the effects of hypoxia (3% O_2) versus normoxia (20% O_2) on two stromal cell lines derived from embryonic day 12 murine placenta. These cell lines significantly support the expansion of hematopoietic cells from adult bone marrow (BM) in normoxia. We show here that the proliferation rate of both placenta stromal cell lines is significantly higher when cultured under hypoxic conditions as compared to normoxia. The hematopoietic growth supportive properties of the placenta cell lines are considerably reduced when the stromal cell lines are co-cultured with hematopoietic cells under conditions of hypoxia. Whereas both normoxia and hypoxia induce differentiation of HPSC to the macrophage lineage, a decreased frequency of macrophages and an increased frequency of hematopoietic progenitors was found in the hypoxic cultures. However, in contrast to the expansion of hematopoietic progenitors in normoxic conditions, HPSC numbers are maintained at input numbers in hypoxia. Vascular endothelial growth factor *(VEGF*), a regulator of vascular development, was found to be upregulated in hypoxic placental stromal cell lines, suggesting that it influences the niche. Taken together, the results of our co-culture system can help us to understand the true role of hypoxia as a niche factor in supportive potential of stromal cells for hematopoietic progenitor and stem cell activity.

Introduction

The placenta has many important functions during pregnancy, including hormone production and gas and nutrient exchange between mother and fetus. In addition, placenta is a hematopoietic organ. The mature placenta consists of fetal- and maternally-derived parts. The fetal part includes the labyrinth (consisting of endothelium-lined fetal capillaries, allantoic mesenchyme, maternal blood sinuses, and trophoblast-lined maternal blood spaces called syncytiotrophoblasts that provide the transport surface between maternal and fetal circulations), spongiotrophoblast layer, and the trophoblast giant cell (TGCs) layer (which borders the maternal decidua) (reviewed in Dunwoodie, 2009; Dzierzak and Robin, 2010; Ottersbach and Dzierzak, 2010).

Early studies suggested that placenta is hematopoietic (Dancis et al., 1977; Till and Mc, 1961), but it was Arvarez-Silva and colleagues who showed that the mouse placenta at embryonic day (E) 9 contains abundant hematopoietic progenitor activity of fetal origin. Up to E12 it contains more hematopoietic activity than fetal liver (Alvarez-Silva et al., 2003). Colony-forming-unit (CFU) activity in placenta peaks at E12.5, containing twice the amount of activity as is found at E11 (Gekas et al., 2005). Interestingly, the allantois and chorion (tissue precursors of the placenta at E8) possess intrinsic hematopoietic potential before the onset of circulation (Corbel et al., 2007; Zeigler et al., 2006). HSCs appear in the murine placenta at E11 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), but it is the aorta-gonad-mesonephros (AGM) region that generates the first definitive HSCs at E10.5. Stromal cell lines derived from the urogenital-ridges and aorta-mesenchyme (AM) region are more potent than stromal cells derived from fetal liver (FL) and BM, thus demonstrating that this embryonic tissue is robust hematopoietic niche (Oostendorp et al., 2002a; Oostendorp et al., 2002b).

In contrast to the AGM (and yolk sac (YS)), the placenta contains large numbers of HSCs. HSC numbers peak at E12 and are 20-fold increased compared to E11 placenta. It harbors 15 times more HSCs than the AGM region. At E13.5 HSC content decreases dramatically in placenta and disappears by E15.5 (Gekas et al., 2005). Since no/few HSCs are detected in the circulating blood, it is suggested that HSCs can be *de novo* generated in the placenta. However, considering the fact that AGM HSC content remains low throughout the embryonic development, the AGM may be an HSC generative niche, while the placenta niche may highly support HSC expansion. Ottersbach and colleagues found that HSCs are highly enriched in the GFP^+ fraction of *Ly6A-GFP* transgenic placentas (fetuses generated by mating *Ly6A-GFP* males with wild type females), confirming that placenta HSCs are of fetal (and not maternal) origin. Moreover, cells co-expressing Ly6A, CD31, and CD34 HSC markers were found located in the vasculature of the labyrinth. Important transcription factors in hematopoiesis, *GATA2* and *Runx1* were found to be expressed in the endothelial cells and in some cells surrounding the vasculature of labyrinth (Ottersbach and Dzierzak, 2005). Also, from week 6 of gestation until the delivery time, the human placenta contains highly potent HSCs of fetal-origin (Ivanovs et al., 2011; Robin et al., 2009). All these observations indicate that the placenta is an important hematopoietic organ and contains a HSC supportive microenvironment during development.

In the adult BM the HSC supportive stromal microenvironment consists of the endosteal and vascular niches (described in detail in Chapter 1). In keeping with this heterogeneity, the placenta microenvironment is also heterogeneous, containing mesenchymal, endothelial, perivascular and syncytiotrophoblast cells. Stromal cells isolated from human placenta express classical mesenchymal stem cell (MSCs) markers, as well as pericyte markers (Crisan et al., 2008; Robin et al., 2009). They have osteogenic, adipogenic, and endothelial lineage differentiation potential, as is expected for MSCs. Based on *in vitro* co-cultures, human placenta MSCs support the maintenance and expansion of human $CD34^+$ umbilical cord blood cells. Similarly, mouse stromal cell lines derived from AGM, placenta, FL, and adult BM has been co-cultured with BM c-Kit^+ Sca-1^+ cells. After one week of co-culture, placental stromal cells supported the expansion of BM cells 284-fold more than AGM, FL, or BM stromal cells (Wang et al., 2011). CFU-GM (granulocyte-macrophage) colonies derived from BM c-Kit^+ Sca-1^+ cells cultured on stromal cells from placenta, FL, AGM, and BM expanded by 14.6, 7.5, 6.1, and 4.6 fold, respectively compared to the input cells. Microscopic analysis of hematopoietic cells cultured on placenta stromal cells revealed that they keep an immature blast-like morphology. Thus, the supportive capacity of placenta stromal cells for hematopoietic progenitors is significantly greater than that of other tissues tested. Both placenta- and BM-derived stromal cells express CD105 and Sca-1 markers (Sca-1 has been shown to be expressed by AGM stromal cells as well (Weisel et al., 2006)). However, placenta stromal cells express CD34 suggesting they are endothelial cells. Microarray analysis confirms high expression of genes involved in endothelial cell formation and differentiation (Tie family receptors, angiopoietins, VEGF, and VEGF receptors 2 and 3). Thus, as a highly vascularized tissue, placenta-derived stromal cells are a model system that can be used to support and study the *ex vivo* expansion of immature hematopoietic progenitors and HSCs.

As discussed in the Introduction chapter, hypoxia and hypoxia inducible factor (HIF) transcription factors play fundamental roles during embryonic development and placenta formation (reviewed in Dunwoodie, 2009). The oxygen level is low in BM niche and HSCs are located in the most hypoxic site of this niche. Also, HPSC activities are improved in hypoxic cultures of BM hematopoietic cells. Yet, little is known about the role of hypoxia on placental HSPC growth and maintenance. In the present study, we raised the question whether hypoxia as a niche factor is required in placenta hematopoiesis. To this end, we derived stromal cells from the fetal part of the E12 embryo and investigated the effects of hypoxia (3% O2 level) on these cells and their supporting potential for hematopoietic progenitor activity of adult BM cells. Placenta stromal cell lines demonstrated an increased rate of cellular growth when cultured under hypoxic conditions and express the hypoxia-sensitive factor VEGF, which is important for vascular and hematopoietic growth. In a co-culture system placenta stromal cells were found to support the expansion of hematopoietic progenitor activity of BM cells, and under hypoxic conditions to inhibit the differentiation of HPSC populations, strongly suggesting that hypoxia represents a hematopoietic niche modulator in the placenta.

Results

Placental stromal cell lines differentiate along osteogenic and adipogenic lineages

Six cell lines were previously derived in our lab from the fetal part of the murine E12 placenta (Master thesis, Ivoune Setiawati Lauw). Their potential to differentiate to mesenchymal cell lineages was tested. Placental (PL) cell lines were cultured in specific medium promoting differentiation to test whether they possess osteogenic, adipogenic and/or chondrogenic lineage potentials. After 14 and 28 days of osteogenic culture, osteogenic potential was detected by alkaline phosphatase (ALP) staining (a widely recognized marker of osteogenic activity (D'Ippolito et al., 1999; Oyajobi et al., 1999; Pittenger et al., 1999) and alizarin red staining (Banfi et al., 2000)) (Figure S1A). The expression of osteogenic lineage related genes, Core-Binding Factor Alpha 1 (*Cbfa1)* and *Osteocalcin,* was detected by RT-PCR (reverse transcriptase – polymerase chain reaction) in the placenta cells after osteogenic differentiation (data not shown). All six lines showed osteogenic potential.

Adipogenic differentiation of the lines was tested by culturing the cells in the presence of insulin and dexamethasone. Cells with typical adipocyte morphology, displaying refractile intracellular vesicles containing lipids were observed starting at day 4 of the culture (Figure S1B). Also RT-PCR analysis showed the expression of Peroxisome proliferator-activated receptor gamma gene (*PPARγ*), an adipocyte lineage related gene (Pittenger et al., 1999). However, the expression of *PPARγ* gene was also detected before specific lineage differentiation culture (in stromal cell lines cultured only in stromal medium). Expression levels were lower as compared to the cells in adipogenic differentiation medium, suggesting that these cells are already partially differentiated or that PPARγ does not represent an exclusive marker for adipocyte differentiation. All six lines were found to be adipogenic. While all six lines possess potential to differentiate towards osteogenic and adipogenic lineage, they varied in the frequency of cells with differentiation potential (Figures S2 and S3). The highest frequency of differentiation to both lineages was observed in the PL8-5 cell line. When tested by micromass culture and pellet formation, no PL cell lines were found to be chondrogenic.

The ability of the placenta cell lines to support immature hematopoietic progenitors was tested in co-culture experiments. An enriched population of bone marrow cells ($CD31^{med}$c-kit^{high}Ly-$6C^{-}$) was sorted and placed in co-culture with the placenta lines. After 7 days of co-culture, cells were harvested and examined by colony forming unit (CFU) assay. The highest CFU-C activity of BM cells was observed in co-cultures with the PL8-5 cell line (data not shown). From the 6 cell lines tested, we chose two cell lines for our study - the PL8-5 cell line which showed the best mesenchymal and hematopoietic supportive characteristics, and PL13-5 as a less supportive control.

Phenotypic and growth characterization of placenta stromal cell lines

We first examined the phenotypic characteristics of the PL8-5 and PL13-5 cell lines. Both cell lines exhibited typical fibroblastic morphology (Figure 1A). To examine whether the mouse placenta cells were expressing markers typical of a hematopoietic supportive niche, we performed flow cytometric analysis for the CD29, Sca-1, CD44, and CD11b surface markers (Figure 1B). PL8-5 and PL13-5 were $CD29^{+}$, $Sca\text{-}1^{+}$, and $CD11b^{-}$ in agreement with the previously described surface phenotype of mouse embryonic stromal cells (mesenchymal cells from AGM and placenta are $CD29^{+}$ and $Sca\text{-}1^{+}$ (Oostendorp et al., 2002a; Wang et al., 2011; Weisel et al., 2006)) and human placenta (mesenchymal cells from human placenta are $CD29^{+}$ and

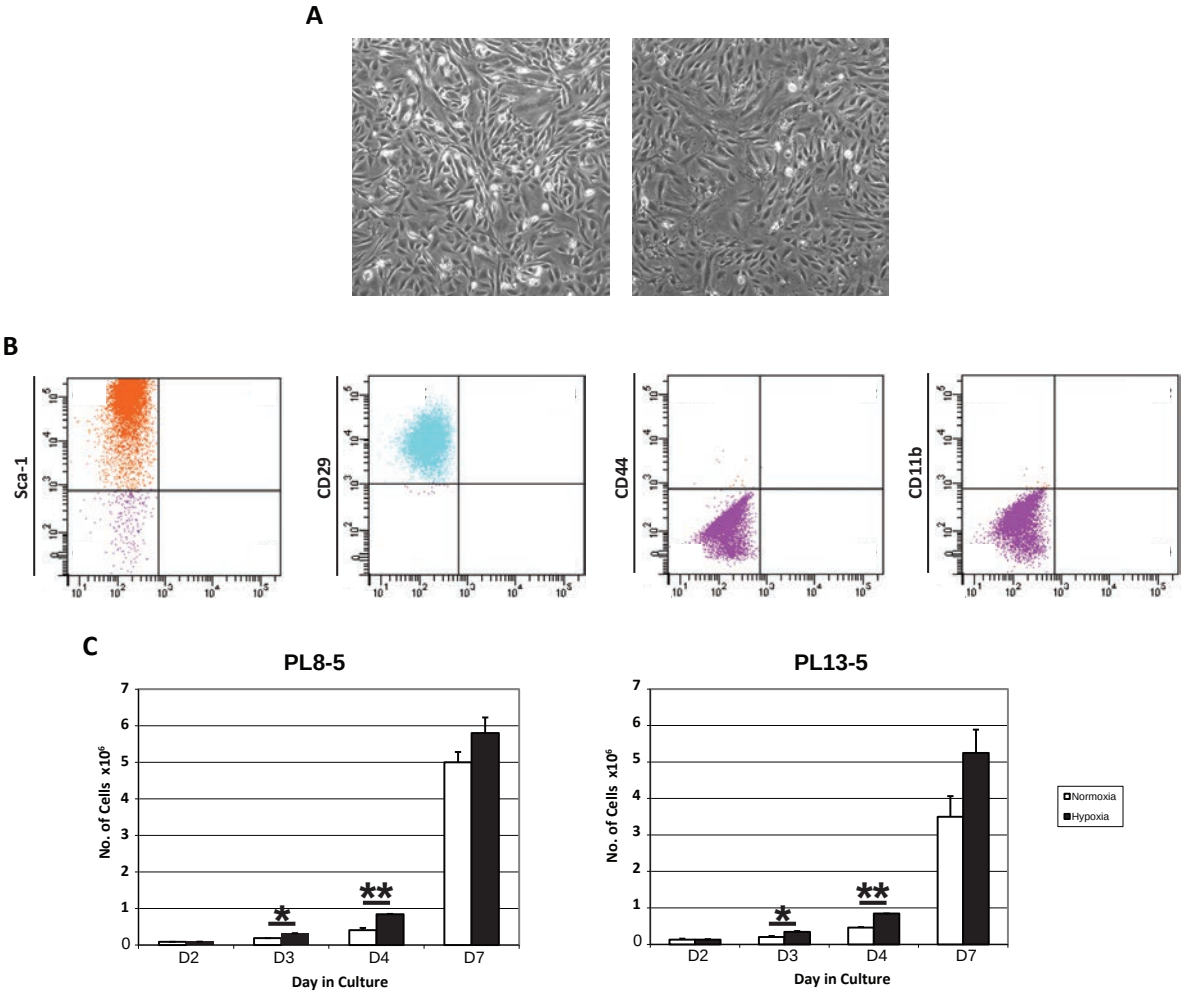


Figure 1. Characterization of placenta-derived stromal cells. (A) Representative phase microscopy of cultured stromal cell lines at passage 6 (4x magnification) showing cells with a fibroblastoid morphology and some proliferating cells. (B) Flow cytometric analysis of PL8-5 cells for Sca-1, CD29, CD44, and CD11b surface markers. (C) Growth rate of placenta stromal cells cultured in normoxia (20% O2) and hypoxia (3% O2) for 7 days. The cell numbers were analyzed by trypan blue exclusion staining at different time points. Data is averaged over 3 independent experiments (mean ± SD). Significant differences are indicated with asterisks (*: p-value ≤ 0.05, **:p-value ≤ 0.005).

CD11b$^-$ (Robin et al., 2009)). However, in contrast to human placenta mesenchymal cells which express CD44, PL8-5 and PL13-5 were CD44$^-$. Thus, while mouse placenta stromal cell lines express classical mesenchymal markers, the expression pattern of these markers is partially different between mouse and human, which may reflect different mesenchymal potentials or species differences.

Next, the cell growth characteristics of the placental cell lines under hypoxic conditions were tested. Previously it was shown that mesenchymal cells derived from adult BM not only can tolerate the oxygen level of <1% *in vitro* but that they also proliferate faster under this condition (reviewed in Das et al., 2010). To investigate the effect of hypoxia on the proliferation of PL8-5 and PL13-5 cell lines, they were cultured under normoxic and hypoxic conditions for 7 days (we define the 3% O2 as hypoxic and 20% O2 as normoxic) and examined their growth rate at different time points during culturing period (Figure 1C). Both cell lines showed significantly increased rates growth of when cultured in hypoxia compared to normoxia. Moreover, the viability of the cells (tested by trypan blue staining) was similar in both conditions (about 90% on average). This data shows that placental mesenchymal cells, like BM mesenchymal cells, survive and proliferate better under hypoxic conditions, supporting the notion that mesenchymal stromal cells are a part of a hypoxic niche in placenta which has adapted to conditions of low oxygen.

Hypoxia induces VEGF expression in mouse placenta cell lines

It has been shown that hypoxic pre-conditioning of BM mesenchymal cells increases the expression of pro-survival and pro-angiogenic factors such as HIF1α, VEGF, and its receptor, FLK-1 (reviewed in Das et al., 2010). To test the oxygen sensing capacity of placenta stromal cell lines, *VEGF* RNA levels of PL8-5 and PL13-5 stromal cells cultured in normoxia and hypoxia at different time points were measured by quantitative RT-PCR. A significant increase in *VEGF* expression was observed in PL8-5 after 5 and 7 days of culturing in hypoxia, but this increase was not significant for PL13-5 (Figure 2A). Increased expression of VEGF receptor 1 (*FLT1*) was detected in PL8-5 cells cultured 7 days in hypoxia. However, we did not detect any induction of VEGF receptor 2 (*FLK1*) expression under hypoxia (data not shown). Surprisingly, the expression of stroma-derived factor 1 (*SDF-1*) was decreased in PL8-5 cells cultured in hypoxia. SDF-1 is secreted by endothelial and osteoblast cells. It is regulated by HIF1α and has a role in homing and mobilization of HPSCs (Ceradini et al., 2004; Ponomaryov et al., 2000; Sipkins et al., 2005). Hypoxia-induced expression of VEGF protein was confirmed in the placenta stromal cells by immunocytochemistry at day 5 and western blotting, in which a strong upregulation of VEGF was observed at day 7 (Figure 2C). Collectively this data shows that the hypoxia-sensitive pro-angiogenic and hematopoietic factor, VEGF, is endogenously expressed in placenta stromal cells and upregulated under hypoxic conditions.

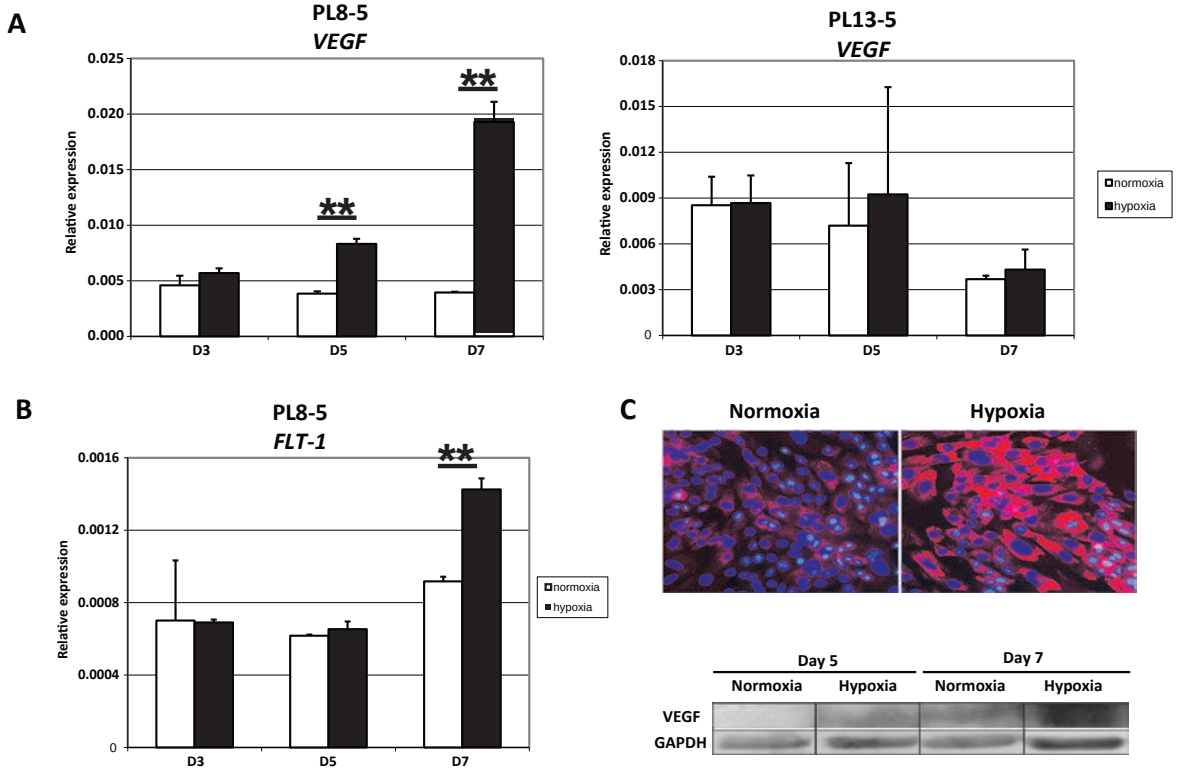
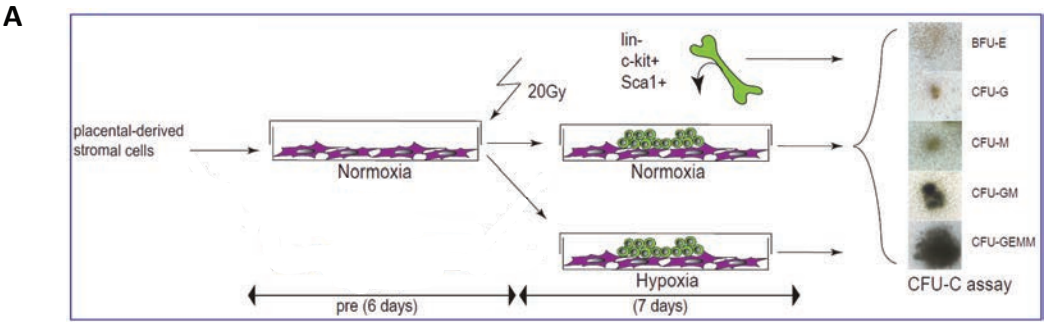


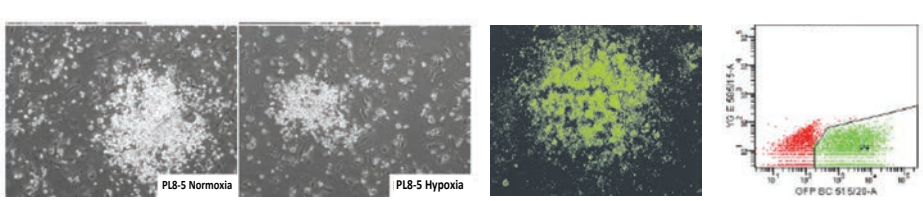
Figure 2. Hypoxia affects the expression of VEGF in placenta stromal cell lines. (A) PL8-5 and PL13-5 cell lines were cultured in normoxia and hypoxia for 7 days and analyzed for the VEGF RNA level by quantitative RT-PCR. (B) The same analysis as A was performed for FLT-1 expression. The data is normalized to β-actin expression level (mean ± SD) (n=3). Significant differences are indicated with asterisks (**p-value ≤0.005). (C) Immunocytochemistry (top panel) and western blotting (bottom panel) for VEGF protein in PL8-5 cells after culturing in normoxia and hypoxia. In the top panel, DAPI-stained and anti-VEGF antibody-stained cells are shown in blue and red, respectively.

Placenta stromal cells support the expansion of hematopoietic cells and hematopoietic progenitor activity

The hematopoietic supportive capacity of PL8-5 and PL13-5 stromal cell lines in normoxia and hypoxia was examined using a co-culture system as illustrated in Figure 3A. Lineage$^-$ c-Kit$^+$ Sca-1$^+$ (LSK) cells, which are enriched in HPSCs, were isolated from adult BM of *actin-GFP* transgenic mice and were used in this experiment. To analyze the effect of hypoxia on either stromal cells supportiveness for hematopoiesis and/or on hematopoietic cells, the stromal cells were pre-cultured in normoxia followed by co-culture with LSK cells in normoxia and hypoxia (Figure 3A). After seven days, the GFP$^+$ cells were sorted and analyzed.

Microscopic analysis of co-cultures showed the formation of big hematopoietic colonies in normoxic cultures, while smaller colonies and more sparsely distributed hematopoietic cells were observed in hypoxic cultures (Figure 3B). After 7 days of co-culture GFP$^+$ cells were quantitatively increased in co-cultures of both cell lines under the normoxic condition.





C

	PL8-5	PL13-5
Normoxia	427	142
Hypoxia	4.2	5.3

D

	PL8-5			PL13-5		
	Mac	Lin⁻	LSK	Mac	Lin⁻	LSK
Normoxia	90.5	5.7	1.1	97	2.2	0.8
Hypoxia	71	16	6.0	79	15.1	4.7

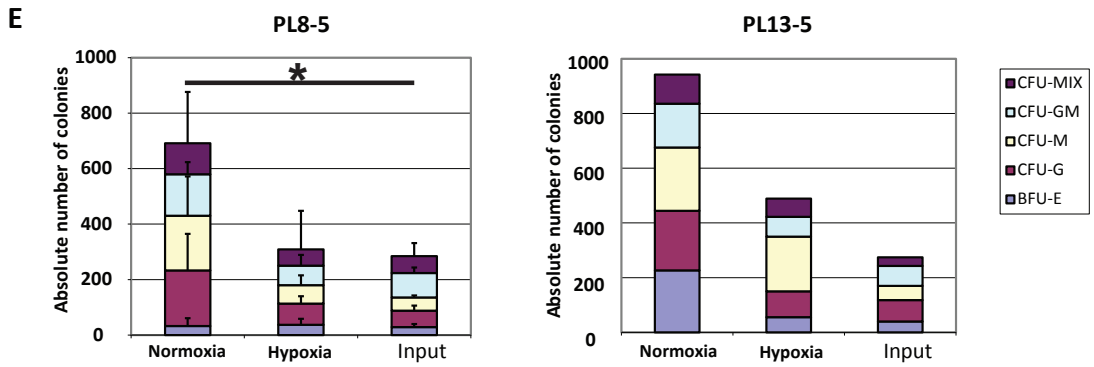


Figure 3. Hematopoietic supportive capacity of placenta stromal cell lines. (A) A schematic diagram of the co-culture procedure is shown. Following the culture of stromal cells in normoxic condition, adult bone marrow Lin⁻ c-Kit⁺ Sca-1⁺ (LSK) cells were seeded on a monolayer of placenta stromal cells (PL8-5, PL13-5). After 7 days of co-culture in normoxia or hypoxia, cells were harvested and Colony Forming Unit (CFU) assay was performed. (B) On the left, the morphology of hematopoietic colonies after 7 days of co-culture is shown. On the right, GFP⁺ hematopoietic cells cultured on stromal cells are shown in green by fluorescence microscopy and flow cytometric analysis. (C) Placenta cell lines support the proliferation of hematopoietic cells. GFP+ cells were counted after 7 days of co-culture in normoxia and hypoxia and the fold changes are summarized here (mean ± SD) (n=3). (D) Summarized data of flow cytometry analysis of hematopoietic cells. After 7 days of co-culture, GFP+ cells were sorted and analyzed for Mac-1, Lin, and LSK markers. Each number is the percentage of cells positive for the associated markers within single viable population (E) Capacity of placenta stromal cell lines to support hematopoietic progenitor activity analyzed in both normoxia and hypoxia. The performance of each line is expressed as the total number of colonies from different types, i.e. BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-mix. Data is averaged over 3 independent experiments (mean ± SD). Significant differences are indicated with asterisks (*: p-value ≤0.05).

PL8-5 and PL13-5 cells support the expansion of hematopoietic cells by 427 and 142 fold, respectively, compared to the initial number of input cells. This is in accordance with the preliminary co-culture data upon which we selected P8-5 and PL13-5 cell lines. In hypoxic condition, the number of hematopoietic cells was only slightly increased as compared to initial number of input LSK cells. These results show that placenta stromal cells support the expansion of hematopoietic cells (Figure 3C). Under both normoxic and hypoxic conditions co-cultures of PL8-5 and PL13-5 supported the differentiation of BM-LSK cells along the macrophage lineage, as demonstrated by the high percentages of $Mac1^+$ (the surface marker for macrophages and granulocytes) cells found by flow cytometry analysis. However, lower percentages of $Mac1^+$ cells were found in the hypoxic co-cultures. Interestingly, under hypoxic conditions, the percentages of Lin^- and LSK^+ cells increased in the co-cultures (summarized in Figure 3D). This data suggest that hypoxic co-cultures favor progenitor maintenance over progenitor differentiation. To test this idea, colony-forming unit-culture (CFU-C) assays were performed to examine progenitor content of the co-cultures.

After co-culture under normoxic and hypoxic conditions, GFP^+ cells were sorted and analyzed for hematopoietic progenitor activity in the CFU-C assay. Compared to input controls, the number of CFU-Cs was increased 2.4 and 1.1 fold in PL8-5 co-cultures, and 3.3 and 1.7 fold in PL13-5 co-cultures under normoxic and hypoxic conditions, respectively (Figure 3E). These data show that placenta stromal cells support hematopoietic progenitor expansion less well in hypoxia than normoxia and that most of the progenitor expansion in the normoxic co-cultures is of progenitors for the macrophage and granulocyte lineages. It is intriguing that hypoxia appears to inhibit the differentiation of hematopoietic cells cultured on placenta stromal cells, implicating either a direct effect of hypoxia on hematopoietic progenitor cells or an indirect effect through the stromal cells. Notwithstanding, these results show that hypoxic co-culture of placental stromal cell lines interferes with their hematopoietic supportive and differentiative capacity.

Hypoxia-sensitive genes are expressed during placental development

VEGF is a well-known angiogenic factor regulated by hypoxia and HIF (Peled et al., 1999; Shweiki et al., 1992) and its two major receptors, FLT1 and FLK1, are expressed on endothelial as well as hematopoietic cells (Hattori et al., 2002; Kabrun et al., 1997). To examine whether hypoxia-targeted genes are expressed in placenta *in vivo*, quantitative RT-PCR for *VEGF*, *FLT-1*, and *FLK1* was performed on fetal placentas at different time points during development from E9 to E18 (Figure 4). High *VEGF* expression was found at E9, decreases thereafter until E12, and increases from E15 onward. The expression kinetics of *FLK1* is similar to *VEGF*, while *FLT1* expression increases from E12 onward. This temporal expression pattern of *VEGF* and *FLK1* could be due to the high level of vasculogenesis after the onset of circulation at E9 and also in the last stages of embryonic development when more oxygen supply is needed for the rapidly growing embryo (Lee et al., 2001). The VEGF receptors play different roles during

embryogenesis that can lead to their different expression patterns. It has been shown that the expression pattern of VEGF is similar to HIF1α and hypoxyprobe by immunostainings on mouse embryo (Lee et al., 2001). Thus, different expression levels of angiogenic factors that are induced by hypoxia may reflect differences in oxygen levels and HIF1α expression in the embryo during development. Of course, the specific cell types in placenta that express the mentioned genes remain to be investigated.

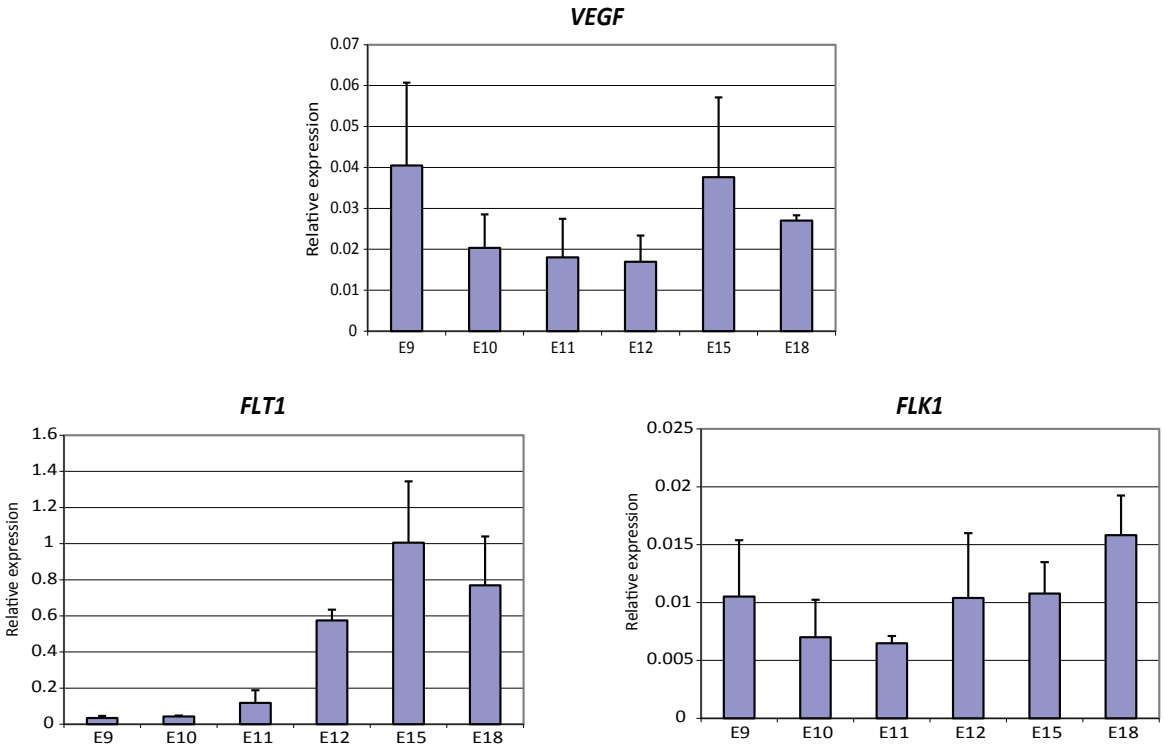


Figure 4. Expression of hypoxia-sensitive genes in murine placenta. E9-E18 fetal placentas were analyzed for expression of vascular endothelial growth factor (VEGF) and VEGF receptor 1 (FLT1), and VEGF receptor 2 (FLK1) by quantitative RT-PCR. The data is normalized to β-actin expression level. Three placentas were analyzed for each stage (mean ± SD).

Placenta contains hypoxic cells

Placenta morphogenesis is regulated by hypoxia. Deletion of HIF transcription factors in mouse leads to failure in placenta formation and embryo lethality by E10.5 (reviewed in Dunwoodie, 2009). Cells with low level of oxygen (≤2%) can be identified by hypoxyprobes, which are 2-nitromidazole drugs such as pimonidazole (Mahy et al., 2003). To detect the hypoxic cells in placenta, we performed pimonidazole staining on E12 murine placenta. The hypoxic regions were detected in fetal labyrinth and TGC layer, and in maternal decidua (Figure 5). In the labyrinth layer, the endothelial cells seem to be hypoxic. Of course, to characterize the hypoxic cell types in placenta (endothelial, mesenchymal, trophoblast, etc.), they should be tested for co-localization with cell-specific markers.

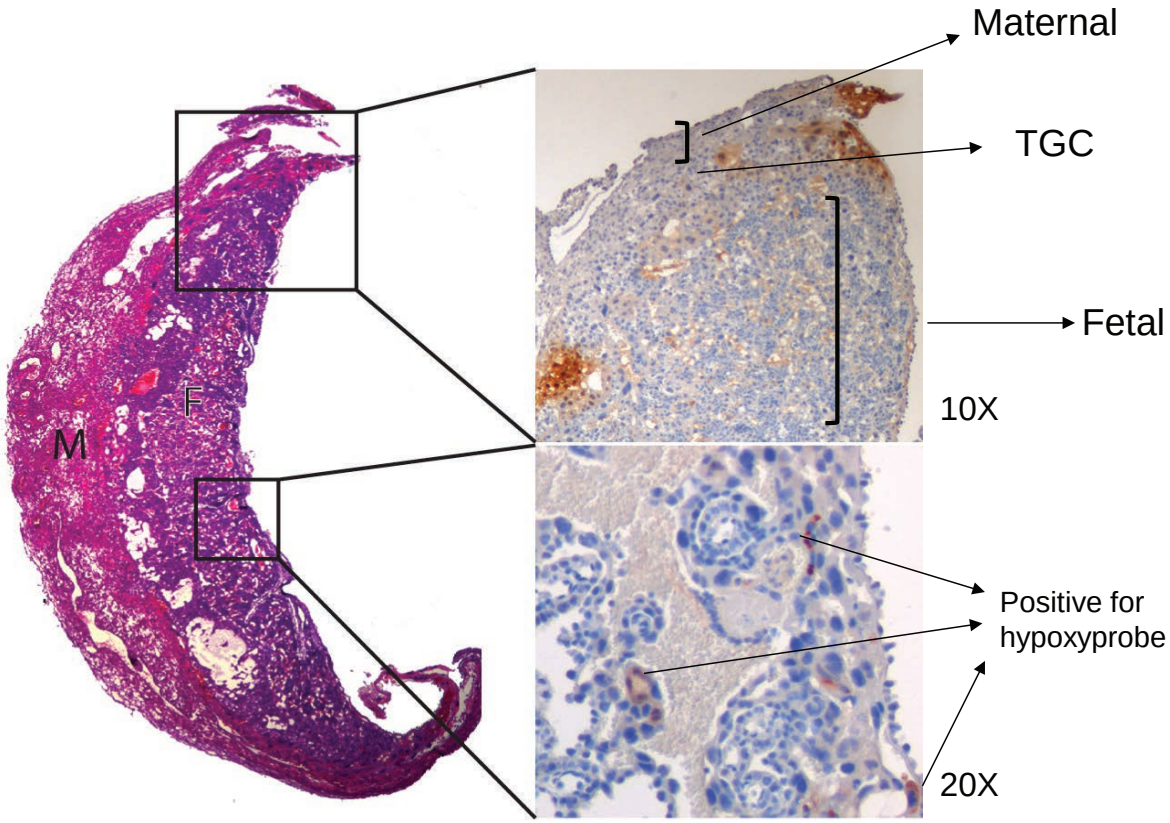


Figure 5. Hypoxic regions within the murine placenta. Hypoxyprobe staining of the E12 murine placenta shows hypoxic cells (in brown) in both fetal and maternal parts. M: maternal, F: fetal, TGC: trophoblast giant cells.

Discussion

In the present study, we showed that the mouse placenta stromal cell lines, PL8-5 and PL13-5, have the capacity to differentiate into osteogenic and adipogenic lineages. Moreover, they have a higher proliferative rate under hypoxic conditions compared to normoxia. This result is in agreement with the increase in proliferation rate of mesenchymal cells from adult BM when cultured in the low oxygen condition (Grayson et al., 2007; Lennon et al., 2001). Thus, our placental cell lines possess the characteristics expected of mesenchymal cells found in the hematopoietic niche.

To mimic the *in vivo* hematopoietic niche of the placenta, we established an *in vitro* system in which BM LSK cells are co-cultured with placenta stromal cells. In such co-cultures we found that the PL8-5 and PL13-5 cell lines support the significant expansion of hematopoietic cells, as compared to input LSK cells cultured under normoxic conditions. The majority of expanding hematopoietic cells are $Mac1^{+}$ after 7 days of co-culture. It remains to be investigated (by checking the hematopoietic cells at earlier times) whether the expansion of hematopoietic

cells is due to the differentiation and subsequent proliferation of the differentiating cells, or whether the HPSCs first expand and then differentiate. In hypoxia, the hematopoietic cell expansion was much reduced. One possible explanation is that, as described in Chapter 1, HSCs cultured under hypoxic conditions are kept in a quiescent state, which improves their long-term repopulating activity (Danet et al., 2003; Eliasson et al., 2010; Hermitte et al., 2006; Ivanovic et al., 2002). An increased rate of apoptosis is another possible explanation for the reduced number of hematopoietic cells in hypoxic co-cultures. Apoptosis assays and cell cycle analysis should be performed to determine the effect of prolonged hypoxia on hematopoietic cells. Moreover, the increased level of VEGF produced under hypoxic conditions suggests that hypoxia may shift the stromal lines to a mesenchymal function related to the support of angiogenesis rather than hematopoiesis.

Co-cultures of LSK cells with PL8-5 cells showed significant expansion of hematopoietic progenitors under normoxia as compared to input progenitor numbers. In hypoxic co-cultures we observed the maintenance and a slight increase in hematopoietic progenitor activity. Co-cultures with PL13-5 gave similar results (to determine the significance more experiments should be performed). Taken together, these data show that placenta stromal cells function in a supportive capacity for hematopoietic activity. In Chapter 1 we described the work of others in which hypoxia either decreases or does not change the hematopoietic progenitor activity of cultured hematopoietic cells (Cipolleschi et al., 2000; Ivanovic et al., 2000; Ivanovic et al., 2002). The discrepancies with our data can likely be attributed to differences in the level of oxygen tension, cytokine combinations, incubation times, background of the donor animals used in different experiments. Optimizing the culturing conditions and also using HSPCs from placenta instead of BM are directions for our future research.

LSK cells differentiate into the macrophage lineage in both normoxic and hypoxic co-culture conditions. However, lower percentages of $Mac1^{+}$ and higher percentages of LSK cells were observed in the hypoxic co-cultures as compared to normoxic cultures. Our data suggests that hypoxia inhibits the differentiation of HPSCs cultured on placenta cells. Input LSK cells undergo less differentiation and hematopoietic progenitor numbers are preserved (same as input numbers). This is in agreement with Jing and colleague's study showing that hypoxia highly maintains the CD34 expression in PB hematopoietic cells cultured on BM stromal cells and keeps them in a primitive state (Jing et al., 2012). Taken together, these results show that hypoxic culture of placental stromal cell lines interferes with their hematopoietic supportive and differentiative capacity. It is interesting to speculate that such hypoxic co-cultures may be better at maintaining the balance between differentiation and maintenance and that the most immature BM-LSK cells, the HSCs, may be well maintained. Transplantation assays should be performed to test this hypothesis.

Some of the observed effects of hypoxia may be due to induction of growth factor or chemokine expression. We detected significant induction of VEGF expression in placenta stromal cells cultured in hypoxia. This is consistent with the findings in some other studies: It has been shown that a local region of low oxygen region in the BM stem cell niche of the mouse increases VEGF expression by mesenchymal cells, which facilitates HPSC migration

through blood vessels (Levesque et al., 2007). Also, Jing et al. showed that hypoxia induces the expression of VEGF in human BM mesenchymal cells alone or in co-culture with human PB $CD34^+$ cells in a HIF1α-dependent manner. Also VEGF plays a role in permeability of stromal cells and distribution of hematopoietic cells within the niche (Jing et al., 2012). In our study, the role of VEGF produced by placenta stromal cells on hematopoietic activity remains to be investigated.

Unexpectedly, we detected decreased SDF-1 expression in hypoxic stromal cells compared to normoxic cells. This is in agreement with Jing and colleague's study which showed decreased SDF-1 expression by hypoxic BM stromal cells. They explained that this observation supports the role of SDF-1 and its receptor, CXCR4, in active cell movement processes (such as stem cell homing). The SDF-1/CXCR4 axis would not be expected to have a role in maintaining hematopoietic cells in their niche in the *in vitro* co-culture system. Although HIF-1α-dependent induction of VEGF and SDF-1 under low oxygen conditions is well known (Ceradini et al., 2004; Prager et al., 2010; Semenza, 2002), SDF-1 has been shown to be regulated independently of HIF-1α in some cases (Lerman et al., 2010). The HIF dependency of SDF-1 and VEGF expression in placenta stromal cells remains to be clarified (by cell-specific HIF1α inhibition) and presently leaves the question whether the observed phenotypes are the result of the direct effect of hypoxia on hematopoietic cells or its indirect effect through stromal cells or both.

In conclusion, our results suggest that low oxygen is an important regulator in the extra-embryonic placental hematopoietic niche both *in vivo* and *in vitro*. Finding out the exact role of hypoxia on hematopoietic progenitor and stem cells of placenta will deepen our knowledge of signals involved in HSC development and may contribute to the expansion of clinically useful human HSCs from this discarded tissue.

Material and methods

Cell lines

Placenta stromal cell lines were previously established in our lab (by Ivoune Lauw). The embryonic day (E) 12 fetal placentas were isolated from embryos generated by matings between wild type (C57BL/10xCBA)F1 females and heterozygous Tag5/Tag11 transgenic male mice. Tag5 mice are transgenic mice that carry the modified form of SV40 large T antigen gene (tsA58) under the β-actin promoter. Tag11 mice carry tsA58 gene under the Phosphoglycerokinase promoter (Oostendorp et al., 2002b).

Mouse strain and embryo generation

Mouse strain C57BL/6 males and females were used to generate embryos. The day of the vaginal plug detection was designated as E0. Pregnant females were sacrificed by cervical dislocation and the embryos were harvested.

For adult bone marrow (BM) cells in co-culture system, Actin-GFP mice with C57BL/6 background were used. All animal procedures were carried out in compliance with Standards for Care and Use of Laboratory Animals.

Placenta tissue, RNA isolation, cDNA synthesis, and quantitative RT-PCR assay

Placentas were dissected from embryos of certain time points during development. The fetal placenta was separated from the maternal part. The placenta tissue was homogenized using a tissue homogenizer machine. Total RNA was isolated with TRIZOL (Invitrogen) according to the manufacturer's protocol and was DNAase-treated with DNase I, Amplification Grade kit (Invitrogen). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instruction. Quantitative PCR was performed using an iCycler (BIO-RAD) and SYBR Green with Platinum Quantitative PCR SuperMix (Invitrogen). The cDNA quantities were normalized to the amount of *β-actin* cDNA.

Protein electrophoresis and western blotting

Cells were collected and boiled for 30 minutes in the sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.56M ß-mercapto-ethanol, 0.005% bromophenol blue, 10% glycerol, 10 mM DTT). The proteins were separated by one-dimensional gel electrophoresis in 10% Bis-Tris acrylamide gels (200V, 1 hr, RT). For western blotting following one-dimensional SDS-PAGE, proteins were electroblotted onto Immobilon P membranes (Millipore Corp, Etten-Leur, The Netherlands) and incubated with specific anti-VEGF (1:1000) and anti-GAPDH (1:10000) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and detected using SuperSignal West Pico chemiluminescent substrate (Pierce Perbio Science, Etten-Leur, The Netherlands).

Immunohistochemistry and hypoxyprobe labeling

Mice were injected with 60mg/kg bodyweight Hypoxyprobe-1 and sacrificed 1 hour later. Dissected embryos were fixed in 4% PFA in PBS at 4°C overnight. Samples were subsequently embedded in paraffin and sectioned at 5 µm. After deparaffination, sections were boiled in 0.01M citrate buffer (pH 6.0) for 15 minutes prior to incubation with primary antibody. Cells were fixed for 10 min in 4% PFA in PBS and washed three times for 5 min in PBS. For immunohistochemistry, 1% BSA in 0.05% Tween (in PBS) was used as block/diluent. Primary antibodies that were used at the indicated dilutions were: Hypoxyprobe-1 MAb1(mouse 1:50; Hypoxyprobe, Inc. Burlington, USA), and VEGF (1:100). Relevant DyLight594 1:800, Jackson Immunoresearch, West Grove, PA, USA) or HRP-conjugated (1:100, DAKO) secondary antibodies were used to detect primary antibodies. Haematoxylin and eosin or DAPI staining

were used as counterstains. Sections were studied and photographed with an Olympus BX40 light microscope. All fluorescent images were taken with an Axio Imager (Zeiss) fluorescence microscope.

Cell culture under low oxygen conditions and medium preparation

Cells were cultured in stromal medium under low oxygen conditions using a low oxygen incubator at 33°c. The oxygen tension was set at 3% by flushing the chamber with a gasenous mixture of 95% N2 and 5% CO2. Because of the presence of the tsA58 gene, all our experiments have been performed at 33°C, where the proliferation and differentiation activities were more efficient compared to 37°C. Stromal medium consists of:

- 50% myeloid long-term (LT)-culture medium for primitive mouse hematopoietic cells/ MyeloCultTM M5300 (StemCells technologies, cat. #5350)
- 35% Minimum Essential Medium (MEM) alpha (Gibco-Invitrogen, cat. #22571-038)
- 15% heat inactivated Fetal Calf Serum (FCS)
- 1% Penicillin and Streptomycin (PS) (Gibco-Invitrogen, cat. #15140-122)
- 1% Glutamax-I (100x) (Gibco-Invitrogen, cat. #35050-038)
- 0.1M β-mercaptoethanol (Sigma, cat. # M-7522) in PBS

Bone marrow cells preparation

The BM was harvested from tibias and femurs of Actin-GFP mice (10 weeks) by flushing with Strican 0.50x16mm syringe and filtered with strainer filter. Next, the cells were ficoll fractionated and washed in PBS/FCS/PS.

Flow cytometry analysis and sorting

For Lin^- c-Kit^+ $Sca\text{-}1^+$ (LSK) isolation from adult, the cells were stained with mouse biotinylated anti-lineage antibody cocktail (CD3e, CD45R/B220, Ly-6G/Gr-1, CD11b/Mac-1, and TER119/ Ly76), straptavidin perCPCy5.5 (secondary antibody), anti-c-Kit APC, and anti-Sca-1 PE antibodies. Anti-Mac-1 FITC antibody was used to analyze the hematopoietic cells. Dead cells were eliminated by Hoechst 33258 labeling (Molecular Probes).

To analyze the stromal cells, anti-CD29 FITC, anti-Sca-1 PE, anti-CD44 FITC, and anti-CD11bPE were used.

All the antibodies were obtained from BD Pharmingen. Flow cytometric analysis and sorting performed using a FACS-Sorp, FACS AriaIII or FACS AriaII cell sorter (BD Biosciences).

Co-culture system

Placenta stromal cell lines were seeded in 0.1% gelatin (J.T.Baker, cat. #25281) pre-coated six-well tissue culture plates, grown to sub-confluency for 3 days in normoxia and hypoxia. Cell seeding densities during expansion period were dependent on the growth curve of the particular cell line. Next, cell lines were irradiated (20 Gy) and the medium was refreshed after irradiation. 1500 LSK cells were seeded onto the irradiated stromal cells in stromal medium. The cultures were maintained in normoxic and hypoxic incubators at 37°c. Every 3 days, half of the medium was removed and non-adherent cells were recovered. After 7 days, both adherent and non-adherent cells were harvested.

In vitro hematopoietic progenitor assay

GFP^+ (single viable) cell suspensions sorted from co-cultures were plated at various dilutions (depending on normoxic or hypoxic conditions) in triplicates in methylcellulose medium (Methocult GF m3434, Stem Cell Technologies Inc.) with 1% PS. The plates were incubated at 37°C in a humidified chamber under 5% CO2 for 12 days. Different hematopoietic colonies were distinguished by morphology and counted with an inverted microscope.

Statistical analysis

Data are presented as mean ± SD. Differences were considered to be significant at p-value calculated by Student's t-test.

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References

Alvarez-Silva, M., Belo-Diabangouaya, P., Salaun, J., and Dieterlen-Lievre, F. (2003). Mouse placenta is a major hematopoietic organ. Development *130*, 5437-5444.

Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R. (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. Experimental hematology *28*, 707-715.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic

gradients through HIF-1 induction of SDF-1. Nature medicine *10*, 858-864.

Cipolleschi, M.G., Rovida, E., Ivanovic, Z., Praloran, V., Olivotto, M., and Dello Sbarba, P. (2000). The expansion of murine bone marrow cells preincubated in hypoxia as an in vitro indicator of their marrow-repopulating ability. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *14*, 735-739.

Corbel, C., Salaun, J., Belo-Diabangouaya, P., and Dieterlen-Lievre, F. (2007). Hematopoietic potential of the pre-fusion allantois. Developmental biology *301*, 478-488.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L.*, et al.* (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell stem cell *3*, 301-313.

D'Ippolito, G., Schiller, P.C., Ricordi, C., Roos, B.A., and Howard, G.A. (1999). Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *14*, 1115-1122.

Dancis, J., Jansen, V., Brown, G.F., Gorstein, F., and Balis, M.E. (1977). Treatment of hypoplastic anemia in mice with placental transplants. Blood *50*, 663-670.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A., and Simon, M.C. (2003). Expansion of human SCID-repopulating cells under hypoxic conditions. The Journal of clinical investigation *112*, 126-135.

Das, R., Jahr, H., van Osch, G.J., and Farrell, E. (2010). The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue engineering Part B, Reviews *16*, 159-168.

Dunwoodie, S.L. (2009). The role of hypoxia in development of the Mammalian embryo. Developmental cell *17*, 755-773.

Dzierzak, E., and Robin, C. (2010). Placenta as a source of hematopoietic stem cells. Trends in molecular medicine *16*, 361-367.

Eliasson, P., Rehn, M., Hammar, P., Larsson, P., Sirenko, O., Flippin, L.A., Cammenga, J., and Jonsson, J.I. (2010). Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. Experimental hematology *38*, 301-310 e302.

Gekas, C., Dieterlen-Lievre, F., Orkin, S.H., and Mikkola, H.K. (2005). The placenta is a niche for hematopoietic stem cells. Developmental cell *8*, 365-375.

Grayson, W.L., Zhao, F., Bunnell, B., and Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochemical and biophysical research communications *358*, 948-953.

Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., Hicklin, D.J., Zhu, Z., Bohlen, P., Witte, L.*, et al.* (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nature medicine *8*, 841-849.

Hermitte, F., Brunet de la Grange, P., Belloc, F., Praloran, V., and Ivanovic, Z. (2006). Very low O2 concentration (0.1%) favors G0 return of dividing CD34+ cells. Stem Cells *24*, 65-73.

Ivanovic, Z., Bartolozzi, B., Bernabei, P.A., Cipolleschi, M.G., Rovida, E., Milenkovic, P., Praloran, V., and Dello Sbarba, P. (2000). Incubation of murine bone marrow cells in hypoxia ensures the maintenance

of marrow-repopulating ability together with the expansion of committed progenitors. British journal of haematology *108*, 424-429.

Ivanovic, Z., Belloc, F., Faucher, J.L., Cipolleschi, M.G., Praloran, V., and Dello Sbarba, P. (2002). Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells. Experimental hematology *30*, 67-73.

Ivanovs, A., Rybtsov, S., Welch, L., Anderson, R.A., Turner, M.L., and Medvinsky, A. (2011). Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. The Journal of experimental medicine *208*, 2417-2427.

Jing, D., Wobus, M., Poitz, D.M., Bornhauser, M., Ehninger, G., and Ordemann, R. (2012). Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. Haematologica *97*, 331-339.

Kabrun, N., Buhring, H.J., Choi, K., Ullrich, A., Risau, W., and Keller, G. (1997). Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development *124*, 2039-2048.

Lee, Y.M., Jeong, C.H., Koo, S.Y., Son, M.J., Song, H.S., Bae, S.K., Raleigh, J.A., Chung, H.Y., Yoo, M.A., and Kim, K.W. (2001). Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. Developmental dynamics : an official publication of the American Association of Anatomists *220*, 175-186.

Lennon, D.P., Edmison, J.M., and Caplan, A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. Journal of cellular physiology *187*, 345-355.

Lerman, O.Z., Greives, M.R., Singh, S.P., Thanik, V.D., Chang, C.C., Seiser, N., Brown, D.J., Knobel, D., Schneider, R.J., Formenti, S.C*., et al.* (2010). Low-dose radiation augments vasculogenesis signaling through HIF-1-dependent and -independent SDF-1 induction. Blood *116*, 3669-3676.

Levesque, J.P., Winkler, I.G., Hendy, J., Williams, B., Helwani, F., Barbier, V., Nowlan, B., and Nilsson, S.K. (2007). Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. Stem Cells *25*, 1954-1965.

Mahy, P., De Bast, M., Gallez, B., Gueulette, J., Koch, C.J., Scalliet, P., and Gregoire, V. (2003). In vivo colocalization of 2-nitroimidazole EF5 fluorescence intensity and electron paramagnetic resonance oximetry in mouse tumors. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology *67*, 53-61.

Oostendorp, R.A., Harvey, K.N., Kusadasi, N., de Bruijn, M.F., Saris, C., Ploemacher, R.E., Medvinsky, A.L., and Dzierzak, E.A. (2002a). Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity. Blood *99*, 1183-1189.

Oostendorp, R.A., Medvinsky, A.J., Kusadasi, N., Nakayama, N., Harvey, K., Orelio, C., Ottersbach, K., Covey, T., Ploemacher, R.E., Saris, C*., et al.* (2002b). Embryonal subregion-derived stromal cell lines from novel temperature-sensitive SV40 T antigen transgenic mice support hematopoiesis. Journal of cell science *115*, 2099-2108.

Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Developmental cell *8*, 377-387.

Ottersbach, K., and Dzierzak, E. (2010). The placenta as a haematopoietic organ. The International journal of developmental biology *54*, 1099-1106.

Oyajobi, B.O., Lomri, A., Hott, M., and Marie, P.J. (1999). Isolation and characterization of human clonogenic osteoblast progenitors immunoselected from fetal bone marrow stroma using STRO-1 monoclonal antibody. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *14*, 351-361.

Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. (1999). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. The Journal of clinical investigation *104*, 1199-1211.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Ponomaryov, T., Peled, A., Petit, I., Taichman, R.S., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Magerus, A., Caruz, A., Fujii, N.*, et al.* (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. The Journal of clinical investigation *106*, 1331-1339.

Prager, G.W., Lackner, E.M., Krauth, M.T., Unseld, M., Poettler, M., Laffer, S., Cerny-Reiterer, S., Lamm, W., Kornek, G.V., Binder, B.R.*, et al.* (2010). Targeting of VEGF-dependent transendothelial migration of cancer cells by bevacizumab. Molecular oncology *4*, 150-160.

Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M., Cerisoli, F., Lauw, I., Kaimakis, P., Jorna, R., Vermeulen, M.*, et al.* (2009). Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. Cell stem cell *5*, 385-395.

Semenza, G. (2002). Signal transduction to hypoxia-inducible factor 1. Biochemical pharmacology *64*, 993-998.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Cote, D., Means, T.K., Luster, A.D., Scadden, D.T., and Lin, C.P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature *435*, 969-973.

Till, J.E., and Mc, C.E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation research *14*, 213-222.

Wang, Y., Nathanson, L., and McNiece, I.K. (2011). Differential hematopoietic supportive potential and gene expression of stroma cell lines from midgestation mouse placenta and adult bone marrow. Cell transplantation *20*, 707-726.

Weisel, K.C., Gao, Y., Shieh, J.H., and Moore, M.A. (2006). Stromal cell lines from the aorta-gonado-mesonephros region are potent supporters of murine and human hematopoiesis. Experimental hematology *34*, 1505-1516.

Zeigler, B.M., Sugiyama, D., Chen, M., Guo, Y., Downs, K.M., and Speck, N.A. (2006). The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. Development *133*, 4183-4192.

Supplementary Figures

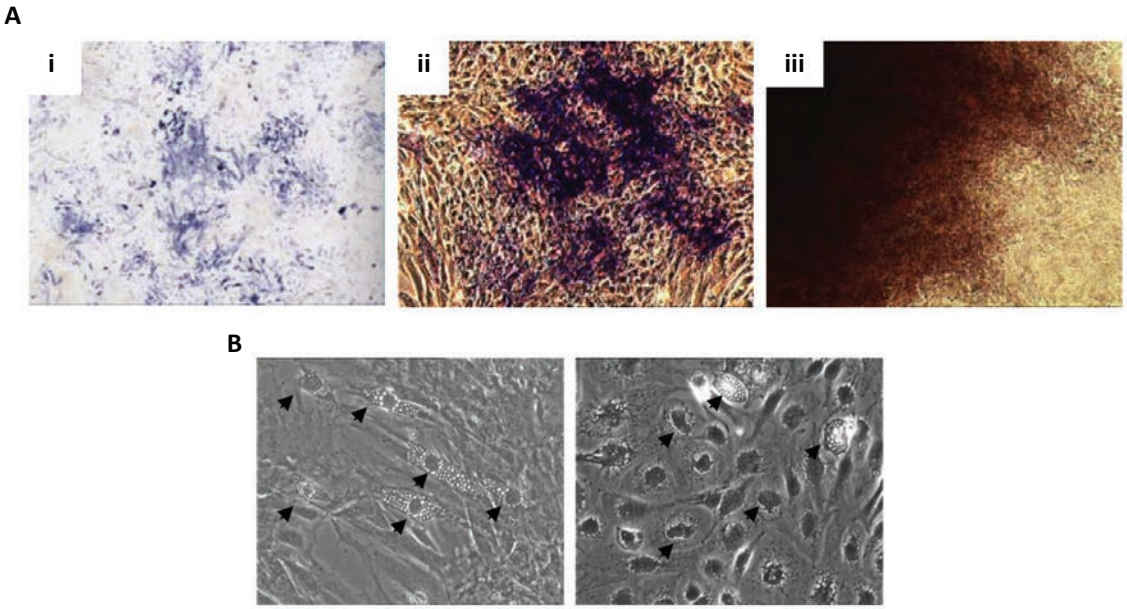
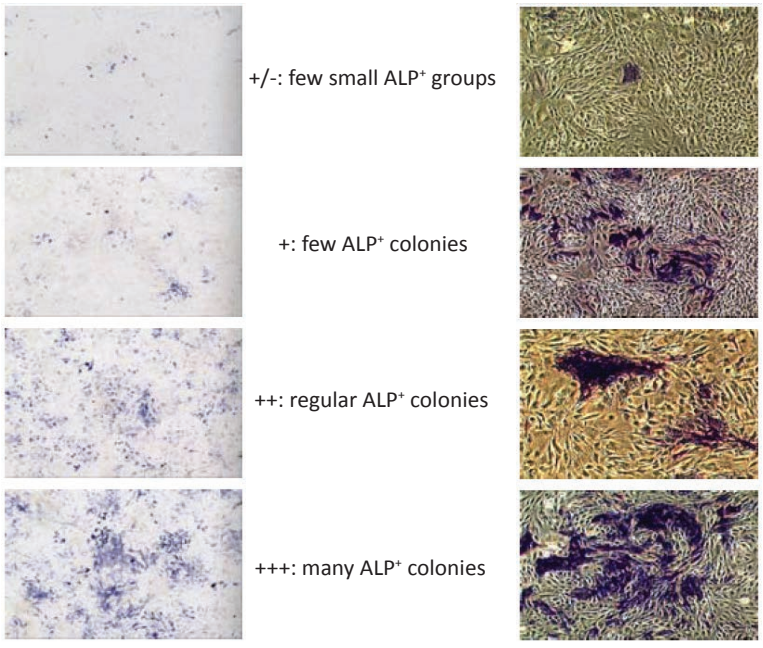
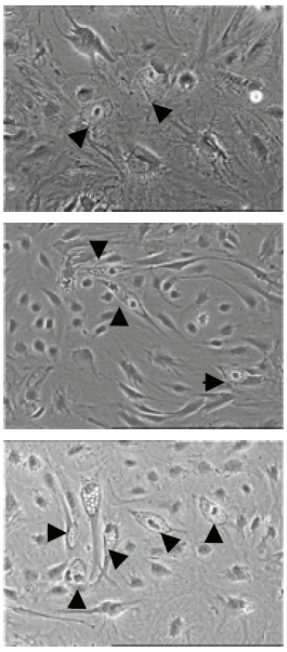


Figure S1. Differentiation of murine placental stromal lines. (A) Osteogenic differentiation of the cell lines is depicted in the top panel. Representative photographic images of placental cell lines for: i. ALP staining of day 10 cultures (1x magnification), ii. ALP staining of day 10 culture (4x magnification), iii. Alizarin red staining of day 28 cultures (4x magnification). (B) Adipogenic differentiation of the cell lines is depicted in the bottom panel. Adipocytes observed on day 10 of differentiation. Arrowheads point to adipocytes.



Line	ALP day 10	ALP day 14
PL1-5	+	+/++
PL2-5	+/-	++
PL7-5	+/-	+
PL8-5	+++	+++
PL9-5	+/-	+
PL13-5	+/-	+

Figure S2. Comparison of the ALP positive cell and colony numbers among the placental stromal cell lines. The scoring was based solely on the ALP staining. The left pictures were taken with no magnification and the right pictures with 4x magnification to show individual colonies. ALP positive cells stain blue. The table summarizes the ALP staining of different lines.



+/-: few adipocytes

+: single adipocytes and small adipocytic colonies

++: regular adipocytic colonies

Line	Adipogenic morphology day 7 AS	Adipogenic morphology day 14
PL1-5	+	+/++
PL2-5	+/-	++
PL7-5	+/-	+
PL8-5	+++	+++
PL9-5	+/-	+
PL13 -5	+/-	+

Figure 3. Comparison of the adipocyte numbers among the placental stromal cell lines. Arrowheads point to adipocytes. All pictures were taken with 10x magnification. The table summarizes the adipogenic differentiation of different lines.

Chapter 4

HIF1α Is a Regulator of Hematopoietic Progenitor and Stem Cell Development

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Abstract

Hypoxia plays a physiological role during fetal organogenesis and is involved in adult bone marrow hematopoietic stem cell (HSC) maintenance. Yet, little is known about the role of hypoxia on embryonic hematopoietic progenitor and stem cells (HPSC) and their microenvironment. Hypoxia inducible factor (HIF) is a key modulator of the transcriptional response to hypoxia. In mammals, oxygen levels range from 2-5% in the uterus, where HIF regulates the genes that play a role in embryo and placenta development. Our hypothesis is that HIF1α is important for the development of HSCs during embryonic stages. To examine the role of HIF1α in the ontogeny of the hematopoietic system, we took a Cre/loxP strategy to delete the *HIF1α* gene in (vascular endothelial cadherin (VEC) expressing) endothelial cells, the precursors to definitive HPSCs. Colony-forming assays (CFU-C) were performed on cells from embryonic hematopoietic tissues: aorta-gonad-mesonephros (AGM) region, fetal liver (FL), yolk sac (YS), and placenta of *+/+:HIF1α$^{fl/fl}$* wild type (WT) and *VEC-Cre/+:HIF1α$^{fl/fl}$* knock out (KO) embryos at different embryonic stages. A significant decrease in the number of CFU-C in embryonic day 10 (E10) KO AGM and placenta, and E11 AGM and FL was observed, as compared to WT controls. Moreover, decreases in the cKit^{+} CD31^{+} HPSC population in KO embryonic tissues was revealed by flow cytometry and corroborated in 3-dimensional whole mount stained KO embryos, showing a 40-50% decrease in the number of aortic hematopoietic clusters at E10. Also, HSC activity was significantly diminished in KO AGMs. These results suggest that HIF1α is necessary for generation and/or expansion of hematopoietic progenitor and stem cells during embryonic development. Adult bone marrow Lin^{-} Sca-1^{+} c-Kit^{+} (LSK) HSCs from KO mice are also affected in their ability to long term repopulate adult irradiated mice – with a lower efficiency than WT cells in the reconstitution of all hematopoietic tissues. In secondary transplantations of KO LSK cells, more peripheral blood chimerism and proliferating (Ki-67 positive) cells were found as compared to WT secondary transplantation controls. Thus, our data show that HIF1α is one of the regulators of HPSC development and function in embryonic hematopoietic tissues beginning from the hemogenic endothelial stage onward.

Introduction

Hematopoietic stem cells (HSCs) localize to particular *in vivo* microenvironments termed ''niches.'' A niche is identified by the anatomical location of HSCs and the surrounding regulatory cells/factors that govern the generation, maintenance, and function of HSCs. In the adult HSCs reside in the bone marrow (BM), where oxygen levels are relatively lower than other tissues (Cipolleschi et al., 1993; Harrison et al., 2002; Levesque et al., 2007). Several studies suggest that long-term repopulating HSCs (LTR-HSCs) are located mainly in the endosteal zones (Calvi et al., 2003; Zhang et al., 2006), which are highly vascularized. The sinusoidal endothelium allows hematopoietic cells to readily pass through this vasculature (Draenert and Draenert, 1980). Thus, the perfusion rate of BM cells in the endosteal zone is limited and the oxygen level is low. Other parts of the BM are populated with hematopoietic cells which consume a high level of oxygen. The localization of HSCs to the endosteal hypoxic niche suggests that hypoxia is a regulator of HSC function.

Intriguingly, HSCs are located in the lowest end of oxygen gradient in BM niche (Kubota et al., 2008; Levesque et al., 2007; Parmar et al., 2007). Parmer et al. applied Hoechst 33342 staining on the BM cells to isolate different hematopoietic subpopulations according to the extent of dye perfusion. HSCs are enriched in the lowest dye uptake fraction, as shown by *in vivo* transplantation analyses. These cells reside in the regions with lowest dye intensity, i.e. the most hypoxic compartment of BM. Similarly, Takubo et al. performed flow cytometric analysis for different subpopulations of BM mononuclear cells (MNCs) based on the intracellular incorporation of hypoxic marker, Pimonidazole (Pimo). The Pimo positive fraction (30% of BM MNCs) was enriched for of LTR-HSCs (68.2%) and quiescent HSCs (Tie2 LSK cells) (Takubo et al., 2010). Altogether, these data reveal that HSCs exist in the most hypoxic microenvironment of the BM niche. Obviously, it is beneficial for HSCs to be localized at a hypoxic niche where the inducer of their senescence, i.e. reactive oxygen species (ROS), is absent.

The master regulator of cellular and systemic hypoxia is hypoxia inducible factor (HIF), which is a heterodimeric bHLH-PAS-type transcription factor consisting of HIFα (HIF1α, HIF2α, and HIF3α) and HIF1β subunits (Dunwoodie, 2009; Wang et al., 1995). HIF1β is constitutively expressed, whereas HIFα expression is regulated by the cellular oxygen concentration. Under normoxic conditions (> 5% oxygen), prolyl hydroxylase domain proteins (Phd1-3) hydroxylate two prolyl residues in the oxygen-dependent degradation domain (ODD) of HIFα enabling the binding of tumor suppressor von Hippel-Lindau protein (VHL), the recognition component of an E3 ubiquitin-protein ligase complex. It subsequently leads to ubiquitylation and proteasomal degradation of HIFα. (Ruas and Poellinger, 2005; Schofield and Ratcliffe, 2005; Semenza, 2007; Simon and Keith, 2008). In situations of hypoxia, the Phds are not active and HIFα proteins are stabilized in the cytoplasm, dimerize to HIF1β, translocate to the nucleus, and thereby bind to hypoxia-responsive elements (HREs) in the promoter regions of almost 200 hypoxia-targeted genes to regulate their transcription (Semenza, 2007; Simon and Keith, 2008). Most transcriptional responses have been attributed to Hif1α and Hif2α. Hif3α is a direct target of Hif1α, but its exact role in response to cellular hypoxia remains to be clarified (Tanaka

et al., 2009). HIF1α is ubiquitously expressed, but the HIF2α expression pattern is slightly limited, serving perhaps a more specialized function than HIF1α (Koh and Powis, 2012; Tian et al., 1997; Wiesener et al., 2003). HIF1α and HIF2α regulate the expression of many common genes, however they have some unique target genes as well (Hu et al., 2003; Keith et al., 2012; Raval et al., 2005).

Germline deletion of *HIF1α* in the mouse leads to lethality by embryonic day 10.5 (E10.5). *HIF1α* knock out (KO) embryos showed failure in placenta development, abnormal neural fold formation, heart developmental defects, and reduced numbers of somites (Cowden Dahl et al., 2005a; Cowden Dahl et al., 2005b; Ryan et al., 1998). The role of HIF1α has been investigated in the regulation of adult HSC function using *MX1-Cre: HIF1α$^{fl/fl}$* conditional KO mice. Absence of HIF1α activity in HSCs was associated with increased cycling, and subsequent senescence and exhaustion of HSCs in serial transplantations, due to increased expression of *Ink4a* locus products (p16 and p19). Furthermore, *MX1-Cre: VHL$^{fl/+}$* animals showed an enhanced quiescent fraction of BM HSCs as compared to wild type (WT) animals due to the over-stabilization of HIF1α. *In vivo* transplantation of *MX1-Cre: VHL$^{fl/+}$* LSK cells resulted in considerably improved BM chimerism of LSK and CD34$^-$ LSK cells (LTR-HSCs). Hence, it appears that HIF1α plays a role in resistance of HSCs against stress conditions and that the VHL/HIF1α regulatory system has a key role in maintaining the quiescent state of HSCs (Takubo et al., 2010). Although it is not clear that how HIF1α acts to maintain quiescence of HSCs, some downstream targets regulating hematopoiesis have been identified.

Vascular endothelial growth factor A (*VEGFA*) gene is a well-known target of HIFα (Plate et al., 1992; Shweiki et al., 1992). VEGFA is essential for the maintenance of HSCs in BM, as shown by the conditional KO of VEGFA (Gerber et al., 2002). Rehn et al. used *Vegf$^{\delta/\delta}$* mouse model in which the HRE is mutated in the *Vegf* promoter region, preventing HIF binding and thus, abolishing the VEGF expression under hypoxic conditions. The number of phenotypic BM HSCs increases in these animals; however, HSC function is significantly abolished as shown by *in vivo* transplantation assays. These data show one of the down-stream pathways through which hypoxia and HIF can regulate hematopoiesis (Rehn et al., 2011).

Oxygen levels range from 1% to 5% in the uterus. Therefore, normal mammalian development takes place in a hypoxic environment and many aspects of embryonic development such as placenta formation, cardiovascular system development, chondrogenesis, etc. are under regulation of HIFs. *HIF1β* germline KO embryos exhibit developmental delays and severe cardiovascular and hematopoietic abnormalities (defects in yolk sac hematopoietic progenitor activity, increased number of apoptotic hematopoietic cells) by E10.5 (Adelman et al., 1999; Ramirez-Bergeron et al., 2006). Using para-aortic splanchpleural (P-Sp) explant cultures to analyse vasculogenesis and hematopoiesis in the KO without the interference of other tissue defects, the vasculogenesis defect was rescued by addition of Sca1$^+$ hematopoietic cells or VEGF protein into the culture (Ramirez-Bergeron et al., 2006). These findings suggest that HIFs regulate development of vascular system by promoting hematopoietic cell survival and the production of their paracrine growth factors affecting vessel development. However, the ubiquitous function of HIF transcription factors and the complication of multi-tissue

developmental deficiencies in germline KO mice preclude examination of the precise role of HIFs specifically in the development of the hematopoietic compartment.

To this aim, we applied a conditional KO approach using Cre/loxp strategy to specifically remove *HIF1α* from endothelial cells that express vascular endothelial cadherin (VEC). The first definitive HSCs appear in the aorta-gonad-mesonephros (AGM) region at E10.5 in developing murine embryos (Medvinsky and Dzierzak, 1996; Sanchez et al., 1996). It has been shown that hematopoietic cells emerge from endothelial cells (VEC^+) lining the major vasculature of the midgestation mouse (Chen et al., 2009; Zovein et al., 2008). *VEC-Cre:HIF1α$^{fl/fl}$* embryo and adult hematopoietic tissues were examined and we show here that: 1) HIF1α regulates HPSC generation and/or expansion during embryonic stages; and 2) HIF1α plays a role in regulating adult BM hematopoietic stem cell function under stress conditions.

Results

HIF1α-deficiency in VEC-expressing cells affects hematopoietic progenitors in embryonic tissues

To analyze the hematopoietic progenitor capacity of conditional knock out (cKO) *VEC-Cre/+: HIF1α$^{fl/fl}$* embryos (E9-E11), we performed the colony-forming unit culture (CFU-C) assay with cells from the aorta-gonad-mesonephros (AGM) region, fetal liver (FL), yolk sac (YS), and fetal placenta. Erythrocyte (BFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte-macrophage (CFU-GM), and granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM) progenitors were quantitated.

We found total CFU-C numbers reduced in cells from para-aortic splanchpleural (P-Sp), fetal placenta, and yolk sac ftissues of E9 cKO embryos compared to controls (Table 1). Although WT tissues always showed more colonies than the cKO tissues, this reduction was not significant due to the large variance in the number of WT CFU-C colonies. At E10 total CFU-C numbers of cKO AGM and placenta are significantly decreased (on average 3 and 2.1 fold, respectively) as compared to WT embryos. Note that in AGM region, the decrease in all colony types except CFU-G is statistically significant. In placenta, the numbers of CFU-M and CFU-MIX are significantly decreased. We did not observe any significant decrease in total CFU-C numbers in cKO YSs compared to controls, but a significant decrease was detected in BFU-E colony numbers (Figure 1A). This is consistent with the results of previously published data on germline *HIF1α* KO mice, which indicated that the KO YS contains fewer BFU-E progenitors (Yoon et al., 2006).

Decreases in total colony numbers of cKO tissues persist at E11, although to a lesser degree when compared to E10. A significant 1.6-fold decrease is observed in AGM CFU-C numbers, with CFU-M and CFU-MIX significantly decreased as compared to WT. Total CFU-C numbers of the cKO FL are significantly decreased by 1.5-fold and in the placenta, a significant decrease is observed for CFU-MIX but not for total CFU-C numbers (Figure 1B). In most cases, the

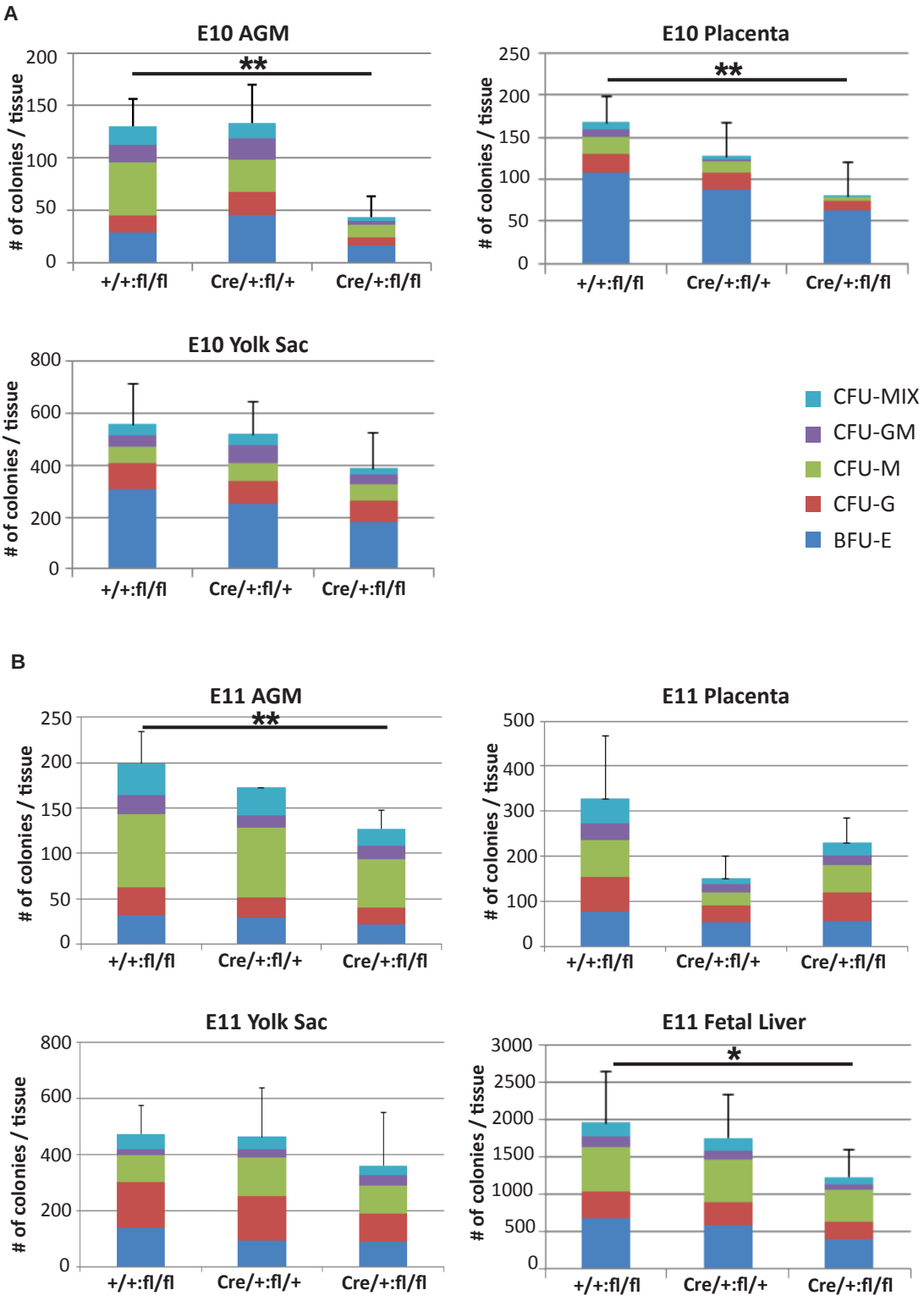


Figure 1. HIF1α affects hematopoietic progenitor generation and/or expansion. (A) CFU-C numbers per AGM, placenta, and yolk sac at E10. (B) CFU-C numbers per AGM, placenta, yolk sac, and fetal river at E11. The colony numbers for each type (mixed, granulocyte=macrophage, macrophage, granulocyte and erythrocytes are shown. Significant differences are indicated with asterisks (*: p-value ≤ 0.05, **:p-value ≤ 0.005). Data are averages derived from 4-7 embryos (3 separate litters) for each genotype (mean ± SD).

heterozygous cKO also showed decreases in CFU-C suggesting a *HIF1α* dosage effect. The summary of all CFU-C data for different stages is shown in Table 1.

Table 1. Summary of CFU-C data for *+/+:HIF1α$^{fl/fl}$* and *VEC-Cre/+:HIF1α$^{fl/fl}$* embryos

AGM						
	SP	# of WT Emb.	CFU-C No.	# of KO Emb.	CFU-C No.	Fold Change
E9 (P-Sp)	20-27	5	4 ± 2.7	4	2.3 ± 3.2	**1.7↓**
E10	30-34	3	130 ± 43.7	4	43.0 ± 19.6	**3.0↓****
E11	43-47	3	199 ± 35.1	3	128 ± 20.5	**1.6↓****

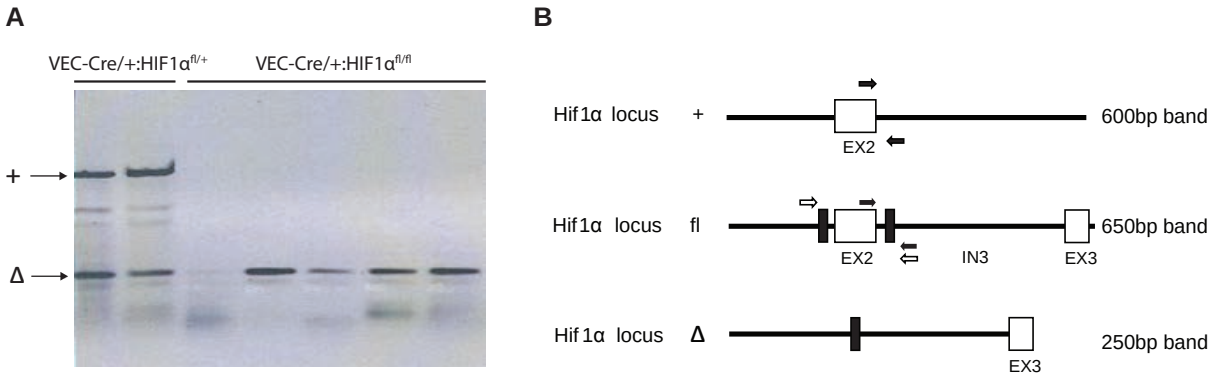
Placenta						
	SP	# of WT Emb.	CFU-C No.	# of KO Emb.	CFU-C No.	Fold Change
E9	20-27	4	14 ± 11.5	4	5 ± 6.9	**2.8↓**
E10	30-34	4	168 ± 31	7	80 ± 38.8	**2.1↓****
E11	43-47	3	329 ± 137	3	230 ± 53	**1.4↓**

Yolk sac						
	SP	# of WT Emb.	CFU-C No.	# of KO Emb.	CFU-C No.	Fold Change
E9	20-27	4	196.3 ± 93.3	4	208 ± 148.4	**1**
E10	30-34	4	558 ± 154.6	8	389 ± 134.5	**1.4↓**
E11	43-47	3	479 ± 99	3	360 ± 189	**1.3↓**

Fetal Liver						
	SP	# of WT Emb.	CFU-C No.	# of KO Emb.	CFU-C No.	Fold Change
E11	44-46	6	1956 ± 691	6	1236 ± 348	**1.5↓***

Hematopoietic progenitor assays were performed for E9 para-aortic splanchnopleural (P-Sp), placenta, and yolk sac, E10 AGM, placenta, and yolk sac, and E11 AGM, placenta, yolk sac, and fetal liver of WT and cKO embryos. The number of embryos analyzed per genotype, stages and somite pairs, total CFU-C numbers per tissue (mean ± SD), fold changes of CFU-C numbers obtained from cKO tissues compared to WT controls, and significance of changes are presented here. Significant differences are indicated with asterisks (*: p-value ≤0.05, **:p-value ≤0.005).

Since CFU-C were not completely eliminated in *VEC-Cre/+: HIF1α$^{fl/fl}$* embryos, we examined the recombination efficiency in the isolated CFU-C. DNA PCR analysis of over 330 individual colonies revealed that 85-100% of (*VEC-Cre/+:HIF1α$^{fl/fl}$)* cells had excised both *HIF1α$^{fl/fl}$* alleles (Figure 2), demonstrating that the recombination efficiency is high in hematopoietic cells derived from endothelial cells. Despite the fact that hematopoietic progenitors are fewer in cKO tissues, all colony types are found and their activity is normal (morphology and size) suggesting that HIF1α does not affect progenitor function. Instead, our data suggest that in the *HIF1α*-deficient embryonic hematopoietic tissues, it is the generation and/or expansion process of hematopoietic progenitors that is defective.



C

Stage	Genotype	# of recombined colonies/total # of picked colonies			
		AGM	Placenta	Yolk Sac	Fetal liver
E10	VEC-Cre/+:HIF1α$^{fl/fl}$	27/30	30/30	26/30	NA
	VEC-Cre/+:HIF1α$^{fl/+}$	20/20	19/20	18/20	NA
	+/+:HIF1α$^{fl/fl}$	0/5	0/5	0/5	NA
E11	VEC-Cre/+:HIF1α$^{fl/fl}$	36/40	35/40	36/40	36/40
	VEC-Cre/+:HIF1α$^{fl/+}$	19/20	20/20	17/20	18/20
	+/+:HIF1α$^{fl/fl}$	0/5	0/5	0/5	0/5

Figure 2. Polymerase chain reaction (PCR) analysis of cells isolated from single CFU-C. (A) Amplified fragments resulting from PCR performed on cells isolated from single CFU-C. Fragments detected are HIF1α WT (+), floxed (fl), and excised (Δ) alleles. (B) The locations of two primers (arrows) used to detect the *HIF1α* alleles are depicted. LoxP sites are shown as black rectangles. (C) Summary table of PCR results for 330 isolated colonies.

Phenotypic hematopoietic stem and progenitor cells are decreased in embryonic *VEC-Cre/+:HIF1α$^{fl/fl}$* tissues

To further examine the hematopoietic deficiencies in *Hlf1α* cKO embryos, flow cytometric analysis (FACS) was performed for hematopoietic markers c-Kit and CD41, and for CD31 which is expressed by endothelial and hematopoietic cells. In the AGM, HPSCs are highly enriched in the ckit+ ((Yokomizo and Dzierzak, 2010) and CD41+ fractions (Robin et al., 2011) and correspond to the vascular hematopoietic cluster cells. All FACS data for embryonic tissues is summarized in Figure 3.

The cKO E10 AGM, shows a 4.7-fold and 1.4-fold decrease in c-Kit$^+$ cells and CD41$^+$ cells, respectively, as compared to WT AGM tissues. Placenta, which contains a greater number of hematopoietic cells at E10 as compared to the AGM, shows a highly significant 3.6-fold decrease in c-Kit$^+$ cells and 3.4-fold decrease in CD41$^+$ cells in the cKO as compared to WT. The E10 cKO yolk sac shows 1.6 and 1.5-fold decrease in c-Kit$^+$ and CD41$^+$ cells, respectively. Overall, the flow cytometry data correspond well to the fold decreases observed in the CFU-C progenitor assay, demonstrating that *HIF1α* cKO hematopoietic tissues lack HPSC, suggesting that there is a deficiency in their formation and/or growth.

AGM

Stage	SP	% c-Kit$^+$ Cells		Fold Change
		WT	KO	
E10	35-37	5.6 ± 3.0	1.2 ± 0.4	**4.7↓***

Stage	SP	% CD41 Cells		Fold Change
		WT	KO	
E10	35-37	1 ± 0.3	0.7 ± 0.2	**1.4↓**

Placenta

Stage	SP	% c-Kit$^+$ Cells (in CD31$^+$ Gate)		Fold Change
		WT	KO	
E10	35-37	43.4 ± 0.5	12.1 ± 4.0	**3.6↓****

Stage	SP	% CD41 Cells (in CD31$^+$ Gate)		Fold Change
		WT	KO	
E10	35-37	24 ± 0.7	7.2 ± 1.2	**3.3↓***

Yolk Sac

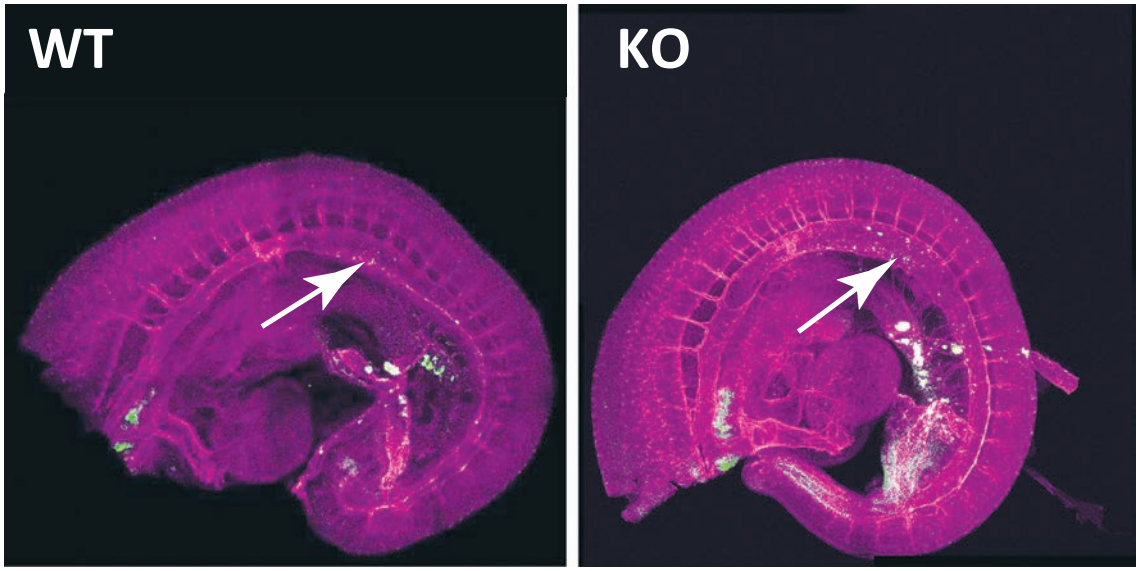
Stage	SP	% c-Kit$^+$ Cells (in CD31$^+$ Gate)		Fold Change
		WT	KO	
E10	35-37	4.5 ± 0.8	3.8 ± 0.5	**1.2↓**

Stage	SP	% CD41 Cells (in CD31$^+$ Gate)		Fold Change
		WT	KO	
E10	35-37	7.1 ± 1.2	5.0 ± 1.5	**1.4↓**

Figure 3. Decreases in phenotypic hematopoietic progenitor and stem cells of *VEC-Cre/+:HIF1α$^{fl/fl}$* embryos. The tables summarize the results of flow cytometric analysis of late E10 (35-37sp) AGM, yolk sac, and fetal placenta tissues stained with specific antibodies against CD31, CD41, and c-Kit markers. The numbers present the percentage of specific population in viable single cells. Three to six embryos were analyzed for each tissue (mean ± SD). Significant differences are indicated with asterisks (*: p-value ≤ 0.05, **: p-value ≤ 0.005).

Hematopoietic cluster cell numbers are decreased in conditional KO embryos

To investigate whether intra-aortic hematopoietic clusters formation is affected by Hif1α, three-dimensional confocal microscopic imaging, using anti-c-Kit and anti-CD31 antibodies, was performed on E10 (32-34 sp) *+/+: HIF-1α$^{fl/fl}$ and VEC-Cre/+: HIF-1α$^{fl/fl}$* embryos. The vasculature throughout the cKO embryo appears normal, as observed by the pattern of CD31 expression. However, there is 20-40% decrease in the number of c-Kit$^+$ cells and there is a 40-50% decrease in the number of hematopoietic clusters in cKO embryos as compared to WT embryos (Figure 4). This data clearly indicates that in the cKO embryos, either the process of HPSC formation from *HIF1α*-deficient hemogenic endothelium is impaired, or the *HIF1α*-deficient HPSCs fail to expand normally after emergence, or both. This agrees with the results of our CFU-C and FACS analysis on embryonic hematopoietic tissues in which hematopoietic progenitor and stem cell populations are decreased in *VEC-Cre/+:HIF1α$^{fl/fl}$* embryos. Our findings suggest that HIF1α activity is essential for HPSC development and/or expansion from hemogenic endothelial cells.



	+/+:HIF1α$^{fl/fl}$	VEC-Cre/+:HIF1α $^{fl/fl}$	
	(33sp)	Embryo 1 (33sp)	Embryo 2 (32sp)
c-Kit^{+}cells	231	182	139
Cluster No.	66	42	38

Figure 4. Number of aortic hematopoietic clusters is reduced in *VEC-Cre/+:HIF1α$^{fl/fl}$* embryos. Confocal images of 32-34sp embryos stained with antibodies against c-Kit (green) and CD31 (magenta). Arrows indicate the hematopoietic clusters in the aorta. The table shows the number of c-Kit^{+} cells and clusters in WT and cKO embryos.

HIF1α-deficiency in VEC-expressing cells affects the robustness of AGM and placenta HSCs in transplantation assays

To test long-term repopulating ability of *HIF1α*-deficient HSC, we performed preliminary transplantation experiments. Two embryo equivalents (ee) of early E11 AGM cells were injected into irradiated adult recipients. Donor cell repopulation was examined in recipients at four months post-transplantation (Figure 5). All recipients with WT AGMs were successfully engrafted, however most of the cKO AGM tissues failed to engraft the irradiated recipients. Thus, the *HIF1α*-deficient AGM has no or very little HSC activity, suggesting either quantitatively lower HSC content or the malfunction/decreased robustness of HSCs, or both.

This result is consistent with our observations of the decreased hematopoietic progenitor activity, decreased phenotypic HPSCs, and decreased number of aortic hematopoietic clusters, which altogether suggest that HIF1α activity is essential for HPSC development and function in the embryo from hemogenic endothelial stage onward.

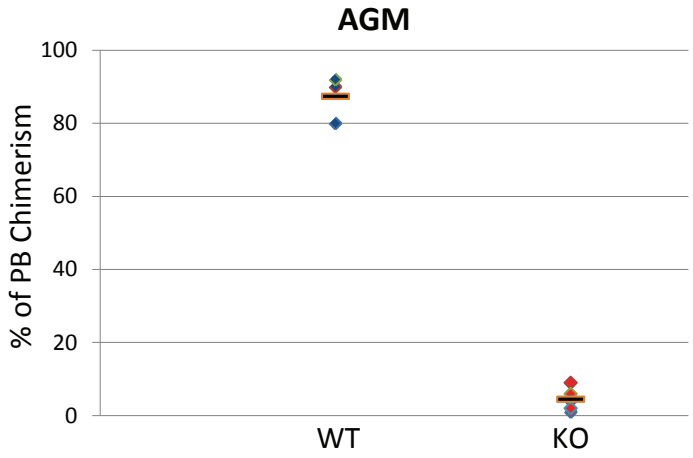
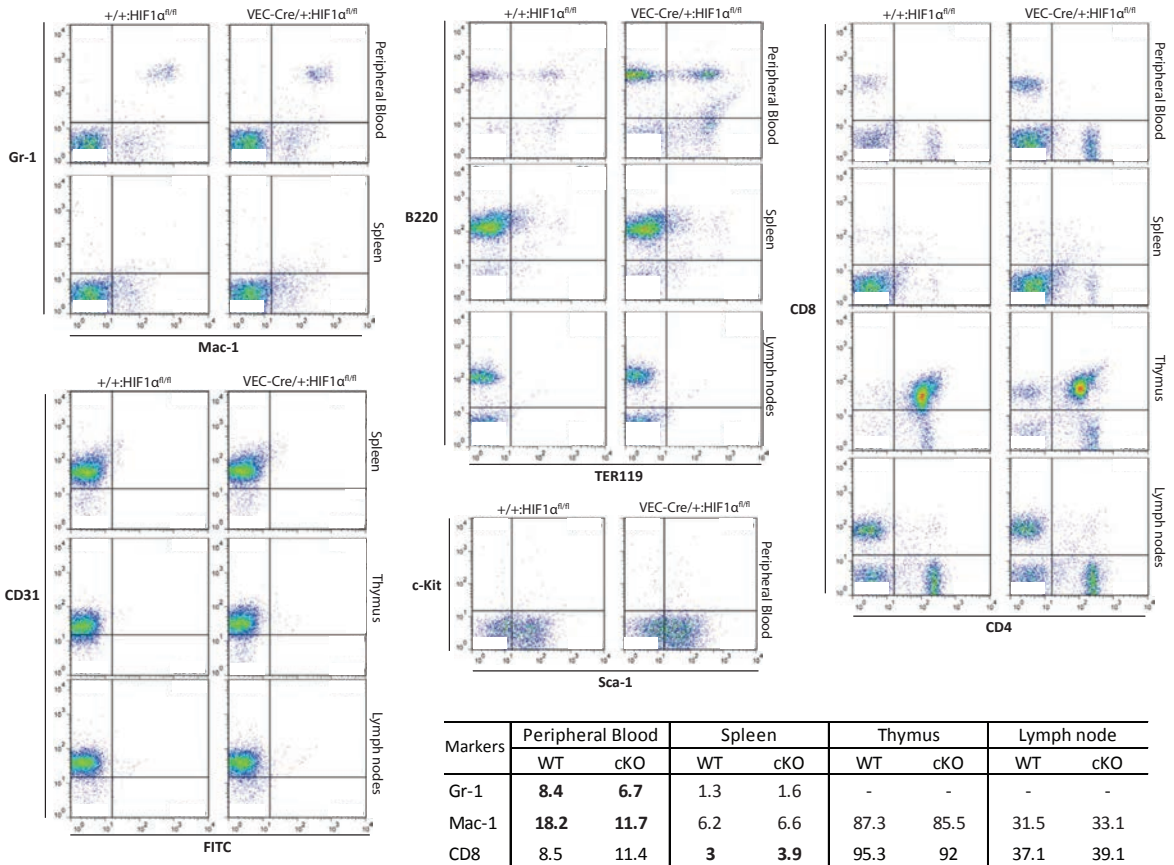


Figure 5. *HIF1α*-deficient HSCs provide less robust hematopoietic engraftment following in vivo transplantation. Long-term reconstitution of adult recipients of 2ee of AGM cells from 46-50sp WT and cKO mouse embryos was analyzed. PB chimerism was assessed by FACS analysis for Ly5.1/Ly5.2 markers 4 months post-transplantation. Three recipients of WT AGM cells and five recipients of KO AGM cells were analyzed. n=2.

HIF1α KO HSC function is defective under stress conditions in adult mice.

In parallel to studies of the embryo, we investigated hematopoiesis in adult *VEC-Cre/+:HIF1α$^{fl/fl}$* mice. Surprisingly, no phenotypic defects were observed in adult cKO animals compared to WT adults. FACS analysis of cells from different hematopoietic tissues, i.e. peripheral blood, spleen, lymph nodes and thymus, of WT and cKO adult animals showed no significant difference in frequency of different hematopoietic cell types under steady state conditions (Figure 6). Nevertheless, the following trends were consistently observed in all cKO analyzed mice as compared to age matched WT controls: In peripheral blood, a decrease in Gr-1$^+$ and Mac-1$^+$ cells and a small increase in B220$^+$, TER119$^+$, and c-Kit$^+$ cells was observed. In spleen, an increase in CD8$^+$ and CD4$^+$ cells was detected.

To assess whether the lack of HIF1α beginning at the earliest (VEC) stage of HSC development affects the repopulating potential of BM HSCs, lethally irradiated recipients were injected with either WT *(HIF1α$^{+/+}$)* or HIF1α-cKO (*HIF1α$^{Δ/Δ}$*) BM Lin$^-$ c-Kit$^+$ Sca1$^+$ (LSK) cells. Similarly, we also transplanted spleen cells of the adult mice. Four months-post transplantation, recipients of WT LSK cells showed on average 48% PB chimerism, whereas for the recipients of HIF1α-cKO cells the chimerim was 33%. Recipients of spleen WT and *HIF1α*-cKO cells showed 54% and 40% PB chimerism respectively (Figure 7A). Although the decreased level of chimerism by *HIF1α* cKO cells was consistently observed in all experiments, this decreased level was not statistically significant, as indicated by the standard t-test. Overall, one can conclude that *HIF1α*-cKO animals have HSCs with potential to repopulate lethally irradiated adult recipients, although this potential is less robust.



Markers	Peripheral Blood		Spleen		Thymus		Lymph node	
	WT	cKO	WT	cKO	WT	cKO	WT	cKO
Gr-1	**8.4**	**6.7**	1.3	1.6	-	-	-	-
Mac-1	**18.2**	**11.7**	6.2	6.6	87.3	85.5	31.5	33.1
CD8	8.5	11.4	**3**	**3.9**	95.3	92	37.1	39.1
CD4	9.1	10.9	**6.2**	**9.4**	-	-	-	-
B220	**61.6**	**66.2**	87.2	85.6	-	-	-	-
TER119	**25.7**	**39.8**	4.9	5.2	-	-	-	-
c-Kit	**1**	**1.2**	-	-	-	-	-	-
Sca-1	50.8	46.2	-	-	-	-	-	-
CD31	-	-	-	-	-	-	-	-

Figure 6. Multicolor flow cytometric analysis of adult hematopoietic tissues. Peripheral blood, spleen, thymus, and lymph nodes of adult *+/+:HIF1α$^{fl/fl}$* and *VEC-Cre/+:HIF1α$^{fl/fl}$* mice were analyzed with specific antibodies against CD4 and CD8 for T-cells, Gr-1, and Mac-1 for myeloid cells, B220 for B-cells, TER119 for erythrocytes, c-Kit and Sca-1 for immature hematopoietic cells, and CD31 for endothelial cells. The table summarizes the outcome of the FACS analysis. The numbers indicate percentage of cells for selected markers in the single viable cell fraction (n=3).

To assess the multi-lineage repopulating capacity of transplanted HSCs, we performed Ly5.1/Ly5.2 FACS analysis on thymus, spleen, bone marrow, and lymph nodes of some primary recipients that had similar chimerism levels (Figure 7B). *HIF1α*$^{\Delta/\Delta}$ HSCs from both BM and spleen reconstituted all hematopoietic tissues at a level comparable to that of WT cells. However, FACS analysis for BM LSK cells showed a significant reduction in the frequency and absolute number of LSK cells in recipients of *HIF1α*-cKO HSCs as compared to the recipients of WT cells (Figure 7C). This finding is consistent with the result reported by Takubo et al. which showed decreased chimerism in the BM LSK fraction of primary recipients of *HIF1α*-cKO LSK cells (Takubo et al., 2010). Hence, HIF1α is essential for maintaining a normal level of LSK cells in stress conditions (i.e. transplantation).

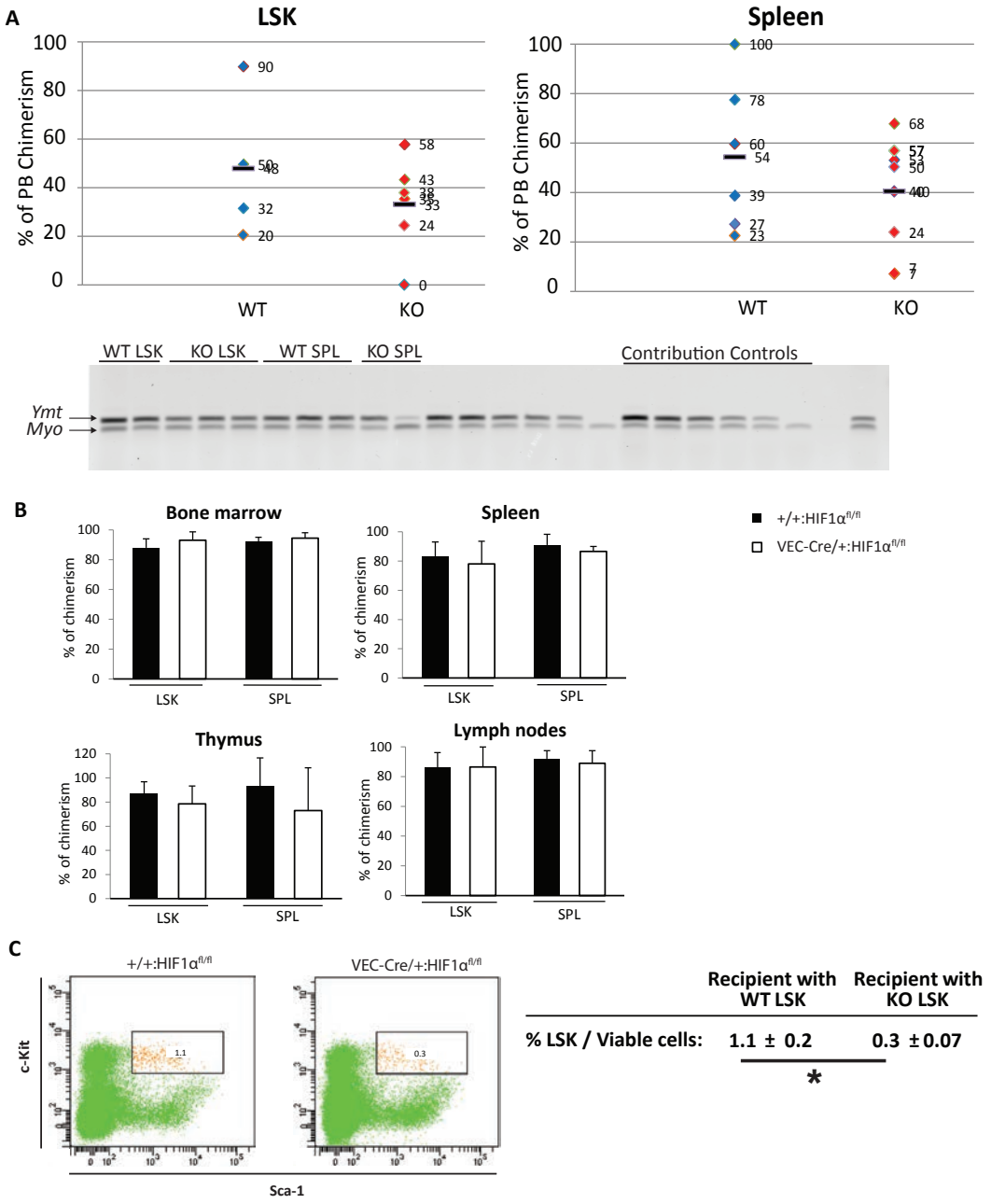


Figure 7. HIF1α is required for the maintenance of adult HSCs under stress condition. Repopulating and multi-lineage potential of *HIF1α*-cKO HSCs was tested by the in vivo transplantation assay. (A) Left panel: 1500 LSK cells isolated from two *+/+:HIF1α$^{fl/fl}$* and three *VEC-Cre/+:HIF1α$^{fl/fl}$* adult male mice were injected into 4 and 6 female recipients, respectively. Four months post-transplantation PB chimerism was tested by *Ymt* PCR. Right panel: The same experiment was performed with 2 x 10^6 spleen cells from WT and cKO animals injected into 6 and 9 female recipients, respectively. Bottom panel: Representative semi-quantitative PCR analysis for *Ymt* gene in the DNA obtained from the recipients' peripheral blood. *Ymt* and *Myo* bands represent the donor and myogenin normalization DNA fragments, respectively. (B) Multi-lineage analysis of bone marrow, spleen, thymus, and lymph nodes from primary recipients of *HIF1α$^{+/+}$* (closed bars) or *HIF-1α$^{Δ/Δ}$* (open bars) HSCs 6 months post-transplantation (mean ± SD) (n=2). (C) LSK cell analysis from primary recipients of *HIF1α$^{+/+}$* and *HIF-1α$^{Δ/Δ}$* LSK cells. A representative FACS plot for each genotype is shown. The mean ±SD of the percentages of LSK cells in the recipients are summarized at the bottom (n=2). Significant differences are indicated with asterisks (*: p-value ≤ 0.05).

HIF1α is required for cell cycle regulation of HSCs

To test whether the self-renewal capacity of *HIF1α*$^{\Delta/\Delta}$ HSCs is affected, secondary transplantations were performed. LSK cells from primary recipients of WT and *HIF1α*$^{\Delta/\Delta}$ LSK cells with similar level of chimerism were injected into lethally irradiated secondary recipients. In addition to that, the same experiment was carried out with the BM mononuclear cells of primary recipients. Surprisingly, PB of secondary recipients of *HIF1α*-cKO BM and LSK cells showed a higher chimerism level at four months post-transplantation (Figure 8A-B). PCR analysis on DNA samples obtained from peripheral blood of primary and secondary recipients of LSK/BM cells showed that the recombination efficiency is high in most of the recipients (70-100%), although as shown in Table 2, a few had a lower efficiency of recombination (45-70%) that may explain some of the differences in chimerism.

To search for a cause for this unexpected increase in chimerism, we examined the cell cycle status (by Ki-67 staining) of the LSK HSCs in the secondary recipients of WT and *HIF1α*-cKO HSCs. A significant decrease in G_0 and increase in G_2/M phase was observed in *HIF1α*-cKO HSC compared to WT cells (Figure 8C). This result indicates that loss of *HIF1α* in HSCs leads to entry into the cell cycle and enhanced proliferation of HSCs. Therefore, HIF1α has a role in cell cycle regulation of HSCs in stress conditions.

Hematopoietic progenitor activity is normal in HIF1α-cKO bone marrow cells.

To evaluate the hematopoietic progenitor capacity of adult cKO animals, we performed CFU-C assay on BM cells. A slight reduction (1.2 fold) in CFU-C frequency was observed in *HIF1α*-cKO BM cells as compared to WT cells, although it was not significant (Figure 9). The mice used in this experiment were nine months old and the BM cellularity of aged cKO animals is significantly decreased by 3.7 fold (Figure 9). As a result, there is no change in the absolute number of CFU-C of cKO mice as compared to WT mice. Based on these results, we conclude that 1) hematopoietic progenitor potential of adult BM cells is comparable to WT; 2) HIF1α has a role in maintaining the BM cellularity of adult mice under aging/stress conditions.

Discussion

In the present study, we demonstrated for the first time, that the loss of *HIF1α* during the earliest stages of definitive HPSC generation leads to defects in hematopoietic progenitor and stem cell generation and growth in embryonic tissues. By using *VEC-Cre* to delete *HIF1α* specifically in endothelial cells (in contrast to previous *Mx1Cre* studies in which *Hlf1α* is ubiquitously deleted) we have shown that HIF1α regulates HPSCs during and after their generation. Deletion of *HIF1α* in VEC expressing cells in the embryonic aorta and vitelline/umbilical arteries leads to a reduction in the number of hematopoietic progenitors, the frequency of phenotypic

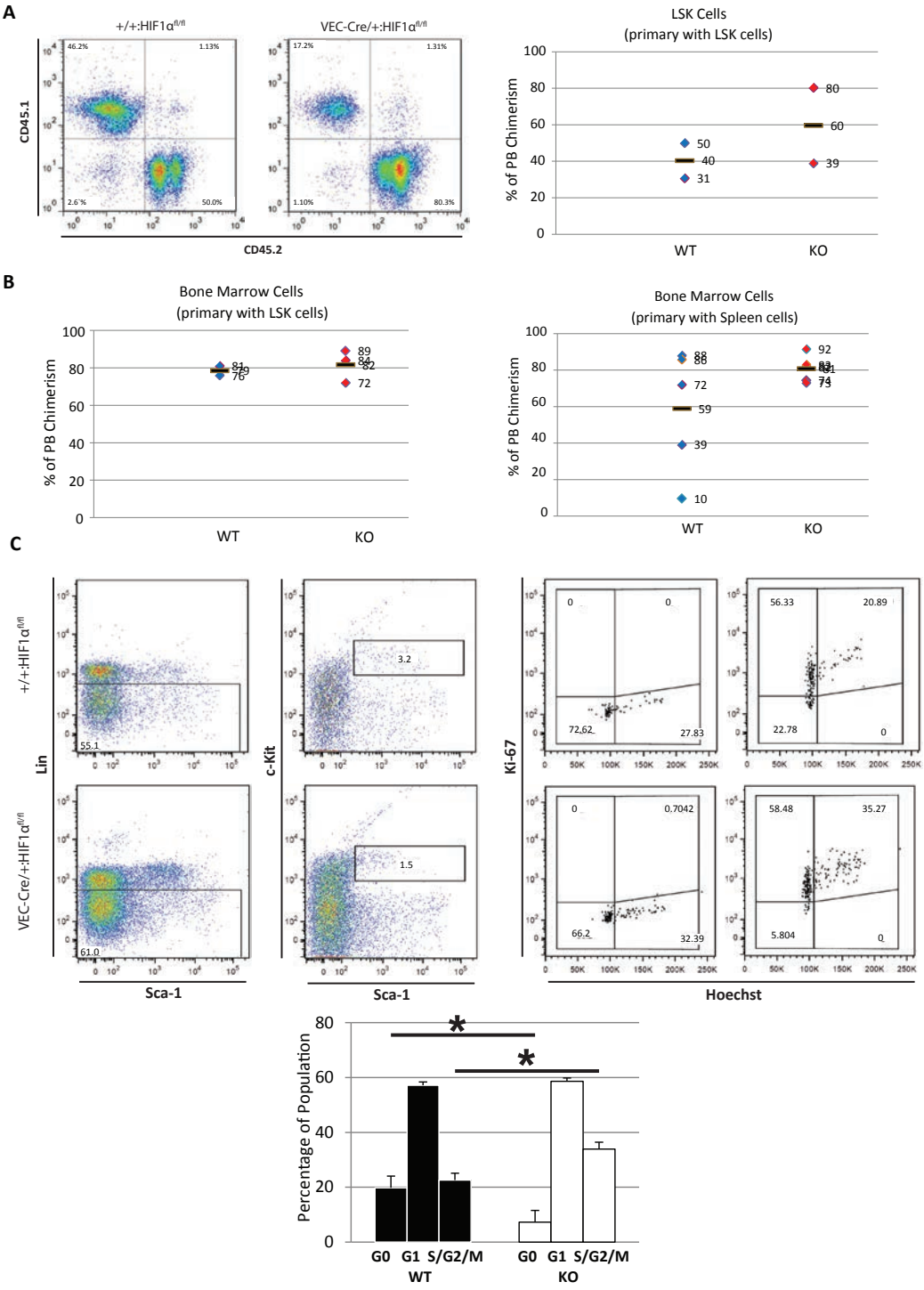


Figure 8. ***HIF1α*-cKO HSCs show a defect in proliferation.** PB chimerism assessed by FACS in secondary recipients 4 months post-transplantation. (A) Left panel: Representative flow cytometric plots of CD45.1/CD45.2 analysis of the secondary recipients of *HIF1α*$^{+/+}$ or *HIF1α*$^{Δ/Δ}$ LSK cells. Right panel: 600 *HIF1α*$^{+/+}$ or *HIF1α*$^{Δ/Δ}$ LSK cells from primary recipients of LSK cells injected into four Ly5.1 recipients. (B) Left panel: 2 x 10^6 ficoll fractionated BM cells from primary recipients of LSK cells injected into five Ly5.1 recipients (bottom panel). Right panel: 2 x 10^6 ficoll fractionated BM cells from primary recipients of *HIF1α*$^{+/+}$ or *HIF1α*$^{Δ/Δ}$ spleen cells injected into nine Ly5.1 recipients. (D) Cell cycle analysis by Ki-67 flow cytometry in LSK fractions from secondary recipients of *HIF1α*$^{+/+}$ or *HIF1α*$^{Δ/Δ}$ LSK cells. Representative FACS plots shown. The bar chart at the bottom shows the mean ± SD of the data. Significant differences are indicated with asterisks (*: p-value ≤ 0.05).

Table 2. Recombination efficiency as analysed in the primary recipients of LSK cells and secondary recipients of LSK/BM cells.

Primary Recipients of KO LSK Cells	
% of PB Chimerism	% of PB Recombination
58	93
43	86
0	52.1
35	96
24	90
38	92

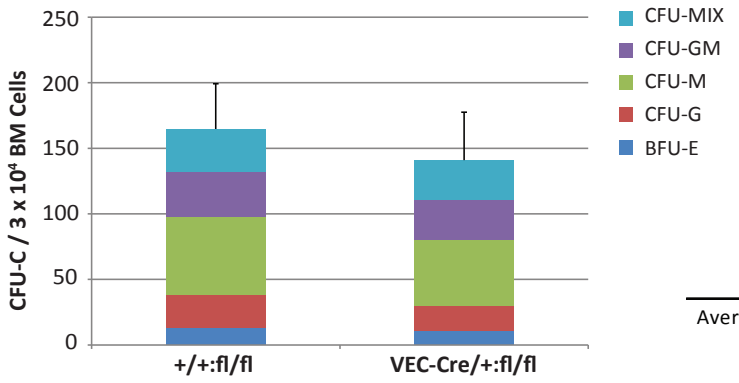
Secondary Recipients of KO LSK Cells (primary with LSK cells)	
% of PB Chimerism	% of PB Recombination
39	48
80	46

Secondary Recipients of KO BM Cells (primary with LSK cells)	
% of PB Chimerism	% of PB Recombination
72	93
84	91
89	86

Primary Recipients of KO Spleen Cells	
% of PB Chimerism	% of PB Recombination
53	96
7	2
68	85
40	98
57	100
7	70
50	97
24	91
57	100

Secondary Recipients of KO BM Cells (primary with spleen cells)	
% of PB Chimerism	% of PB Recombination
74	98
82	99
73	98
92	100

The percentages of recombination for the KO alleles are calculated by DNA analysis from PB of the recipients. PCR assay was performed with the primers defined in Figure 2C to detect WT, floxed (fl), and recombined (Δ) *HIF1α* alleles. The intensity of the band of each fragment was measured by Quantum Image software, the ratio was calculated and the percentage of recombination was calculated by dividing the Δ band intensity by the summation of Δ, fl, and WT band intensities, i.e. Δ /(Δ+fl+WT).



Total BM Cells	
WT	KO
7.9×10^7	2.7×10^7
5.8×10^7	2.4×10^7
9.0×10^7	1.4×10^7
-	1.6×10^7
Average: $7.6 \times 10^7 \pm 1.6 \times 10^7$	$2.0 \times 10^7 \pm 0.6 \times 10^7$

**

Figure 9. CFU-C data of bone marrow cells from *+/+:HIF1α$^{fl/fl}$* and *VEC-Cre/+:HIF1α$^{fl/fl}$* mice. 3 x 10^4 BM cells from WT and cKO mice were cultured in methylcellulose medium in triplicate plates. The data is averaged over 3 WT and 4 cKO mice. On the left, the number of total BM cells of each animal is shown (mean ± SD). Significant differences are indicated with asterisks (**: p-value ≤ 0.005).

HPSCs, the number of aortic hematopoietic clusters/c-Kit$^+$ cells, and the reduction/absence of functional stem cell activity of embryonic hematopoietic tissues. We have shown that adult BM HSCs are also affected. Under stress conditions of serial transplantation, *VEC-Cre HIF1α*$^{-/-}$ BM HSCs undergo an unscheduled entry into the cell cycle and proliferate. Hence, HIF1α plays multiple roles in regulating HPSC throughout all stages of development.

The major embryonic vasculature is known to be the source of all definitive HPSC, as shown by lineage tracing experiments with *VEC-Cre* induced marker recombination ((Chen et al., 2009; Zovein et al., 2008). *VEC-Cre* expression and activity begins at about E7.5 in blood islands of yolk sac, chorionic mesoderm, and vitelline artery. At E9.5, *VEC-Cre* activity is observed in the whole vasculature including aorta and umbilical artery. As definitive progenitor cells and HSCs are first detected at E8.5 and E10.5 respectively in the aorta, vitelline and umbilical arteries, the loss of *HIFα* is likely to affect their generation from the subset of hemogenic endothelial cells lining these vessels. Whole embryo confocal imaging showed clear quantitative decreases in vascular hematopoietic clusters, suggesting that either the endothelial-to-hematopoietic transition is affected or the growth/expansion of the emerging HPSCs is affected. The receptor tyrosine kinase Flk1 is known to be expressed on endothelial cells of the midgestation vaculature and moreover, on emerging aortic hematopoietic cluster cells (Yokomizo and Dzierzak, 2010). Since *VEGF* is a direct target of HIF1α, it is likely that the activation of this signaling pathway may be affected in Flk1 expressing cells in the absence of HIF1α. Whereas, Flk1 is known to play an essential role in vasculogenesis and vascular permeability, we found no morphological defects in the vasculature of cKO embryos by 3-D confocal imaging. It is as yet unclear what specific process(es) in the endothelial to hematopoietic transition is (are) affected by *HIF1α* deficiency.

Our analysis of *VEC* cKO adult mice showed that HIF1α also plays a role in regulating BM HSC function under stress conditions. Steady state hematopoiesis in adult cKO mice appears to be normal. The differentiation capacity of cells in the adult hematopoietic tissues was maintained, but HSCs were slightly less robust in the reconstitution of irradiated recipients. The effects of *HIF1α* deficiency were only revealed upon examination of the BM of the primary recipients – HSC content (LSK cells) as measured by flow cytometric analysis was significantly decreased. Transplantation of these *VEC HIF1α*$^{\Delta/\Delta}$ BM HSCs into secondary recipients resulted in higher peripheral blood donor cell chimerism, as compared to WT control secondary recipients. We found that there were fewer quiescent and more cycling HSCs in the LSK fraction of BM of *VEC HIF1α*$^{\Delta/\Delta}$ secondary recipients as compared to WT.

Self-renewal and differentiation potential are two defining characteristics of hematopoietic stem cells. Despite the fact that HSCs divide and differentiate to progenitors, a primary property of these cells is their ability to remain in a quiescent state, i.e. non-dividing G_0 state of the cell cycle. It has been shown that almost 75% of bone marrow LTR-HSCs are quiescent and less than 5% of them are actively cycling (in S/G_2/M phase) (Simon et al., 2002; Wilson et al., 2008). The balance between proliferation and quiescence of HSCs is highly regulated and maintaining the HSCs in a quiescent state is crucial for their long-term repopulating function

(Jang and Sharkis, 2007). Quiescent HSCs are maintained in a hypoxic niche at a lower oxidative stress to prevent their differentiation and exhaustion (Hooper et al., 2009). LTR-HSCs utilize cytoplasmic glycolytic metabolism that is associated with HIF1α up-regulation (Semenza, 2007). HIF1α, as the master regulator of hypoxia, modulates HSC cycling and its deficiency leads to loss of quiescent HSCs in the BM of adult mice. As shown by Takuba and colleagues in studies of adult *MX1-Cre:HIF1α*$^{fl/fl}$ mice, HIF1α is required to maintain normal number of HSCs. Under stress condition such as serial transplantations, aging, and myelosuppression, *HIF1α* deficient HSCs lose their quiescence (Takubo et al., 2010). Our observations for adult BM HSCs are in general agreement with the published study and support the fact that HIF1α is essential for HSC regulation.

One slight discordancy in the results of the two studies is that we observed a higher chimerism level in the secondary recipients as compared to the primary recipients, whereas the *MX1-Cre* study showed more the chimerism in primary recipients. It should be noted that the two studies differ in the developmental time and tissues in which *HIF1α* is deleted: In *MX1-Cre:HIF1α*$^{fl/fl}$ mice, the deletion process takes place in adult mice and the deletion of floxed gene occurs in some non-hematopoietic tissues such as heart and kidney (Lee and Simon, 2012). In *VEC-Cre:HIF1α*$^{fl/fl}$ mice, *HIF1α* deletion is initiated in early stages of embryonic development when *VEC-Cre* expression begins specifically in endothelial cells. The deletion of the targeted gene is restricted to endothelial and hematopoietic cells. Moreover, the recombination efficiencies differ. Although the recombination efficiency in *MX1-Cre:HIF1α*$^{fl/fl}$ study was not tested, another study showed an almost 100% deletion of the targeted allele in the spleen by *MX1-Cre* (Lee and Simon, 2012). We observed 80-90% recombination efficiency in the donor BM cells in primary transplantation assay. Thus, the chimerism differences between our study with *VEC-Cre* mediated *HIF1α* deletion and the *MX1-Cre* study may be accounted for by an escaped fraction of non-recombined cells in our study. Alternatively, HIF1α is redundant during development in *VEC-Cre:HIF1α*$^{fl/fl}$ mice and is compensated by other regulatory factors such as HIF2α. It has been shown that HIF2α is abundantly expressed in Lin^{-} cells of BM (Takubo et al., 2010), although its role is not clear yet.

In conclusion, HIF1α plays different roles in hematopoiesis during embryonic and adult stages. HIF1α is required for HPSC generation and/or expansion during embryonic development. The most important time point for HIF1α activity is E10 which corresponds to the time when HPSC emerges from hemogenic endothelium in AGM region. In adult mice, HIF1α is dispensable for HSC function under steady state conditions. However, under stress conditions (transplantation, aging) HIF1α is required to maintain the normal number of HSCs and keep them in a quiescent state. Understanding the precise role of HIF1α and physiological weak oxygenation, or “in situ normoxia,” in regulating hematopoiesis can broaden our view of in vitro gene manipulation and culturing conditions for hematopoietic stem cells that may allow the efficient ex vivo expansion of these cells for clinical applications.

Material and methods

Mice: strains and generation

The background strain of the transgenic animals used in this study was C57BL/6. The transplantation recipients were either Ly5.1 or C57BL/6 adult mice (8-20 weeks). *HIF1α$^{fl/fl}$* mice (Ryan et al., 1998) were purchased from Jackson Laboratories. *VEC-Cre* mice were kindly provided by Dr. Nancy Speck's group from University of Pennsylvania (Iyer et al., 1998). To obtain *VEC-Cre/+:HIF1α$^{fl/fl}$* animals, the *VEC-Cre/+* mice were crossed to *HIF1α$^{fl/fl}$* mice and the resulting *VEC-Cre/+:HIF1α$^{fl/+}$* offspring were crossed to *HIF1α$^{fl/fl}$* mice. The animals' genotypes were determined by DNA PCR. All animal procedures were carried out in compliance with Standards for Care and Use of Laboratory Animals.

Embryo generation and cell preparation

Mouse embryos were generated from timed matings. The day of vaginal plug was counted as day 0. Pregnant mice were sacrificed by cervical dislocation and the embryos were harvested. Since embryos within the same litter can be of different developmental stages, the number of somite pairs was used to stage embryos. AGM, yolk sac, placenta (fetal part), and fetal liver were dissected as described in (Robin and Dzierzak, 2005). Dissections were performed quickly to preserve the viability of the tissues.

For obtaining single cell suspensions of embryonic materials, tissues were collagenase (0.125%) treated for 45 minutes at 37°C (except for FL whose dissociation was performed with pipetting), washed with phosphate-buffered saline PBS (ginco Inc.), 10% heat-inactivated filtered fetal calf serum (FCS) (GibcoBRL), penicillin/streptomycin (PS) (PBS/FCS/PS), and collected in the same medium (Robin and Dzierzak, 2005). For placenta, the fetal part was treated with collagenase for 1 hour, the cells were washed and ficoll fractionated, and mononuclear cells were collected in PBS/FCS/PS and filtered with a nylon 40µm strainer filter. The YS was filtered as well. Next, the viable cells were counted by trypan blue in counting chamber and kept in PBS/FCS/PS at 4°C for further procedures. For transplantations and CFU-C experiments the embryos were genotyped by KAPA fast genotyping kit (Kappa Biosystems).

For adult mice, the bone marrow was harvested from tibias and femurs of animals by flushing with Strican 0.50X16mm syringe and filtered with strainer filter. Next, the cells were ficoll fractionated and washed in PBS/FCS/PS. After collection, peripheral blood erythrocytes were lysed (lysing buffer from BD Biosceince) and remaining leukocytes were washed in PBS/FCS/PS. The spleen, thymus, and lymph nodes were crushed through cell strainer and washed in PBS/FCS/PS. Cells were counted and were used for progenitor colony assay, flow cytometry analysis, and transplantations.

In vitro hematopoietic progenitor assay

Single cell suspensions from AGM, placenta, YS, and FL of E9, E10, and E11 embryos were plated at various dilutions in triplicate in methylcellulose medium (Methocult GF m3434, Stem Cell Technologies Inc.) with 1% PS. The plates were incubated at 37°C in a humidified chamber under 5% CO_2 for 12 days. Different hematopoietic colonies were distinguished by morphology and counted with an inverted microscope. The same procedure was performed for adult BM cells.

Flow cytometry analysis and sorting

For embryonic tissues, the cells were stained with anti-CD31 PE-Cy7, anti-c-Kit APC, and anti-CD41 PE antibodies, and incubated for 30 minutes at 4°C in the dark, and then washed in PBS/10%FCS. For adult tissues, the cells were stained with anti-Mac-1 FITC, anti-Gr-1 PE, anti-CD4 PE, anti-CD8 PE, anti-c-Kit PE, anti-Sca-1 FITC, anti-B220 PE, and anti-TER119 FITC, and then incubated as described above. For LSK sorting, BM cells were stained with mouse anti-lineage PE antibody cocktail (CD3e, CD45R/B220, Ly-6G/Gr-1, CD11b/Mac-1, and TER119/Ly76). To investigate the chimerism level in the recipient mice, the cell suspensions from hematopoietic tissues were stained with anti-CD45.1 PE and anti-CD45.2 FITC antibodies. All the antibodies were obtained from BD Pharmingen. Dead cells were eliminated by Hoechst 33258 labeling (Molecular Probes). Flow cytometric analysis and sorting was performed using a FACS-Sorp, FACSAriaIII or FACSAriaII cell sorter (BD Biosciences).

Whole-mount immunostaining

Whole-mount immunohistochemistry was performed as described previously (Yokomizo and Dzierzak, 2010; Yokomizo et al., 2011). Embryos were stained with biotinylated rat anti-mouse anti-CD31 (MEC13.3, BD Biosciences) and rat anti-mouse anti-c-Kit (2B8, BD Biosciences). Secondary antibodies were streptavidin-Cy3 (Jackson ImmunoResearch) and goat anti-rat IgG-Alexa647 (Invitrogen), respectively. Samples were analyzed with a confocal Zeiss LSM 510 Meta microscope.

In vivo transplantation assay

Female recipient mice were sub-lethally irradiated (split dose of 9 Gy of γ-irradiation, 2.5 hours apart) and received the test cells via tail vein injection. All C57BL/6 donor cells were of the Ly5.2 type (contained the CD45.2 allele). Most recipients were of the Ly5.1 type, allowing assessment of donor cell engraftment FACS analysis. We used donor cells from male animals

in the case of Ly5.2 recipients and then assessed donor chimerism by semiquantitative PCR for the *Ymt* gene. The percentage of chimerism was calculated by a standard curve of control DNA dilutions with 0% to 100% donor marker DNA. Myogenin PCR was performed for DNA quantity normalization. Recipients were considered repopulated when the donor chimerism was ≥ 10%.

For transplantations of embryonic cells, 2ee of AGM cells or 1ee of placenta cells were co-transplanted with 2 x 10^5 spleen cells (recipient background) to support short-term survival. Cells were intravenously injected into the tail vein of recipients and analyzed for donor cell hematopoietic contribution at four months post-transplantation. For transplantation of adult cells, 2 x 10^6 spleen cells or 1500 LSK cells were co-transplanted with 3 x 10^5 BM mononuclear cells. Primary recipients were analyzed four months post-transplantation for multilineage donor cell chimerism in the bone marrow, thymus, spleen, and thymus. For secondary transplantation, 2 x 10^6 ficoll fractionated BM cells or 600 LSK cells from primary recipients were co-injected with 2 x 10^5 spleen cells (recipient background) into recipients. Blood and BM was analysed at 4 months post-transplantation.

Cell cycle analysis

For cell cycle analysis, harvested BM cells were stained with anti-lineage PE antibody cocktail as described before. The Lin^- cells were sorted and stained for anti-c-Kit APC and anti-Sca-1 PE-Cy7. Next, the cells were fixed with 2% paraformaldehyde for 1 hour, washed, and incubated overnight in 0.2% Triton. The cells were washed and incubated with KI76-FITC for 2 hours. IgG-FITC was used for the negative control (FITC mouse anti-Ki-67 set from BD pharmigen). Next, the cells were washed and incubated with Hoechst 33258 for a maximum of 2 hours. The cells were analyzed by FACS.

Statistical analysis

Data are presented as mean ± SD. Differences were considered to be significant at p-value ≤0.05 calculated by Student's t-test.

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References

Adelman, D.M., Maltepe, E., and Simon, M.C. (1999). Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes & development *13*, 2478-2483.

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*, 841-846.

Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E., and Speck, N.A. (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature *457*, 887-891.

Cipolleschi, M.G., Dello Sbarba, P., and Olivotto, M. (1993). The role of hypoxia in the maintenance of hematopoietic stem cells. Blood *82*, 2031-2037.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P., and Simon, M.C. (2005a). Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Molecular and cellular biology *25*, 10479-10491.

Cowden Dahl, K.D., Robertson, S.E., Weaver, V.M., and Simon, M.C. (2005b). Hypoxia-inducible factor regulates alphavbeta3 integrin cell surface expression. Molecular biology of the cell *16*, 1901-1912.

Draenert, K., and Draenert, Y. (1980). The vascular system of bone marrow. Scanning electron microscopy, 113-122.

Dunwoodie, S.L. (2009). The role of hypoxia in development of the Mammalian embryo. Developmental cell *17*, 755-773.

Gerber, H.P., Malik, A.K., Solar, G.P., Sherman, D., Liang, X.H., Meng, G., Hong, K., Marsters, J.C., and Ferrara, N. (2002). VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature *417*, 954-958.

Harrison, J.S., Rameshwar, P., Chang, V., and Bandari, P. (2002). Oxygen saturation in the bone marrow of healthy volunteers. Blood *99*, 394.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K.*, et al.* (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell stem cell *4*, 263-274.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., and Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Molecular and cellular biology *23*, 9361-9374.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y.*, et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes & development *12*, 149-162.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Keith, B., Johnson, R.S., and Simon, M.C. (2012). HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nature reviews Cancer *12*, 9-22.

Koh, M.Y., and Powis, G. (2012). Passing the baton: the HIF switch. Trends in biochemical sciences *37*, 364-372.

Kubota, Y., Takubo, K., and Suda, T. (2008). Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. Biochemical and biophysical research communications *366*, 335-339.

Lee, K.E., and Simon, M.C. (2012). From stem cells to cancer stem cells: HIF takes the stage. Current opinion in cell biology *24*, 232-235.

Levesque, J.P., Winkler, I.G., Hendy, J., Williams, B., Helwani, F., Barbier, V., Nowlan, B., and Nilsson, S.K. (2007). Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. Stem Cells *25*, 1954-1965.

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

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proceedings of the National Academy of Sciences of the United States of America *104*, 5431-5436.

Plate, K.H., Breier, G., Weich, H.A., and Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature *359*, 845-848.

Ramirez-Bergeron, D.L., Runge, A., Adelman, D.M., Gohil, M., and Simon, M.C. (2006). HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. Developmental cell *11*, 81-92.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Molecular and cellular biology *25*, 5675-5686.

Rehn, M., Olsson, A., Reckzeh, K., Diffner, E., Carmeliet, P., Landberg, G., and Cammenga, J. (2011). Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. Blood *118*, 1534-1543.

Robin, C., and Dzierzak, E. (2005). Hematopoietic stem cell enrichment from the AGM region of the mouse embryo. Methods in molecular medicine *105*, 257-272.

Robin, C., Ottersbach, K., Boisset, J.C., Oziemlak, A., and Dzierzak, E. (2011). CD41 is developmentally regulated and differentially expressed on mouse hematopoietic stem cells. Blood *117*, 5088-5091.

Ruas, J.L., and Poellinger, L. (2005). Hypoxia-dependent activation of HIF into a transcriptional regulator. Seminars in cell & developmental biology *16*, 514-522.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. The EMBO journal *17*, 3005-3015.

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*, 513-525.

Schofield, C.J., and Ratcliffe, P.J. (2005). Signalling hypoxia by HIF hydroxylases. Biochemical and biophysical research communications *338*, 617-626.

Semenza, G.L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. Science's STKE : signal transduction knowledge environment *2007*, cm8.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by

hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Simon, M.C., and Keith, B. (2008). The role of oxygen availability in embryonic development and stem cell function. Nature reviews Molecular cell biology *9*, 285-296.

Simon, M.C., Ramirez-Bergeron, D., Mack, F., Hu, C.J., Pan, Y., and Mansfield, K. (2002). Hypoxia, HIFs, and cardiovascular development. Cold Spring Harbor symposia on quantitative biology *67*, 127-132.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell stem cell *7*, 391-402.

Tanaka, T., Wiesener, M., Bernhardt, W., Eckardt, K.U., and Warnecke, C. (2009). The human HIF (hypoxia-inducible factor)-3alpha gene is a HIF-1 target gene and may modulate hypoxic gene induction. The Biochemical journal *424*, 143-151.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes & development *11*, 72-82.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America *92*, 5510-5514.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W.*, et al.* (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *17*, 271-273.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Yokomizo, T., and Dzierzak, E. (2010). Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. Development *137*, 3651-3661.

Yokomizo, T., Ng, C.E., Osato, M., and Dzierzak, E. (2011). Three-dimensional imaging of whole midgestation murine embryos shows an intravascular localization for all hematopoietic clusters. Blood *117*, 6132-6134.

Yoon, D., Pastore, Y.D., Divoky, V., Liu, E., Mlodnicka, A.E., Rainey, K., Ponka, P., Semenza, G.L., Schumacher, A., and Prchal, J.T. (2006). Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. The Journal of biological chemistry *281*, 25703-25711.

Zhang, J., Grindley, J.C., Yin, T., Jayasinghe, S., He, X.C., Ross, J.T., Haug, J.S., Rupp, D., Porter-Westpfahl, K.S., Wiedemann, L.M.*, et al.* (2006). PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature *441*, 518-522.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C.*, et al.* (2008). Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell stem cell *3*, 625-636.

Chapter 5

General Discussion and Future Prospects

The work in this thesis focused on the development of the adult hematopoietic system and the placenta hematopoietic supportive niche in both mouse and human. We showed that human placenta contains hematopoietic progenitor and stem cells (HPSCs) continuously through different stages of development until term. Human placenta stromal cells were found to be pericyte-like cells and were found to support human hematopoiesis. Therefore, we conclude that human placenta is a potent niche that supports HPSCs. We also showed that mouse placenta stromal cells support the expansion of bone marrow (BM) hematopoietic cells and hematopoietic progenitors. In our evaluation of the role of hypoxia, as a niche factor, we showed that hypoxic culture of placental stromal cell lines interferes with their hematopoietic supportive and differentiative capacity. By conditionally deleting the *HIF1α* gene (the major regulator of hypoxia) in endothelial cells at early stages of hematopoietic development, we demonstrated that in the developing embryo HIF1α regulates the HPSCs during and after their emergence from vasculature. HIF1α-deficiency in the embryonic aorta and vitelline/umbilical arteries results in a decrease in the number of hematopoietic progenitors, the frequency of phenotypic HPSCs, the number of aortic hematopoietic clusters/c-Kit^{+} cells, and the reduction/absence of functional HSCs in embryonic hematopoietic tissues. In adulthood, we found that HIF1α is dispensable for HSC regulation under steady state, but is essential in cell cycle regulation and maintaining the normal content of HSCs under stress conditions of serial transplantations. These observations demonstrate that HIF1α plays multiple roles during mouse development, being important in HPSC emergence, in the placental niche for HSPC maintenance and in adult BM HSC under stress conditions.

HSCs and their niche in human placenta

Previously, it has been shown in the mouse embryo that the development of the hematopoietic system takes place in various anatomical sites. The first definitive adult-repopulating hematopoietic stem cells (HSCs) are generated in the aorta-gonad-mesonephros (AGM) region of the E10.5 embryo (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Hematopoietic cells emerge from endothelial cells (expressing vascular endothelial cadherin (VEC)) lining the major vasculature of the midgestation mouse embryo (Chen et al., 2009; Ivanovs et al., 2011). Confocal live-imaging of the dorsal aorta has demonstrated the real-time emergence of hematopoietic cells from endothelial cells lining the ventral part of dorsa aorta called “hemogenic endothelium” (Boisset et al., 2010). Later at E11-11.5, HSCs appear in the yolk sac (YS), placenta, and fetal liver (FL) (Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). Thereafter, FL remains the main hematopoietic tissue for colonization and expansion of HSCs until birth. Prior to birth, HSCs start to migrate to the bone marrow (BM), where they reside throughout adulthood (Christensen et al., 2004).

Mesenchymal cells from these mouse embryonic tissues provide a hematopoietic supportive microenvironment. It has been shown that mesenchymal stromal cell (MSC) lines derived from AGM, YS, and FL support hematopoietic progenitor and stem cells (HPSCs)

in vitro (Moore et al., 1997; Oostendorp et al., 2002a; Oostendorp et al., 2002b; Yoder et al., 1994). The fact that the E12 mouse placenta harbors many more HPSCs than the AGM region (Gekas et al., 2005) suggests the placenta provides a robust hematopoietic niche during development. Wang and colleagues showed that placental stromal cells support the expansion of BM hematopoietic cells and progenitors appreciably more than AGM, FL, or BM stromal cells (Wang et al., 2011b). These observations encouraged us to examine the human placenta for existence of HSCs throughout its developmental stages, and also to study stromal cells derived from mouse and human placenta to extend our knowledge of signals involved in HSC development in this promising niche.

We showed that the human placenta contains all types of hematopoietic progenitors throughout development, and more importantly that it contains *bona fide* HSCs starting at week 6 of gestation until term. This was an unexpected result since previous data in the mouse term placenta had shown almost no adult repopulating HSCs were present (Gekas et al., 2005). CFU-GM (colony-forming unit-granulocyte macrophage) and CFU-Mix progenitors were found in the $CD34^-$ fraction of placenta cells at week 6 and then switched to the $CD34^+$ fraction by week 15. Similarly, HSCs were in both $CD34^+$ and $CD34^-$ fractions of week 6 placentas. Later at week 16-20, HSCs were more enriched in the $CD34^+$ fraction. This data suggests the developmental regulation of the CD34 marker on HPSCs, or the generation of discrete CD34+ and CD34- subsets of hematopoietic cells. Interestingly, we found that the HSC content of the human placenta (when using the three enzyme treatment to dissociate cells from the tissue) reached almost 10% of the number of HSCs contained in one unit of umbilical cord blood (UCB) (Bhatia et al., 1997; Wang et al., 1997). Thus, the human placenta, similar to mouse placenta, is replete with potent HSCs that can be considered as an additional source for HPSCs together with UCB for banking and potential clinical use. Obviously, the cost-effectiveness and speed of harvesting HSCs from human placenta needs to be improved. Thus, this routinely discarded tissue is now appreciated as a new source of HSCs with potential use clinically and in studies of the signals and factors regulating HPSC generation/growth and function.

In our examination of the placenta microenvironment, we found that placenta stromal cells express classical MSC markers and showed the mesenchymal differentiation potential of human placental stromal cells along osteo- and adipogenic lineages (beginning at gestational week 6 onward). These results are in agreement with previously reported placenta MSC cell lines (Fukuchi et al., 2004; Igura et al., 2004; Li et al., 2005; Miao et al., 2006; Parolini et al., 2008; Portmann-Lanz et al., 2006; Wulf et al., 2004; Yen et al., 2005; Zhang et al., 2006). Placenta stromal cells are pericyte-like cells as they expressed CD146 and NG2 markers. They also support the expansion of cord blood $CD34^+$ cells and immature hematopoietic progenitors in co-culture systems. Hence, our study introduces a new source for young MSCs which can be stored for regenerative medicine purposes. Needless to mention, characterization of such a favorable niche for emergence and/or expansion of HSCs will broaden the knowledge required to achieve our final goal, i.e. efficient *ex vivo* expansion of HSCs.

Hypoxia and hematopoietic niche in mouse placenta

Hypoxia plays a fundamental role during embryogenesis and placenta morphogenesis through its major response regulator, hypoxia inducible factors (HIFs) (reviewed in Dunwoodie, 2009). Furthermore, in the murine BM, low oxygen levels are to be considered as a niche factor that plays a fundamental role in HSC regulation (Parmar et al., 2007; Takubo et al., 2010) and is crucial to long-term repopulating activity of HSCs (Cheshier et al., 1999; Wilson et al., 2008). Interestingly, HIF1α has been shown to affect the HSC behavior in the adult BM (Takubo et al., 2010). This raised the question whether HIF1α contributes to the *in vivo* regulation of HPSC generation/maintenance/function in the mouse embryo.

Previously in our lab, the temporal and spatial distribution of MSCs was found to be associated with mouse hematopoietic sites such as the AGM, FL, and neonatal BM (Mendes et al., 2005), suggests that stromal cells play a role in maintenance and expansion of HPSCs at early developmental stages. MSC lines derived from these tissues have osteogenic, adipogenic, and chondrogenic differentiation potential (Durand et al., 2006; Zipori, 2004). More importantly, they support hematopoietic cells *in vitro* (Moore et al., 1997; Oostendorp et al., 2002a). Different hematopoietic tissues possess different capacities in terms of generation or expansion of HSCs suggesting that the hematopoietic programs and the microenvironments that support these programs are different among these tissues. Since the mouse placenta contains a large number of adult-reconstituting HSCs (15 times more than the AGM region) (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), it is expected that this tissue will also yield hematopoietic-supportive MSC lines and exhibit similar differentiation potentials.

Considering the proven role of hypoxia in embryogenesis and placenta formation, as well as in BM HSC regulation, we raised the question in this thesis whether hypoxia, as a niche factor, has any effect on the placenta stromal cells and their hematopoietic supportiveness. Our study demonstrated that the mouse placenta stromal cells have mesenchymal differentiation potential. Hypoxia significantly improves the cellular growth of placenta stromal cells. Also, hypoxic placenta stromal cells showed increased VEGF expression (by RNA and protein analysis) consistent with the previous observation in hypoxic culture of BM stromal cells (Jing et al., 2012; Levesque et al., 2007). This shows that hypoxia improves the expression of pro-angiogenic and pro-survival factors in mesenchymal cells.

We showed that placenta stromal cells provide support for expansion of BM hematopoietic cells and hematopoietic progenitors as compared to input number. This support was reduced in hypoxia - as compared to normoxia. The hematopoietic cells and progenitors were maintained in hypoxia and showed a slight increase compare to the input numbers. This data is in agreement with the previously published data showing that hypoxia either decreases or does not change the hematopoietic progenitor activity of cultured hematopoietic cells (Cipolleschi et al., 2000; Ivanovic et al., 2000; Ivanovic et al., 2002). The reduced number of BM cells in hypoxia compared to normoxia could be due to the fact that HSCs are kept in a low cycling state under hypoxic conditions (Danet et al., 2003; Eliasson et al., 2010; Hermitte et al., 2006; Ivanovic et al., 2002). Another explanation for the decreased rate of hematopoietic cell

expansion could be that hypoxia induces apoptosis of hematopoietic progenitors and/or stem cells. Investigations should be performed to test whether any of these possibilities explain such observations.

Although LSK cells differentiate into the macrophage lineage in both normoxic and hypoxic co-culture conditions, lower percentages of $Mac1^+$ and higher percentages of Lin^- and LSK cells were observed in the hypoxic co-cultures. This suggests that hypoxia inhibits the differentiation of HPSCs cultured on placenta cells, which is in agreement with the previous finding that hypoxia keeps the peripheral blood (PB) hematopoietic cells cultured on BM cells in a primitive state (Jing et al., 2012). We propose that hypoxia conditioning of HSCs shifts the balance between differentiation and self-renewal in favor of the latter. Testing this calls for *in vivo* transplantation assay.

Collectively, these results show that hypoxic culture of placental stromal cell lines interferes with their hematopoietic supportive and differentiative capacity. Whether this interference is mediated through stromal cells only, or both stromal and hematopoietic cells, remained to be investigated.

Further investigation is needed to realize the optimal culturing conditions that can reveal a true understanding of what the role of hypoxia is in HPSC generation, maintenance and expansion in the placenta niche. Furthermore, the underlying molecular mechanisms mediating the effect of hypoxia on HPSCs remain to be elucidated. Characterization of such mechanisms will help us to design and manipulate HSC culturing systems in order to improve long-term repopulating capacity of HSCs or *ex vivo* HSC expansion.

In addition to hypoxia, there are other niche components and signals which may favor extensive expansion of HSCs in both human and mouse placenta. Adult BM microenvironment is a complex structure composed of mesenchymal cells, endothelial cells, osteoblasts, adipocytes, the extracellular matrix, growth factors, cytokines, and adhesive molecules that provide support for HPSC homing, self-renewal, maintenance, and differentiation. The hematopoietic niche of the placenta within the labyrinth is likely to be similar, but also consists of placenta-specific syncytiotrophoblast cells. It is known that the placenta is rich in growth factors and cytokines that are secreted by various placental cells, suggesting a unique set of molecules that may affect hematopoiesis. An interesting direction for future research is to investigate which of the aforementioned specific niche factors support hematopoiesis and how.

HIF1α in HPSC regulation during embryonic development

It has been shown that long-term repopulating (LTR)-HSCs reside in the most hypoxic region of the BM niche of the adult mouse (Parmar et al., 2007; Takubo et al., 2010). HIF1α is the major regulator of cellular and systemic hypoxia (Wang et al., 1995), which is regulated post-translationally under control of oxygen level (Jaakkola et al., 2001; Maxwell et al., 1999; Ruas and Poellinger, 2005; Schofield and Ratcliffe, 2005). Germline deletion of *HIF1α* in the

mouse leads to lethality by E10.5 (Cowden Dahl et al., 2005a; Cowden Dahl et al., 2005b; Ryan et al., 1998). Using *MX1-Cre: HIF1α$^{fl/fl}$* conditional knockout (cKO) mice, HIF1α has been shown to regulate adult HSC function. HIF1α deficiency increases the cycling and subsequent senescence and exhaustion of HSCs in serial transplantations due to increased expression of *Ink4a* locus products. Hence, it appears that HIF1α plays a role in the resistance of HSCs against stress conditions and maintains the quiescent state of HSCs (Takubo et al., 2010).

The ubiquitous function of the HIF1α transcription factor prohibits evaluating its precise role particularly in the development of the hematopoietic compartment. To this end, we applied a conditional KO approach using Cre/loxp strategy to specifically remove *HIF1α* from VEC-expressing endothelial cells. Our examination of *VEC-Cre:HIF1α$^{fl/fl}$* embryos demonstrated that HIF1α regulates HPSCs during and after their generation. Deletion of *HIF1α* in VEC-expressing cells in the embryonic aorta and vitelline/umbilical arteries leads to a decrease in the number of hematopoietic progenitors, the frequency of phenotypic HPSCs, the number of aortic hematopoietic clusters/c-Kit^{+} cells, and the reduction/absence of functional stem cell activity of embryonic hematopoietic tissues. The most important time point for HIF1α activity appears to be E10, which corresponds to the time when HPSCs emerge from hemogenic endothelium in the AGM region. Whole embryo confocal imaging showed no morphological defects in the vasculature of cKO embryos, and flow cytometric analysis showed similar frequency of the cells positive for endothelial marker.

3-D confocal imaging showed obvious quantitative reduction in aortic hematopoietic clusters, suggesting that either the endothelial-to-hematopoietic transition or the growth/expansion of the emerging HPSCs is affected. Additionally, *HIF1α*-deficient AGM cells failed to engraft the irradiated animals. However, both cKO embryos and adults looked normal and adult BM had the potential to reconstitute the hematopoietic system of irradiated recipient. This implies that there are other tissues harboring HSCs during embryonic development which are not affected/less affected by *HIF1α* deficiency and are sufficient to establish the whole hematopoietic system.

To verify our hypothesis that the loss of *HIF1α* affects the generation of HPSCs from hemogenic endothelial cells lining the vasculature in the *VEC-Cre:HIF1α$^{fl/fl}$* embryos, we need to investigate the particular process(es) that regulate the emergence of HPSCs from endothelial cells and are mediated through *HIF1α*. The VEGF/FLK1 axis and the integrins are the two candidates that may be affected (hypoxia induces the expression of some integrins and their ligands such as Itga5 (Cowden Dahl et al., 2005b; Kilburn et al., 2000)).

HIF1α in HSC regulation of adult BM

We observed no phenotypic defects in hematopoietic tissues of adult *VEC-Cre:HIF1α$^{fl/fl}$* mice, whereas BM HSC activity was affected under stress conditions of serial transplantations. In the primary transplantation, slightly lower chimerism levels were observed in the PB of the KO HSC recipients, implying that KO HSCs are slightly less robust in reconstitution capacity.

Moreover, the HSC content is reduced in the recipients of *HIF1α*$^{-/-}$ HSCs showing the defect in HSC maintenance under stress conditions. In the secondary recipients, *HIF1α*$^{-/-}$ BM HSCs experienced an unscheduled cell cycling status and lost their quiescence state. Hence, our observations for adult BM HSCs are in support of the fact that HIF1α is essential for HSC regulation, as shown by Takubo and colleagues (Takubo et al., 2010). It has been shown that almost 75% of bone marrow LTR-HSCs are quiescent, whereas less than 5% of them are actively cycling (Simon et al., 2002; Wilson et al., 2008). The balance between proliferation and quiescence of HSCs is tightly regulated and maintaining the HSCs in a quiescent state is crucial for their long-term repopulating function (Jang and Sharkis, 2007). Our results along with the findings of the aforementioned studies suggest that HIF1α is one of the regulators that ensures the proper balance between the proliferation and quiescence of stem cells.

To summarize, in adult mice, HIF1α is dispensable for HSC function under steady state conditions. However, under stress conditions of serial transplantations, HIF1α is required to maintain the normal number of HSCs and their quiescent state. Hence, HIF1α plays multiple roles in regulating HPSC throughout embryonic and adult development.

It has been shown that HIF2α is highly expressed in Lin$^-$ cells of BM (Takubo et al., 2010). Although its role is not clear yet, in our case it may partially compensate for the lack of HIF1α in KO HSCs of BM. This can be proposed as a reason for the milder effects of HIF1α deficiency in the adult compared to embryonic life. We discussed in Chapter 1 that the regulation of specific genes by HIF1α and HIF2α seems to be context-dependent. Additionally, they can replace each other in some specific functions (Fujiwara et al., 2007; Warnecke et al., 2004). It has been shown that HIF2α is hydroxylated at a lower efficiency than HIF1α, which causes stabilization and activation of HIF2α in a higher oxygen level compared to HIF1α. Also, in some cell lines HIF1α is active in short periods of severe hypoxia or anoxia (<0.1% O_2), while HIF2α is active in mild hypoxia (<5 % O_2) and remains active for long periods of hypoxia (Holmquist-Mengelbier et al., 2006). Therefore, it seems that HIF1α drives the initial response to hypoxia, but later in chronic hypoxic condition, HIF2α comes into play and has the main role in regulating the response to hypoxia (Holmquist-Mengelbier et al., 2006; Koh et al., 2011). Considering this information in conjunction with our findings, we can hypothesize that embryonic and adult hematopoietic tissues represent different contexts in terms of HIF regulation. No comparative study between embryonic tissues and adult BM in terms of oxygen level has been performed; but one can assume they differ in oxygen level and cellular components, leading to different isoform-specific regulation of HIF transcription factors during development. Therefore to understand the distinct role of HIF1α in HSC regulation, the overlapping/complementary and unique roles of HIF1α and HIF2α in HSCs during both embryonic and adult life needs to be studied. Moreover, understanding the molecular mechanisms through which HIF directs the stress resistance in adult HSCs will be another important research task for future.

Finally, an understanding of the role of HIF1α is not only crucial in normal physiological processes to facilitate growth of the hematopoietic system, but also it is important to understand the role of hypoxia in pathophysiological conditions, such as leukemia. It has been shown that in normoxia HIF1α signaling is selectively activated in the stem cells of mouse

lymphoma and human acute myeloid leukemia (AML) having a crucial function in maintaining cancer stem cells (Wang et al., 2011a). In chronic myeloid leukemia (CML), HIF1α acts in the survival maintenance of cancer stem cells (Zhang et al., 2012). These observations may provide an effective approach to target cancer stem cells for therapeutic applications. Therefore, continued research on this topic has a two-fold impact: on one hand, it defines the cues that govern the *ex vivo* expansion of HSCs for potential clinical application, and on the other hand, it characterizes inhibitors of HIF transcription factors or their downstream effectors which could have pharmaceutical applications in treatment of hematological malignancies.

References

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proceedings of the National Academy of Sciences of the United States of America *94*, 5320-5325.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature *464*, 116-120.

Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E., and Speck, N.A. (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature *457*, 887-891.

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. Proceedings of the National Academy of Sciences of the United States of America *96*, 3120-3125.

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

Cipolleschi, M.G., Rovida, E., Ivanovic, Z., Praloran, V., Olivotto, M., and Dello Sbarba, P. (2000). The expansion of murine bone marrow cells preincubated in hypoxia as an in vitro indicator of their marrow-repopulating ability. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *14*, 735-739.

Cowden Dahl, K.D., Fryer, B.H., Mack, F.A., Compernolle, V., Maltepe, E., Adelman, D.M., Carmeliet, P., and Simon, M.C. (2005a). Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Molecular and cellular biology *25*, 10479-10491.

Cowden Dahl, K.D., Robertson, S.E., Weaver, V.M., and Simon, M.C. (2005b). Hypoxia-inducible factor regulates alphavbeta3 integrin cell surface expression. Molecular biology of the cell *16*, 1901-1912.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A., and Simon, M.C. (2003). Expansion of human SCID-repopulating cells under hypoxic conditions. The Journal of clinical investigation *112*, 126-135.

Dunwoodie, S.L. (2009). The role of hypoxia in development of the Mammalian embryo. Developmental cell *17*, 755-773.

Durand, C., Robin, C., and Dzierzak, E. (2006). Mesenchymal lineage potentials of aorta-gonad-mesonephros stromal clones. Haematologica *91*, 1172-1179.

Eliasson, P., Rehn, M., Hammar, P., Larsson, P., Sirenko, O., Flippin, L.A., Cammenga, J., and Jonsson, J.I. (2010). Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-

reconstituting hematopoietic stem cells during in vitro culture. Experimental hematology *38*, 301-310 e302.

Fujiwara, S., Nakagawa, K., Harada, H., Nagato, S., Furukawa, K., Teraoka, M., Seno, T., Oka, K., Iwata, S., and Ohnishi, T. (2007). Silencing hypoxia-inducible factor-1alpha inhibits cell migration and invasion under hypoxic environment in malignant gliomas. International journal of oncology *30*, 793-802.

Fukuchi, Y., Nakajima, H., Sugiyama, D., Hirose, I., Kitamura, T., and Tsuji, K. (2004). Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells *22*, 649-658.

Gekas, C., Dieterlen-Lievre, F., Orkin, S.H., and Mikkola, H.K. (2005). The placenta is a niche for hematopoietic stem cells. Developmental cell *8*, 365-375.

Hermitte, F., Brunet de la Grange, P., Belloc, F., Praloran, V., and Ivanovic, Z. (2006). Very low O2 concentration (0.1%) favors G0 return of dividing CD34+ cells. Stem Cells *24*, 65-73.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K*., et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer cell *10*, 413-423.

Igura, K., Zhang, X., Takahashi, K., Mitsuru, A., Yamaguchi, S., and Takashi, T.A. (2004). Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. Cytotherapy *6*, 543-553.

Ivanovic, Z., Bartolozzi, B., Bernabei, P.A., Cipolleschi, M.G., Rovida, E., Milenkovic, P., Praloran, V., and Dello Sbarba, P. (2000). Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. British journal of haematology *108*, 424-429.

Ivanovic, Z., Belloc, F., Faucher, J.L., Cipolleschi, M.G., Praloran, V., and Dello Sbarba, P. (2002). Hypoxia maintains and interleukin-3 reduces the pre-colony-forming cell potential of dividing CD34(+) murine bone marrow cells. Experimental hematology *30*, 67-73.

Ivanovs, A., Rybtsov, S., Welch, L., Anderson, R.A., Turner, M.L., and Medvinsky, A. (2011). Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. The Journal of experimental medicine *208*, 2417-2427.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J*., et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Jing, D., Wobus, M., Poitz, D.M., Bornhauser, M., Ehninger, G., and Ordemann, R. (2012). Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. Haematologica *97*, 331-339.

Kilburn, B.A., Wang, J., Duniec-Dmuchowski, Z.M., Leach, R.E., Romero, R., and Armant, D.R. (2000). Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. Biology of reproduction *62*, 739-747.

Koh, M.Y., Lemos, R., Jr., Liu, X., and Powis, G. (2011). The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor

growth and invasion. Cancer research *71*, 4015-4027.

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*, 4891-4899.

Levesque, J.P., Winkler, I.G., Hendy, J., Williams, B., Helwani, F., Barbier, V., Nowlan, B., and Nilsson, S.K. (2007). Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. Stem Cells *25*, 1954-1965.

Li, C.D., Zhang, W.Y., Li, H.L., Jiang, X.X., Zhang, Y., Tang, P., and Mao, N. (2005). Isolation and Identification of a Multilineage Potential Mesenchymal Cell from Human Placenta. Placenta.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

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

Mendes, S.C., Robin, C., and Dzierzak, E. (2005). Mesenchymal progenitor cells localize within hematopoietic sites throughout ontogeny. Development *132*, 1127-1136.

Miao, Z., Jin, J., Chen, L., Zhu, J., Huang, W., Zhao, J., Qian, H., and Zhang, X. (2006). Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. Cell biology international *30*, 681-687.

Moore, K.A., Ema, H., and Lemischka, I.R. (1997). In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood *89*, 4337-4347.

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.

Oostendorp, R.A., Harvey, K.N., Kusadasi, N., de Bruijn, M.F., Saris, C., Ploemacher, R.E., Medvinsky, A.L., and Dzierzak, E.A. (2002a). Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity. Blood *99*, 1183-1189.

Oostendorp, R.A., Medvinsky, A.J., Kusadasi, N., Nakayama, N., Harvey, K., Orelio, C., Ottersbach, K., Covey, T., Ploemacher, R.E., Saris, C*., et al.* (2002b). Embryonal subregion-derived stromal cell lines from novel temperature-sensitive SV40 T antigen transgenic mice support hematopoiesis. Journal of cell science *115*, 2099-2108.

Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Developmental cell *8*, 377-387.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proceedings of the National Academy of Sciences of the United States of America *104*, 5431-5436.

Parolini, O., Alviano, F., Bagnara, G.P., Bilic, G., Buhring, H.J., Evangelista, M., Hennerbichler, S., Liu, B., Magatti, M., Mao, N*., et al.* (2008). Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. Stem Cells *26*, 300-311.

Portmann-Lanz, C.B., Schoeberlein, A., Huber, A., Sager, R., Malek, A., Holzgreve, W., and Surbek, D.V. (2006). Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. American journal of obstetrics and gynecology *194*, 664-673.

Ruas, J.L., and Poellinger, L. (2005). Hypoxia-dependent activation of HIF into a transcriptional regulator. Seminars in cell & developmental biology *16*, 514-522.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. The EMBO journal *17*, 3005-3015.

Schofield, C.J., and Ratcliffe, P.J. (2005). Signalling hypoxia by HIF hydroxylases. Biochemical and biophysical research communications *338*, 617-626.

Simon, M.C., Ramirez-Bergeron, D., Mack, F., Hu, C.J., Pan, Y., and Mansfield, K. (2002). Hypoxia, HIFs, and cardiovascular development. Cold Spring Harbor symposia on quantitative biology *67*, 127-132.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M.*, et al.* (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell stem cell *7*, 391-402.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America *92*, 5510-5514.

Wang, J.C., Doedens, M., and Dick, J.E. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood *89*, 3919-3924.

Wang, Y., Liu, Y., Malek, S.N., and Zheng, P. (2011a). Targeting HIF1alpha eliminates cancer stem cells in hematological malignancies. Cell stem cell *8*, 399-411.

Wang, Y., Nathanson, L., and McNiece, I.K. (2011b). Differential hematopoietic supportive potential and gene expression of stroma cell lines from midgestation mouse placenta and adult bone marrow. Cell transplantation *20*, 707-726.

Warnecke, C., Zaborowska, Z., Kurreck, J., Erdmann, V.A., Frei, U., Wiesener, M., and Eckardt, K.U. (2004). Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 1462-1464.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Wulf, G.G., Viereck, V., Hemmerlein, B., Haase, D., Vehmeyer, K., Pukrop, T., Glass, B., Emons, G., and Trumper, L. (2004). Mesengenic progenitor cells derived from human placenta. Tissue engineering *10*, 1136-1147.

Yen, B.L., Huang, H.I., Chien, C.C., Jui, H.Y., Ko, B.S., Yao, M., Shun, C.T., Yen, M.L., Lee, M.C., and Chen, Y.C. (2005). Isolation of multipotent cells from human term placenta. Stem Cells *23*, 3-9.

Yoder, M.C., Papaioannou, V.E., Breitfeld, P.P., and Williams, D.A. (1994). Murine yolk sac endoderm- and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells. Blood *83*, 2436-2443.

Zhang, H., Li, H., Xi, H.S., and Li, S. (2012). HIF1alpha is required for survival maintenance of chronic myeloid leukemia stem cells. Blood *119*, 2595-2607.

Zhang, X., Mitsuru, A., Igura, K., Takahashi, K., Ichinose, S., Yamaguchi, S., and Takahashi, T.A. (2006). Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering. Biochemical and biophysical research communications *340*, 944-952.

Zipori, D. (2004). Mesenchymal stem cells: harnessing cell plasticity to tissue and organ repair. Blood cells, molecules & diseases *33*, 211-215.

Summary

The differentiation and maintenance of the hematopoietic system is founded in the rare hematopoietic stem cells (HSCs) that are defined by two pivotal characteristics, self-renewal and multi-lineage differentiation. The development of HSCs commences in embryonic life. In mouse, the first definitive adult-repopulating HSCs are generated in the aorta-gonad-mesonephros (AGM) region of the embryonic day (E) 10.5 embryo. Hematopoietic cells emerge from endothelial cells (expressing vascular endothelial cadherin (VEC)) lining the major vasculature of the midgestation mouse embryo. Later at E11-11.5, HSCs appear in the yolk sac (YS), placenta, and fetal liver (FL). In contrast to the AGM and YS, the placenta contains large numbers of HSCs (15 times more than the AGM region). In late stages of development, FL remains the main hematopoietic tissue for colonization and expansion of HSCs until birth. Prior to birth, HSCs start to migrate to the bone marrow (BM), where they reside throughout adulthood. In the adult BM, the majority of HSCs are quiescent. However, because of the limited life span of circulating hematopoietic cells, some HSCs are active and contribute to the replacement of old hematopoietic cells with new ones. This demanding job does not lead to exhaustion of HSCs because the balance between the quiescent and proliferative states of HSCs is strongly regulated by the surrounding niche. Understanding the niche components and their regulatory factors in both embryonic and adult hematopoietic tissues provides valuable insight into the mechanisms governing generation and expansion of hematopoietic progenitor and stem cells (HPSCs).

Oxygen levels are relatively low in BM compared to other tissues and HSCs are located in the lowest end of oxygen gradient in the BM niche. Here, the HSCs are kept in a quiescent state to prevent their differentiation and exhaustion, which is crucial for their long-term repopulating function. Hypoxia inducible factor (HIF)1α, as the master regulator of hypoxia, modulates HSC cycling and its deficiency leads to loss of quiescent HSCs in the BM of adult mice. Oxygen levels are also low in the uterus and many aspects of embryonic development, such as placenta formation, are regulated by hypoxia and HIFs. Mesenchymal stem cells (MSCs) derived from the AGM, FL, and neonatal BM tissues have osteogenic, adipogenic, and chondrogenic differentiation potential and more importantly, they support hematopoietic cells *in vitro*. Yet, little is known about the role of hypoxia on HPSC growth and maintenance in murine embryo.

The fact that the E12 mouse placenta harbors many more HPSCs than the AGM region suggests the placenta provides a robust hematopoietic niche during development. This observation encouraged us to examine the human placenta for existence of HSCs throughout its developmental stages and to study stromal cells derived from mouse and human placenta to extend our knowledge of signals involved in HSC development in this promising niche. We show that the human placenta contains all types of hematopoietic progenitors throughout development, and more importantly that it contains *bona fide* HSCs starting at week 6 of gestation until term. This was an unexpected result since previous data in the mouse term placenta had shown almost no adult repopulating HSCs were present. We found that the

HSC content of the human placenta reached almost 10% of the number of HSCs contained in one unit of umbilical cord blood (UCB). Furthermore, mesenchymal cells derived from human placenta have the potential to differentiate along osteogenic, adipogenic, and enotheilial lineages and support the expansion of cord blood $CD34^+$ cells and immature hematopoietic progenitors. Hence, our study introduces an additional source for HPSCs together with UCB for banking and potential clinical use and a new source for young MSCs which can be stored for regenerative medicine purposes.

To study the HPSC niche in mouse placenta, we analyzed the E12 placenta-derived stromal cells and demonstrated that they have the osteogenic and adipogenic differentiation potential and support expansion and progenitor activity of the BM hematopoietic cells. Also, the role of hypoxia (as a niche factor) on stromal cells and their supportiveness for HPSC maintenance/expansion was analyzed. Faster proliferation of placenta stromal cells and the induction of hypoxia-sensitive gene expression under hypoxic conditions show that the placenta HPSC niche has the potential to respond to oxygen-deficits. Also, hypoxia interferes with placenta stromal cells' hematopoietic supportive and differentiative capacity. Therefore, placenta contains a hematopoietic supportive niche and hypoxia may be involved in HPSC generation/expansion in placenta.

Importantly, to understand the role of HIF1α in HPSC development, we analyzed the effects of *HIF1α*-deletion in conditional knockout (cKO) *VEC-Cre:HIF1α$^{fl/fl}$* mouse embryos. We showed that HIF1α is essential in HPSC generation and/or expansion in the mouse embryo, as the hematopoietic progenitor activity and HSC repopulating potential of *HIF1α* cKO embryonic tissues are diminished. Moreover, phenotypic HPSCs and the number of aortic hematopoietic clusters/c-Kit^+ cells are reduced in cKO embryos. Whole-mount imaging of cKO embryos showed clear quantitative reduction in aortic hematopoietic clusters, suggesting that either the endothelial-to-hematopoietic transition or the growth/expansion of the emerging HPSCs is affected. In adult mice, HIF1α is required to maintain the normal number of HSCs and to keep them in a quiescent state under stress conditions. Collectively, HIF1α plays multiple roles in HPSC regulation throughout life. Continued characterization of the role of hypoxia and HIFs on HPSC regulation will help us to design and manipulate HSC culturing systems so as to improve long-term repopulating capacity of HSCs or *ex vivo* HSC expansion.

Samenvatting

De uitrijping en het onderhoud van het hematopoietische systeem is gebaseerd op de zeldzame hematopoietische stamcellen (HSC's), die worden gedefinieerd door twee cruciale kenmerken, zelfvernieuwing en uitrijping naar verschillende bloedcel types. De ontwikkeling van HSC's begint in embryonale leven. In muizen worden de eerste definitieve volwassen HSC's gegenereerd in de aorta-gonaden-mesonephros (AGM) gedeelte van de embryonale dag (E) 10,5 embryo. Hematopoietische cellen komen uit endotheelcellen (die vasculaire endotheliale cadherine (VEC) to expressie brengen) langs de grote bloedvaten van de midgestation muis embryo. Later op E11-11,5, verschijnen HSC's in de dooierzak (YS), placenta, en foetale lever (FL). In tegenstelling tot de AGM en YS bevat de placenta een groot aantal HSC's (15 keer meer dan de AGM regio). In latere stadia van ontwikkeling, speelt FL steeds een belangrijker rol in kolonisatie en de expansie van HSC's tot aan de geboorte. Voorafgaand aan de geboorte beginnen de HSC's te migreren naar het beenmerg (BM), waar zij verblijven voor de rest van het leven. In de volwassen BM zijn de meeste HSC's in rust. Alhoewel, vanwege de beperkte levensduur van circulerende hematopoietische cellen, sommige HSC's actief zijn en helpen bij de vervanging van oude hematopoïetische cellen. Deze veeleisende taak leidt niet tot uitputting van HSC's, omdat het evenwicht tussen de in rust zijnde- en proliferatieve toestand van HSC's is sterk gereguleerd door de omringende niche. Inzicht in de niche componenten en hun regulerende factoren in zowel de embryonale en volwassen hematopoietische weefsels geeft een waardevol inzicht in de mechanismen die generatie en uitbreiding van hematopoïetische voorloper- en stamcellen (HPSCs) regulieren.

Zuurstofgehalte is relatief laag in BM ten opzichte van andere weefsels en HSC's bevinden zich in de lagere kant van zuurstof gradiënt in de BM niche. Hier worden de HSC bewaard in een rusttoestand om hun uitrijping en uitputting, wat cruciaal is voor de lange termijn repopulatie functie. Hypoxia inducible factor (HIF) 1α, de hoofd regulator van hypoxie, moduleert HSC cyclus en de deficiëntie ervan leidt tot verlies van rustende HSC's in de BM van volwassen muizen. Zuurstof niveaus zijn ook laag in de baarmoeder en de vele aspecten van de embryonale ontwikkeling, zoals placenta vorming, worden gereguleerd door hypoxie en HIF's. Mesenchymale stamcellen (MSC's) verkregen uit de AGM, FL, en neonatale BM weefsels hebben osteogene, adipogene en chondrogene uitrijping potentiaal en ondersteunen hematopoietische cellen *in vitro*. Toch is er weinig bekend over de rol van hypoxie op HPSC groei en het onderhoud in muis embryo.

Dat er in de E12 muis placenta zich veel meer HPSC's bevinden dan in de AGM gebied suggereert dat de placenta tijdens de ontwikkeling een robuuste hematopoietische niche is. Deze observatie moedigde ons aan om de menselijke placenta voor het bestaan van HSC's te besturen voor gedurende haar verschillende ontwikkelingsstadia, en om stromale cellen afkomstig van muis en mens placenta te onderzoeken ter uitbreiding van onze kennis van de signalen die betrokken zijn bij HSC ontwikkeling in deze veelbelovende niche. We laten zien dat de menselijke placenta alle soorten van hematopoietische voorlopercellen bevat tijdens gedurende haar ontwikkeling, en wat nog belangrijker is dat het vanaf week 6 van de

zwangerschap tot bevalling bevat *bona fide* HSC bevat. Dit was een onverwacht resultaat, omdat eerdere gegevens in de muis terminale placenta kon nagenoeg geen volwassen repopulatie-capabele HSC's aantonen. We vonden dat de HSC inhoud van de menselijke placenta bijna 10% van het aantal HSC's in één eenheid van navelstrengbloed (UCB) bereikt. Bovendien mesenchymale cellen uit menselijk placenta hebben het potentieel om te differentiëren langs osteogeen, adipogene en enotheilial celtypes en de expansie van $CD34^+$ navelstrengbloed cellen en onvolwassen hematopoëtische voorlopers. Onze onderzoek introduceert een extra bron for HPSC's samen met UCB voor weefselbanken en potentiele klinische toepassingen en een nieuwe bron voor jonge MSC's dat kan worden opgeslagen voor regeneratieve geneeskunde doeleinden.

Voor het bestuderen van HSPC niche in de muis placenta hebben we uit E12-placenta geisoleerde stromale cellen geanalyseerd. We laten zien that deze cellen osteogene en adipogene uitrijpingspotentiaal hebben en ondersteuning bieden voor expansie en voorloper activiteit voor BM hematopoietische cellen. Ook werd de rol van hypoxie (als niche factor) op stromale cellen en hun ondersteuning voor HPSC onderhoud/expansie geanalyseerd. Snellere proliferatie van placenta stromale cellen en de inductie van hypoxie-gevoelige genexpressie onder hypoxische omstandigheden laat zien dat de placenta HPSC niche de potentieel bezit om te reageren op zuurstof tekort. Hypoxie interfereert ook met hematopoietische ondersteunende en differentiative capaciteit van de placenta stromale cellen. De placenta bevat daarom een hematopoietische ondersteunende niche en hypoxie speel mogelijk een rol bij HPSC generatie/expansie in placenta.

Om de rol van HIF1α in HPSC ontwikkeling beter te begrijpen, analyseerden we de effecten van *HIF1α*-deletie in conditionele knockout (cKO) *VEC-Cre: HIF1α$^{fl/fl}$* muis embryo's. We toonden aan dat HIF1α essentieel is voor het genereren en/of expansie van HPSC's in de muis embryo, aangezien de hematopoietische activiteit en HSC repopulatie potentieel van *HIF1α* cKO embryonale weefsels is aangetast. Bovendien zijn fenotypische HPSC's en het aantal aorta hematopoietische clusters / c-Kit+ cellen gereduceerd in cKO embryos. Whole-mount imaging van cKO embryo's toonde duidelijke kwantitatieve vermindering van de aorta hematopoietische clusters, wat suggereert dat ofwel de endotheel-naar-hematopoietische overgang of de groei/expansie van de uitgroeiende HPSC's is aangedaan. Bij volwassen muizen is HIF1α vereist voor het behouden van het normale aantal HSC's en voor in het rusttoestand houden van HSC's onder stressomstandigheden. Samengevat, HIF1α speelt meerdere rollen in HPSC regulatie gedurende het hele leven. Verdere karakterisatie van de rol van hypoxie en HIF's op HPSC regulatie zal ons helpen bij het ontwerpen en manipulatie van HSC kweeksystemen, om de langetermijn repopulatie capaciteit van HSC's of ex vivo expansie van HSC's te verbeteren.

Abbreviations

AGM	Aorta-Gonad-Mesonephros
ALDA	Aldolase A
AM	Aorta-mesenchyme
AML	Acute myeloid leukemia
Ang	Angiopoietin
Arnt	Aryl hydrocarbon receptor nuclear translocator
BFU-E	Erythroid burst-forming unit
bHLH	Basic helix-loop-helix
BM	Bone marrow
BMP	Bone morphogenetic protein
BMPRIA	BMP receptor type IA
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BNIP3L/NIX	BCL2/adenovirus E1B 19kDa interacting protein 3-like
CA-IX	Carbonic anhydrase 9
CFU-C	Colony forming unit in culture
CFU-G	Colony forming unit - granulocyte
CFU-GEMM	Colony forming unit - granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM	Colony forming unit - granulocyte, macrophage
CFU-M	Colony forming unit - macrophage
CFU-S	Spleen colony forming unit
CFU-S8	Colony forming unit-spleen day 8
CHIP	Carboxyl terminus of Hsp70-interacring protein
cKO	conditional KO
CML	Chronic myeloid leukemia
DMEM	Dulbecco’s modified Eagle’s medium
E	Embryonic day
EB	Embryoid bodies
EDTA	Ethylenediaminetetraacetic acid
ee	embro equivalent
EPAS1	Endothelial PAS protein 1
EPO	Erythropoietin
ES	Embryonic stem
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Fih	Factor inhibiting HIF
FITC	Fluorescein isothiocyanate
FL	Fetal liver
fl	floxed
FLT	Fms-like tyrosine kinase
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HAF	Hypoxia associated factor
hdm2	Human double minute 2
HIF	Hypoxia inducible factor

HLF	HIF1α-like factor
HPSC	Hematopoietic progenitor and stem cells
HRE	hypoxia-responsive element
HRE	Hypoxia responsive element
HRF	HIF-related factor
HSC	Hematopoietic stem cells
Hsp	Heat shock protein
IL	Interleukine
IPAS	Inhibitory PAS
IRE	Iron-responsive element
KO	knockout
LDHA	Lactate dehydrogenase A
LN	Lymph node
LSK	Lin^{-} $cKit^{+}$ $Sca\text{-}1^{+}$
LTR	Long-term repopulating
MCT4	Monocarboxylate transporter 4
MEM	Minimum essential medium
MMP	Matrix metalloproteinases
MNC	Mononuclear cells
MOP2	Member of PAS family 2
MP	mitochondrial potential
Mpl	Myeloproliferative leukemia virus oncogen
MSC	Mesenchymal stem cell
NEPAS	Neonatal and embryonic PAS
NF	Nuclear factor
ODD	Oxygen-dependent degradation domain
PAS	Per-Arnt-Sim
PB	Peripheral blood
PBS	Phoshate Buffered Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFK	Phosphofructokinase
PGK	Phosphoglycerate kinase
PHD	Prolyl hydroxylase domain protein
PI3-K	phosphatidylinositol 3-kinase
PL	Placenta
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Penicillin and streptomycin
PSp	Para-aortic splanchnopleura
PTH	Parathyroid hormone
PVP	Polyvinylpyrolidone
RACK1	Receptor of activated protein kinase C
ROS	Reactive oxygen species
SCF	Stem cell factor
SDF	Stromal-derived factor
SLAM	Signaling lymphocyte activation molecule

SP	Spleen
STR	Short-term repopulating
TAD	Transactivation domains
TGC	Trophoblast giant cell
Th	Thymus
TPO	Thrombopoetin
UCB	Umbilical blood cord
UTR	Untranslated region
VCAM	Vascular cell adhesion molecule
VEC	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VHL	von Hippel-Lindau tumor suppressor protein
WT	Wild type
YS	Yolk sac

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List of Publications

Imanirad, P., Solaimani Kartalaei, P., Crisan, M., Yamada-Inagawa, T., va der Linden, R., Vink, C., Speck, N. and Dzierzak, E., *HIF1α is a regulator of hematopoietic progenitor and stem cell development*, In Preparation.

Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M**.,** Cerisoli, F., Lauw, I., Kaimakis, P., Jorna, R., Vermeulen M., Kayser, M., van der Linden, R., **Imanirad, P**., Verstegen, M., Nawaz-Yousaf, H., Papazian, N., Steegers, E., Cupedo, T., Dzierzak, E. *Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development*. Cell Stem Cell. 5(4), 385-395, 2009

Meyer N.C., Alasti, F., Nishimura, C.J., **Imanirad, P.**, Kahrizi, K., Riazalhosseini, Y., Malekpour, M., Kochakian, N., Jamali, P., Van Camp, G., Smith*, R.J.H., Najmabadi, H. *Identification of three novel TECTA mutations in Iranian families with autosomal recessive nonsyndromic hearing impairment at the DFNB21 locus*. American Journal of Medical Genetics A. 143A(14), 1623-1629, 2007.

Conference Presentations

Oral presentations

Role of hypoxia in hematopoietic progenitor and stem cell generation and function during mouse ontogeny, 41st Annual Meeting of ISEH – Society for Hematology and Stem Cells, Amsterdam, Netherlands, August, 2012.

Poster Presentations

Role of hypoxia in hematopoietic progenitor and stem cells in developing mouse placenta. Stem Cells in Development and Disease. Amsterdam, Netherlands, October, 2010.

Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. Royan International Stem Cell Meeting. Tehran, Iran, September, 2010.

Role of hypoxia in hematopoietic progenitor and stem cells in developing mouse placenta, Medical Genetics Centre (MGC) Ph.D. Workshop. Cologne, Germany, June, 2010.

Role of hypoxia in hematopoietic progenitor and stem cells in developing mouse placenta. Erasmus Stem Cell Institute (ESI) Opening Symposium. Rotterdam, Netherlands, May, 2010.

Ph.D. Portfolio

Name of Ph.D. student:	Parisa Imanirad
Erasmus MC department:	Cell Biology
Research school:	Molecular Genetic Center
PhD period:	September 2008 – February 2013
Promoter:	Dr. Elaine Dzierzak

Courses

Literature training course	2011
Radiation safety course	2011
In vivo Imaging: from molecule to organism	2009
Flow Cytometry and FlowJo FACS Analysis	2009
Development, Stem Cells and Disease	2009
Molecular and Cell Biology	2009
Laboratory animal science (Article 9)	2008

International Conferences

41st Annual Meeting of ISEH – Society for Hematology and Stem Cells, Amsterdam, Netherlands (Oral Presentation)	2012
Nederland Institute of Regenerative Medicine (NIRM) Meeting, Amsterdam, Netherlands	2010
Royan International Stem Cell Meeting, Tehran, Iran	2010
Stem Cell in Development and Diseases (SCDD) Meeting, Amsterdam, Netherlands	2010
Stem Cell in Development and Diseases (SCDD) Meeting, Amsterdam, Netherlands	2009

National Conferences

Dutch Society for Developmental Biology (DSDB) Meeting, Utrecht, Netherlands	2012
Dutch Society for Stem Cell Research (DSSCR) Meeting, Utrecht, Netherlands	2010
Dutch Society for Stem Cell Research (DSSCR) Meeting, Rotterdam, Netherlands	2009
Erasmus MC Medical Genetics Centre (MGC) Meeting, Rotterdam, Netherlands	2009
Erasmus MC Medical Genetics Centre (MGC) Meeting, Rotterdam, Netherlands	2008

Workshops

Erasmus Stem Cell Institute Retreat, Nijverdal, Netherlands (Oral Presentation)	2011
Erasmuc MC Medical Genetics Centre (MGC) Workshop, Cologne, Germany	2010
EUrythron Meeting, Lisbon, Portugal	2009

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