Ex vivo Expansion of Hematopoietic Stem Cells

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To my parents and
my grandmother

Contents

Chapter 1

Introduction

Hematopoiesis

Hematopoiesis is a complex cellular differentiation process resulting in the formation of all blood cell types. In this process, hematopoietic stem cells (HSCs) reside at the top of the hematopoiesis hierarchy and have the capacity to differentiate into all blood cell lineages (multipotency) as well as maintaining themselves (self-renewal) during the lifespan of an individual [1, 2] (Fig.1). Mouse primitive HSCs are first found in the blood islands of the extra-embryonic yolk sac at day 7.5 of gestation. At day 10.5 of gestation the earliest HSCs can be detected in the dorsal aorta region of aorta gonad mesonephros (AGM) [3]. The first HSC precursors cluster in the aortic endothelium and it has been shown that these definitive HSCs originate from specialized heamogenic endothelial cells (ECs) of the mouse embryonic aorta [4] (Fig.2). Recently, it has been reported that similar to the AGM at day 10.5-11.5, ECs of mouse embryonic head also have hemogenic capacity and give rise to HSCs [5]. HSCs then migrate from these initial sources into the fetal liver around day 11, where they undergo dramatic expansion during fetal development [6]. At next stage, around day 12 to16, HSCs are mobilized from the fetal liver into the bone marrow and spleen; although the fetal liver remains an important organ of definitive hematopoiesis during the embryonic period. From birth and throughout adult life, the bone marrow becomes the major hematopoetic tissue [7].

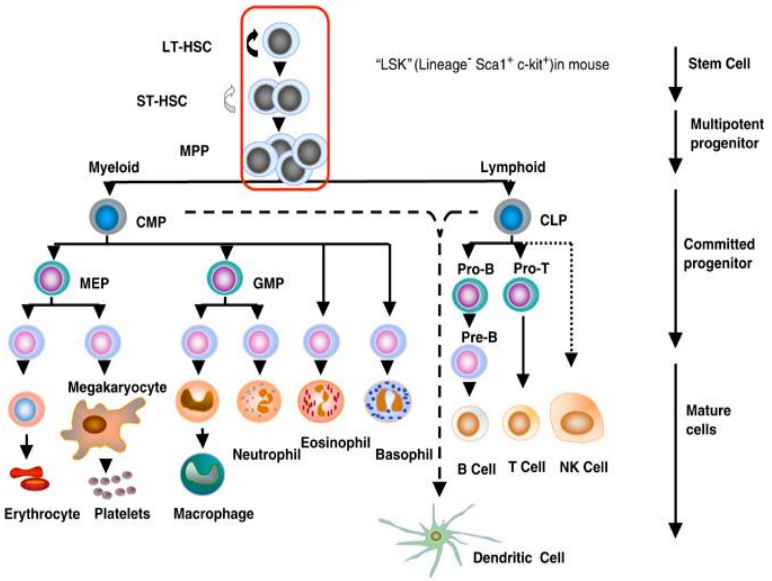

Figure 1: The hierarchy of hematopoietic cells. LT-HSC, long-term repopulating HSC; ST-HSC, short-term repopulating HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte–macrophage progenitor. The encircled pluripotent population, LT-HSC, ST-HSC and MPP are Lin^-, $Sca\text{-}1^+$, $c\text{-}kit^+$ as shown (Taken from: Larsen et al. Oncogene 2005) [8]

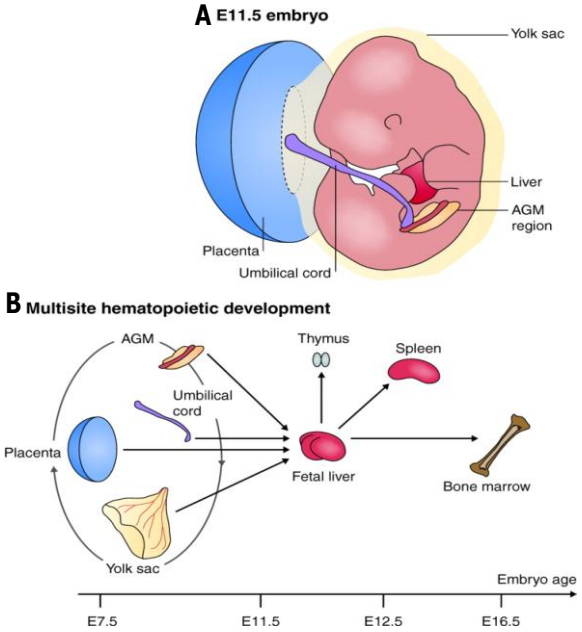

Figure 2: Continuity in embryonic hematopoietic development. **(A)** A schematic representation of an E11.5 mouse embryo (anterior uppermost), showing development of embryonic hematopoietic stem cell (HSC) niches: placenta, umbilical cord and aorta-gonad-mesonephros (AGM) region. The yolk sac expands and encompasses the embryo. **(B)** A model of multisite hematopoietic development, showing hematopoietic progenitors and definitive. HSCs from the sites shown in A colonizing the liver rudiment and each other (as shown by the circular arrow). Disagreement exists about which of these sites is the genuine source of dHSCs and the adult hematopoietic system. After expansion in the fetal liver, dHSCs colonize the bone marrow, spleen and thymus. In adulthood, the thymus and spleen are colonized by bone marrow progenitors (not shown) (Taken from: Medvinsky A et al. Development 2011) [7].

Murine hematopoietic stem cells

Murine HSCs were initially identified based on their ability to form colonies in the spleens of lethally irradiated mice following bone-marrow transplantation [2, 9, 10]. Subsequently, the long-term repopulating (LTR) assay has been introduced to examine the HSC potential of lifelong reconstitution of all blood cell lineages after transplantation into lethally irradiated recipients. In the most stringent version of this assay, known as serial transplantation, HSC-containing donor bone marrow can be re-transplanted into secondary, and tertiary, recipients while maintaining both self-renewal and multi lineage differentiation capacity. These functional assays provided opportunities to identify the cell-surface phenotype of mouse HSCs, allowing their purification by fluorescence-activated cell sorting (FACS) [2, 11].

The cell surface profile of murine hematopoietic stem cells

Lineage Markers. Primitive hematopoietic cells, including HSCs, do not express the surface markers that are associated with the terminal maturation of specific blood cell types.

Lack of expression of these lineage (lin) markers (i.e., CD3, B220, Gr1, Mac1, and Ter119), which includes all of the mature hematopoietic cell types (T-cells, B-cells, granulocytes, monocytes, and RBCs) can be used to isolate immature cells from the more differentiated cells. Further, murine bone marrow (BM) HSCs were distinguished as cells that do not express cell-surface markers of lineage-committed hematopoietic cells (Lin-) but do express high levels of stem-cell antigen 1 (Sca-1) and c-Kit, which is known as the LSK (Lin– Sca-1+c-Kit+) subset. Because only some phenotypic LSK HSCs have LTR activity, they can be further subdivided into long-term (LT)-HSCs, which are CD34–, fms-related tyrosine kinase 3 (FLT3)– CD150+, and short-term (ST)-HSCs, which are CD34+FLT3– and have only limited self-renewal capacity [12].

Human hematopoietic stem cells

Although, it has been shown that HSCs can proliferate and differentiate *in vivo*, there is as yet no *in vitro* assay that specifically detects HSCs. They divide poorly in semisolid media. They are phenotypically separable from most of the cells able to form mature myeloid colonies, erythroid, and/or megakaryocyte progeny *in vitro*. These colony-forming cells (CFCs) are considered to comprise a large, intermediate progenitor compartment that spans the entire stepwise process of lineage restriction. Long-term culture initiating cell (LTC-IC) assays can detect some [13, 14] but not all HSCs, which are more primitive than CFCs. For detecting human HSCs, the use of immunodeficient mice can be also a helpful assay [15]. The NOD/SCID mouse assay has provided a useful tool in the *in vivo* analysis of human hematopoietic stem cells, although only the short-term repopulating ability of stem cells can be analyzed due to the development of lymphomas and leakiness of murine B and T cells in aging NOD/SCID mice.

The cell surface profile of human hematopoietic stem cells

HSCs and primitive progenitors in human, like those in mice, are also distinguished from the majority of the cells in hematopoietic tissues by their lack of expression of various markers specific to maturing blood cells (i.e., CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD56, and CD66b).

CD34 was the first differentiation marker to be recognized on primitive human hematopoietic cells and is still the most commonly used marker to obtain enriched populations of human HSCs and progenitors for research or clinical use. CD34 is expressed on ~1-4% of the nucleated cells in normal human bone marrow (BM) aspirate samples and <0.1% of nucleated cells in steady-state human peripheral blood [16]. Human HSCs can also be purified based on functional markers, such as the detoxifying enzyme aldehyde dehydrogenase

(ALDH) [17]. Hess et al. showed that Lin^- $ALDH^{high}$ from umbilical cord blood cells (UCB) can repopulate and result in human cell engraftment in NOD-SCID mice whereas UCB-Lin^- $ALDH^{low}$ hardly repopulate [17].

Hematopoietic stem cell nice

The stem cell niche is a term occasionally used to describe the stem cells location, however, the niche is actually composed of cellular components surrounding stem cells and signaling molecules provided by supporting cells [2, 18].

Within the human body, stem cell niches maintain adult stem cells in a quiescent state, but at situations like tissue injury, the surrounding microenvironment actively signals to stem cells to either increase self renewal or differentiation to form new tissue.

When stem cells are cultured in vitro, they differentiate, change their morphology and lose their self-renewal ability. Hence there is a lot of activity to study the various components of the niche to try to replicate the in vivo niche conditions in vitro. Several factors have been shown to be important for the regulation of stem cell characteristics within the niche but it is as yet not possible to effectively maintain/expand HSCs in vitro [2, 19, 20].

Interactions between stem cells and neighbor differentiated cells

The bone marrow consists of hematopoietic and non-hematopoietic (stromal) cells. It has been demonstrated that stromal cells have an active role in the regulation of HSCs differentiation into all blood cell lineages[21]. In 1978 Schofield predicted that there is a specific hematopoietic stem cell niche in BM, which "fix" the stem cells in place and prevent their maturation, allowing the stem cell to proliferate and retain its stemness [22]. In 2003, two groups reported that the size of the HSC pool *in vivo* is influenced by the bone-forming osteoblast cells, identifying that osteoblast cells are the critical component of the HSC niche [23, 24]. In these studies it was shown that the high level expression of bone morphologenetic protein (BMP) receptor type IA and notch ligand jagged 1 on oesteoblast cells increase the number of HSCs through stimulating the BMP pathway and Notch signaling, respectively [23, 24].

Products of osteoblasts that have been shown to be positive regulators of HSCs include angiopoietin-1 (Ang1), thrombopoietin (TPO) and Jagged-1 (under conditions of parathyroid hormone/parathyroid hormone-related peptide receptor (PPR) activation) whereas osteoblast-associated negative regulators of the HSC niche include osteopontin (OPN) and dikkopf1 [25-30].

Osteoblasts also abundantly express CXCL12, a chemokine that is involved in chemotaxis, homing, survival and maintenance of HSCs in the bone marrow. However, it should be noted that other non-osteoblast "reticular" cells in the marrow also express high levels of CXCL12 and may participate in the niche [31, 32]. Osteoblastic cells are a heterogeneous population thought to derive from multipotent mesenchymal progenitors. The specific role of each subset of osteoblastic cells in the niche has yet to be determined.

In this context, other cell types derived from mesenchymal progenitors, such as adipocytes and osteoclasts are also involved in the regulation of HSCs activity (Fig.3). In one study it was demonstrated that adipocytes secret adiponectin molecule that increases proliferation of HSCs, while maintaining their immature state [33]. In contrast, another study showed that the content of adipocytes in the marrow negatively correlates with the hematopoietic activity [34]. Osteoclasts also play a role in the mobilization of hematopoietic progenitors in the circulation through the cathepsin K-mediated cleavage of CXCL12. Furthermore, the osteoclastic bone absorption actively changes the Ca+2 concentrations in the BM microenvironment, which is important in HSC engraftment. HSCs deficient in the calcium sensing receptor were unable to engraft bone marrow [35]. Besides osteoclasts, other cell types participate in HSC regulation in the marrow, such as endothelial cells[36]. All blood vessels are covered by endothelial cells and in the BM they build a barrier between the developing hematopoietic cells and the blood [37,38]. BM endothelial cells constitutively express cytokines, such as CXCL12, and adhesion molecules, such as endothelial-cell (E)-selectin and vascular cell-adhesion molecule 1 (VCAM1), which are important for HSC mobilization, homing, and engraftment.

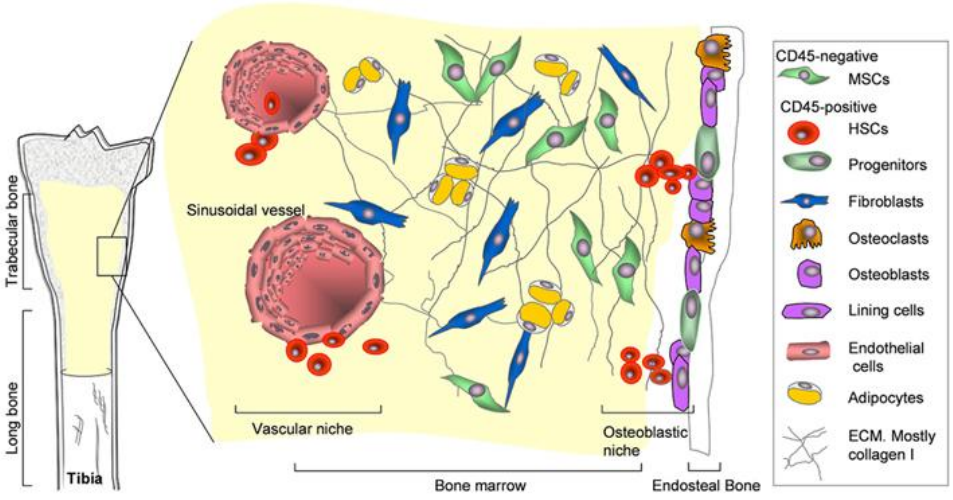

Figure 3. The stem cell niches in bone marrow. In the bone marrow, HSCs and their progeny populate the vascular niche which is surrounded by stromal cells derived from MSCs. Naïve MSCs with true stem cell attributes are part of the stroma while MSCs which are committed osteoprogenitor cells reside in the osteoblastic niche (Taken from Grassel et al. FBS 2007).

The sympathetic nervous system (SNS) also plays an important role in regulation of HSC mobilization between BM and bloodstream [39, 40]. It was shown that the SNS neurotransmittor, norepinephrine (NE), controls G-CSF-induced osteoblast suppression, bone CXCL12 downregulation, and HSC mobilization [39]. Additional studies in the mouse revealed

that the cyclical release of HSCs and expression of CXCL12 in the bone marrow microenvironment was regulated through circadian NE secretion by the SNS [40, 41]. Neurotransmittors also have roles in human HSC mobilization, proliferation and differentiation [41, 42].

Based on all these studies, it is clear that the HSC niche is much more complex than consisting of one or two cell types and further studies will be required to fully clarify how each of these cell types contributes to the HSC niche.

Extracellular matrix components

The concept of extracellular matrix (ECM) regulating primitive cells is a longstanding debate. As part of the bone marrow niche, it has been shown that ECM proteins influence human haematopoiesis.

One *in vitro* study explored the capacity of human HSCs adhesion to the ECM secreted by human marrow fibroblasts, including fibronectin, and discovered that adherence to the matrix varied both with the cell lineage and maturation stage of the progenitor cell [43]. A study on the spatial location of ECM proteins including fibronectin, collagen types I, III and IV, and laminin in murine femoral bone marrow (BM) by immuno-fluorescence labeling has revealed distinct locations of each protein, supporting the notion that they have an important role in the homing and lodgment of transplanted cells [44]. Whether there is a preference for a specific subset of primitive hematopoietic cells to adhere to certain ECM proteins within the adult BM remains to be explored. Many reports have documented the importance of β1 integrins, particularly α4β1 (VLA-4) and α5β1 (VLA- 5), in modulating adhesive interactions between HSCs, cellular and ECM components that comprise the stem cell niche [45-47]. One study demonstrated that VLA-5 is expressed on mouse and human long-term repopulating hematopoietic cells. It binds to fibronectin in the ECM and disruption of this binding can lead to decreased engraftment in the BM [48]. The deletion of α4 integrin induced by Mx1-Cre has shown that α4 integrin-deficient HSCs accumulate in the peripheral blood [49]. Furthermore, in transplantation studies, $\alpha 4^{-/-}$ cells displayed impaired homing to the BM, and short-term engraftment was severely delayed [49].

Another study on the cell–matrix interaction focused on laminins, a group of ECM proteins expressed in BM, that bind HSCs through their cognate receptors [50]. Specific expression of p67 laminin receptor was found on erythroid HPCs and blocking p67 binding on donor cells with antifunctional antibody leads to reduced BM homing of BFU-Es [51]. Whether non-erythroid HSCs can interact with laminins through other receptors is still unanswered.

Growth factors

Stem cell factor (SCF)

The gene encoding stem cell factor (SCF) is found in the Sl locus in mice and on chromosome 12q22-12q24 in humans [52]. SCF is produced by fibroblasts and endothelial cells and is active in both soluble and trans-membrane forms [53, 54]. SCF binds to c-kit (SCF

receptor, CD117) and induces the homodimerization of c-kit receptor, activating the intracellular domain of the receptor, and triggering cellular pathways that regulate HSC activity [55]. Even before identifying the SCF and c-kit as a receptor-ligand pair, mouse studies revealed that mutation in c-kit kinase domain reduces colony-forming units spleen/HSCs activity [55].

The role of c-kit was also examined in adult mice by applying the anti c-kit antibody, ACK2, which resulted in a striking decrease in the number of HSCs and other hematopoietic progenitor cells in the bone marrow [56]. It has been shown that SCF promotes HSCs survival and migration but not proliferation by activating the AKT and ERK1/2 pathways. c-Kit, is a type III receptor protein-tyrosine kinase and activates the phosphoinositide 3-kinase (PI3K) pathway, which indirectly causes AKT phosphorylation followed by phosphorylation and inhibition of death proteins (FOXO3 and BAD). SCF also can effectively promote growth and survival signaling pathways by downstream effectors of the PI3K/AKT signaling pathway like mTOR, which has downstream effectors such as p70S6K and 4E-binding protein 1. Parallel to the AKT pathway, ERK1/2 is phosphorylated by a kinase cascade (RAF to MEK to ERK1/2) that is activated by RAS [57].

Thrombopoietin (TPO)

The human and mouse thrombopoietin (TPO) gene is located on chromosome 3 (q26.3-27) and 16, respectively. TPO is produced primarily in the liver and its receptor, Mpl, is expressed on platelets, megakaryocytes and the HSC compartment, including long-term HSCs (Fig.4). Approximately 50% of the murine fetal liver stem cell-enriched population, 70% of the murine marrow LSK cells, and 70% of the human bone marrow $CD34^{+}CD38$- express c-mpl [58]. TPO production is constant and the concentration of available circulating TPO is controlled by the Mpl receptors available on the surface of megakaryocytes and platelets to internalize the cytokine [59-62]. Transplantation assays also revealed that LSK cells from wild type mice engraft almost 7-fold better than LSK cells from c-mpl deficient mice [58]. The distinct role of TPO in postnatal HSC maintenance is accompanied by accelerated HSC cell-cycle kinetics in $Tpo^{-/-}$ mice [62] and reduced expression of the cyclin-dependent kinase inhibitors $p57^{Kip2}$ and $p19^{INK4D}$ as well as multiple Hox transcription factors. Thus, TPO regulates post-transplantation HSC expansion as well as the maintenance of adult quiescent HSCs. TPO is also the primary regulator of platelet production. Taken together, these observations highlight the key role for TPO signaling in control of platelet number and regulation of HSCs [59,62,63].

TPO works through inducing receptor dimerization which results in tyrosine phosphorylation, and a series of signaling events including activation of JAK/STAT, Shc/Ras/MAPK and PI3K/Akt. These pathways overlap with those induced by other cytokines , but the differences that lead to the unique biological effects of TPO that can maintain long term reconstitution cells without combination with other growth factor, have not been well characterized [27, 64, 65].

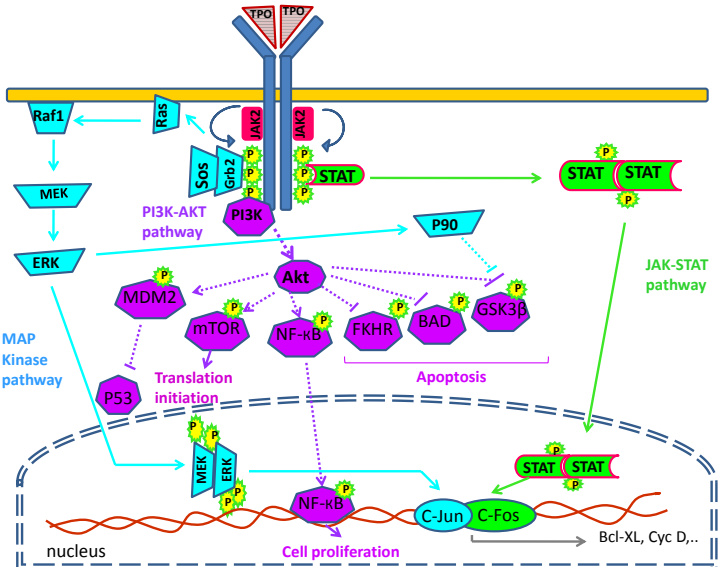

Figure 4: signalling pathway of TPO in HSCs. In the absence of ligands, cytokine receptor (Mpl) is preassembled with tyrosine kinase JAK in inactive complexes. Cytokine binding to extracellular domains of receptors induces a conformational change which allows the appended JAKs to cross- phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become phosphorylated themselves and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatydylinositol-3-kinase, PI3K, and Akt) (Taken from Stefan Constantinescu Signal transduction and molecular hematology group [Structure and function of cytokine receptors]).

Fms-related tyrosine kinase 3 ligand (Flt3L)

Fms-related tyrosine kinase 3 ligand (Flt3L) belongs to a small group of growth factors that regulates proliferation of early hematopoietic cells. The chromosomal location of the Flt3L gene is preserved on chromosome 7 in mouse and chromosome 19 in human [66, 67]. Flt3L is expressed in bone marrow fibroblasts and stromal cells of adherent layers of long-term bone marrow culture (BMC). While multiple Flt3L isoforms have been identified the transmembrane isoform is predominant.When proteolytically cleaved, the transmembrane isoform becomes soluble and biologically active [66]. The Flt3L receptor, CD135, is a tyrosine kinase type III receptor and is predominantly expressed on immature hematopoietic stem and progenitor. When this receptor binds Flt3L, it forms a homodimer and activates signaling through second messengers and supports precursors survival in different lineages of blood-forming cells such as CFU-GM, CFU-GEMM, and the very primitive high proliferative potential colony-forming cells, HPP-CFC [68]. Although, Flt3L alone cannot stimulate proliferation, it synergizes well with other CSFs and interleukins (G-CSF, GM-CSF, M-CSF, IL3, IL6, IL7, IL11,

IL12 and SCF) to induce growth and differentiation [69, 70]. In adult murine BM cells FLt3L is a marker of short term hematopoietic stem cells ST-HSCs. ST-HSCs have more limited self-renewal capacity and are capable of giving rise to blood lineages for 8–12 weeks [71]. However, using Flt3L-deficient mice it was shown that HSC expansion in fetal liver or after transplantation is Flt3L independent. Moreover characterization of HSCs in Flt3(-/-) mice revealed that the FLT3 receptor is not required for HSC steady-state maintenance or expansion after transplantation [72], suggesting that Flt3 receptor and ligand are not critical regulators of mouse HSCs, neither in steady state nor during fetal or posttransplantation expansion [72].

Insulin like growth factor 2

Insulin-like growth factor-2 (IGF-2), also known as somatomedin A, is a member of the insulin family of polypeptide growth factors. The Mouse Igf2 gene on chromosome 7 and human IGF-2 gene on chromosome 11 both extend over 30 kb, containing 8 exons (5 noncoding and 3 coding exons).

In rodents IGF-2 expression is widespread in prenatal tissues and dramatically diminishes after birth, therefore, IGF- 2 functions as a fetal growth factor. In humans however, IGF-2 is expressed in various tissues throughout life and helps to maintain self-renewal and pluripotency of stem cells [73-75].

IGF2 activates both the IGF-1 receptor and a splice variant of the insulin receptor (IR) known as IR-A. IGF2 action through the IR-A, promotes the self-renewal of stem/progenitor cell. IGF2 binds to the IGF-2R, which mediates IGF2 shuttling to lysosomes for degradation. Although, its role in signaling has not been clearly demonstrated [76, 77], Zhang *et al*. reported that HSC-supportive mouse fetal liver $CD3^+$ cells express IGF2 and the angiopoietin like protein family that can stimulate HSCs expansion [78]. The recent study by Chou *et al.*, clarified that these cytokines are expressed from SCF^+DLK^+ stromal cells of fetal liver and not from $CD3^+$ cells [79]. IGF-2 in combination with other growth factors, promoted expansion of day-15 fetal liver $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ long-term (LT)-HSC numbers about ~ 2-fold [80].

Fibroblast growth factor-1 (FGF-1)

The human Fibroblast growth factor-1 (FGF-1) gene maps to Chromosome 5 at position q31 [81]. Acidic fibroblast growth factor is derived from beta-endothelial cell growth factor by posttranslational processing [82]. FGF-1 belongs to the families of FGFs of which, to date, 22 FGFs and four FGF receptors have been identified in vertebrate genomes. All FGFs have a high affinity for heparin and for cell surface heparin sulfate proteoglycan. This complex is crucial for high-affinity binding of FGF to its receptors [83].

FGF-1 is a potent stimulator of stem cell activity *in vitro*. All long-term repopulating HSC activity is contained in the lineage-depleted, FGF receptor (FGFR)-positive cell population in mouse bone marrow (BM). Initial studies demonstrated that FGF-1 induces granulopoiesis and megakaryocytopoiesis [84]. Both FGF-1 and FGF-2 can sustain the proliferation of hematopoietic progenitor cells and maintaining their primitive phenotype [85-87]. FGF-1 was

shown to be involved in the expansion of multilineage, serially transplantable long-term repopulating HSCs. Most noticeable is that the culturing of unfractionated mouse bone marrow in serum-free media supplemented with only FGF-1 results in a significant and sustained expansion of cells with both lymphoid and myeloid repopulating capacity [88].

Although the mechanism of FGF-induced signal transduction is not well-defined, FGF causes rapid FGFR dimerization on the cell surface, resulting in intracellular polypeptides phosphorylation, e.g. MAP kinases which correlates with cell proliferation induction, and inducing tyrosine phosphorylation of Src during the entire G1 period, which correlates with migration [89]. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment [90].

Angiopoietin like protein 3

Angiopoietin like protein 3 (Angptl3) is a 455 amino acid protein, encoded by seven exons on mouse chromosome 4, and human chromosome 1. This polypeptide has the characteristic structure of Angiopoietin: a signal peptide, an extended helical domain to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen homology domain (FHD). However, unlike the other angiopoietins, Angptl3 does not bind to the tyrosine kinase receptor Tie-1 or Tie-2, and was suggested that Angptl3 functions differently from the other family members [91].

Several members of the Angptl family play roles in regulating lipid metabolism and angiogenesis [92]. In particular, Angptl3 inhibits lipoprotein lipase activity *in vitro* and *in vivo*, and mice deficient in Angptl3 display defects in lipid metabolism[92, 93]. In addition, it was shown that Angptl3 is capable of binding to the cell-surface integrin αVβ3, and stimulating the adhesion and migration of endothelial cells and inducing blood vessel formation. Angptl3 also activates protein kinase B and increases the permeability of glomerular endothelial cells [93, 94]. Several studies suggested that Angptl3 plays a role in the regulation of HSC activity [78, 95].

HSCs in Angptl3$^{-/-}$ mice were decreased in numbers, quiescence, and repopulating activity. In addition Angptl3, which is secreted by BM endothelial and other stromal cells, binds directly to the HSC cell surface and regulates HSC activity by repressing the expression of the Ikaros transcription factor. Its over-expression diminishes the repopulation activity of HSCs [95].

Recently, it has been shown that the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several ANGPTLs including Angptl3[96]. Therefore, Angptl3 as an extrinsic factor directly binds to HSCs and supports expansion of repopulating cells in coculture experiments. As a consequence, combination of saturated levels of SCF, TPO, IGF2, FGF1 and Angptl3 were able to promote expansion of LT-HSCs up to ~30-fold [78].

Physiochemical nature of the niche environment

Ca $^{2+}$concentration

The osteoblast lineage, responsible for bone formation, is one of the key cellular components of the HSC niche [23,97, 98]. The endosteum, where active bone modeling and remodelling takes place, has a high extracellular concentration of calcium-ions, reaching up to 40mM near the resorbing osteoclasts. This is more than 20-fold the calcium-ion concentration in serum[99]. Hence, a unique features of bone that contribute to the microenvironmental niche for stem cells might include the high concentration of calcium ions at the HSC-enriched endosteal surface [99, 100].

Because Ca^{2+} Receptor (CaR) is also expressed on haematopoietic cells, including monocyte/ macrophage lineage cells, it has been postulated that CaR might affect stem cell responses to the unique endosteal microenvironment [35, 101]. To prove this hypothesis, $CaR^{-/-}$ mice were studied, which revealed that these mice have abundant primitive HSCs in their spleen and in the circulation but few HSCs in BM. However, the number of HSCs in fetal liver was normal. In addition, the numbers of progenitor cells were relatively low compared to other blood lineages in BM of $CaR^{-/-}$ mice. $CaR^{-/-}$ mice do not have any problem in transporting HSCs from fetal liver to BM but that they cannot home properly in the endosteal niche. Taken all together while the calcium concentration proved to be an important factors in HSCs niche biology, but the mechanism still needs to be elucidate [35].

Oxygen gradients

The oxygen concentration within the human bone marrow has a gradient from below 1% in hypoxic niches up to 6% in the sinusoidal cavity [102]. Most slow cycling hematopoietic stem cells are found in the hypoxic zones close to the bone surface and distant from capillaries, raising the possibility that these hypoxic niches are important for diminished HSC proliferation [103]. That quiescent HSCs situated in a hypoxic environment has recently been confirmed in mice by injecting Hoechst 33342 dye which increases the *in vivo* level of oxygen. Bone marrow cells from injected mice with Hoechst 33342 dye were isolated and divided according to dye diffusion gradients. To investigate the stem cell characteristics, cells with low and high Hoechst fluorescence were transplanted into lethally irradiated mice. The results showed that the bone marrow fraction with the lowest Hoechst-dye uptake, referred as hypoxic, had the highest amount of long-term repopulating cells [104].Therefore, it was postulated that hypoxic niches in the bone marrow microenvironment provide optimal conditions for HSC maintenance.

The oxygen level in the hypoxic niche at the molecular level regulates the heterodimer transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 consists of 2 subunits, α and β, of which α is oxygen sensitive and β is oxygen insensitive. Three regulatory HIF subunits were characterized as HIF-1α, HIF-2α, and HIF-3α. At oxygen levels above 5%, hydroxylation of the proline residues 402 and 564 of HIF-1α enables binding of the ubiquitination ligase von Hippel-Lindau tumor suppressor protein, which leads to HIF-1α proteosome degradation

[105]. In contrast, at oxygen levels below 5%, hydroxylation is inhibited leading to HIF-1α stabilization. Consequently, HIF-1 heterodimer transcription factor targets several genes that are involved in HSC maintenance, including erythropoietin (EPO), erythropoietin receptor (EPO-R) transferrin and its receptor, VEGF and its receptor VEGF-R (Flt1) [106, 107].

It has also been proposed that oxidative stress suppresses N-cadherin-mediated HSC adhesion to osteoblasts and induces HSCs to exit the niche [108].

Hematopoietic stem cell transplantation (HSCT)

Hematopoietic deficiencies are potentially curable with hematopoietic stem cell transplantation (HSCT) replacing defect HSCs with healthy HSCs. Subsequently these healthy HSCs generate different hematopoietic lineages in the patients' body for lifelong [109-111]. Stem cell transplantation can be performed with cells from a family member or an unrelated (matched) volunteer (allogeneic transplantation) or with stem cells previously collected from the patient (autologous transplantation) [111, 112].

In autologous transplantation the cells from the patient are used, and there is no need to look for a human leukocyte antigen (HLA) match and no risk for graft-versus-host disease (GvHD). In case of leukemias, however the disadvantage of an autologous transplant is the possibility that the graft contains malignant cells and the lack of a graft-versus-tumor effect. Therefore autologous transplantation is not the best alternative for patients with a malignant disease. For these patients an allogenic transplantation is the most suitable treatment [113, 114]. However, the outcome of allogenic transplantation is strongly affected by the degree of HLA-matches which can result in a higher or lower chance of graft rejection (Host-versus-Graft reaction (HvG)) and Graft-versus-Host Disease (GvHD)[115, 116]. Furthermore, patients who receive allogenic transplantation require a longer recovery period. This is due to the interaction of the donor-host immune system and immune suppression treatment prior to transplantation, resulting in an increased infections risk in allogenic compared to autologous transplantation [116]. Taken together, allogenic and autologous transplantation each has its own advantages and disadvantages. Hence, a decision on the type of HSCT is made in each case based on various factors [116].

Sources and procurement of HSC

The sources of HSC are BM, peripheral blood, or umbilical cord blood (UCB) for autologous or allogeneic transplantation.

Bone marrow

Bone marrow is the internal tissue of large bones and consists of two types of stem cells, hematopoietic stem cells and stromal stem cells. Hematopoietic stem cells give rise to all different blood cells and stromal stem cells generate fat, cartilage and bone. On average there is about 2.6kg of bone marrow in adult humans.

For transplantation BM is obtained from the posterior iliac crests of the donor under epidural or under general anesthesia. If the amount of bone marrow aspirate is insufficient, the anterior iliac crest or sternum can also be used [117]. BM aspirate can be used immediately for transplantation or alternatively be stored at 4°C for 24 hours with no loss of stem cell viability [117]. Generally, 2×10^8 nucleated BM cells per kg of patients' body is sufficient to provide a stable long-term engraftment, although this number can be reduced to 1×10^8/kg [117, 118].

Peripheral blood stem cells

Peripheral blood stem cells (PBSCs) are currently the most widely used source of HSCs for allogenic transplantation. PBSCs are isolated from donor peripheral blood by apheresis. In this method peripheral blood is withdrawn and after isolation of a particular constituent, PBSCs in this case, the remainder is returned to the donor. Prior to apheresis, donors are treated with several sub-cutaneous injection of granulocyte-colony stimulation factor to increase the number of HSC and mobilize them from BM to PB [119-121]. It is known that PBSCs engraft faster than BM-derived stem cells. For instance neutrophil and platelet engraftment after PBSC transplantation is seen 14 and 18 days, respectively, compared to 19 and 25 days in case of BM transplantation [119, 122-124].

Umbilical cord blood

Umbilical cord blood (UCB) has a higher HSC frequency compared to immobilized adult peripheral blood. Hence, UCB is an important HSC source to support myeloblative or non-myeloblative therapies. UCB can be accessed rapidly and easily and has a lower GVHD incidence regardless of HLA disparity. Laghlin *et al*. showed that HLA-mismatched UCB transplanted in adult patients or children required a longer period of time to engraft compared to an HLA-matched sibling or unrelated marrow transplant, whereas a UCB transplant resulted in a lower incidence of GvHD [125]. because UCB provide a greater flexibility of HLA disparity than BM or PBSC grafts, finding UCB donors in minority populations that are currently under-represented in donor registries is a high priority [126-128]. These special properties make UCB an attractive option when BM or PBHSC donors are not the best possible alternative [109]. Although the use of UCB as a stem cell source has increased significantly in recent years, especially in children and early adulthood, it is not without downsides. One of the most important drawbacks of UCB for HSC therapy is the limited available HSCs for transplantation [129, 130]. The outcome of HSC transplantation is correlated with the total nucleated cell (TNC) number transplanted per kilogram (kg) of recipients body weight [131]. As a consequence, UCB transplantation (UCBT) is likely more successful in children. To overcome the low HSC problem, two approaches have been introduced. One has utilized more than one UCB unit in order to achieve a higher number of available TNC for infusion [128, 132-134] while the second approach has attempted to expand UCB units *ex vivo* [129, 135-137].

Combining more than one cord blood unit may overcome the drawback of low cell dose in a single cord blood unit [138]. However, combining UCB units could raise concerns such as

a higher chance of GvHD or a lack of engraftment as a result of immune reactivity among the transplanted units [131, 139]. Moreover, another concern of double-unit UCB transplantation is 'graft-versus-graft' effect. In an early double-unit UCB transplantation attempt both UCB units engrafted [140]; however, only one unit contributed to long-term hematopoiesis repopulation [141, 142].

The second approach to resolve the issue of limited available HSCs for transplantation in UCB units is *ex vivo* expansion of HSCs [129, 135-137]. Many clinical applications of HSCs would become feasible if there would be a culture system that expands HSC numbers while maintaining stem-cell pluripotency [143-145]. In particular, the *ex vivo* expansion of human cord blood HSCs would make this important source of cells useful for adult applications. During the past two decades several methods have been used for *ex vivo* UCB cell expansion, such as liquid suspension culture application [146].

In liquid suspension culture, isolated UCB stem cells are exposed to a cocktail of cytokines, and growth factors for a specific period of time[129]. To establish an optimum growth factor cocktail for *in vitro* expansion of human HSCs, different combinations of growth factors have been studied. In this regard, the analysis of 16 cytokines on stroma-free cultures of $CD34^+CD38^-$ bone marrow cells indicated that the combination of FLT3L, SCF and IL3 yield the highest (30-fold) expansion of starting long-term culture-initiating cells (LTC-IC) [147]. In another study addition of Flt-3 maintained the ability of human $CD34^+$ cells to sustain long-term HSCs [148]. TPO has also been reported to increase the multi lineage growth of $CD34^+CD38^-$ bone marrow cells under stroma-free conditions when added to the cytokine combination of Flt3-L and SCF [149]. Levac *et al.* reported that the combination of SCF, TPO, and Flt3L (STF) can successfully expand UCB-derived primitive HSCs [150]. IGF2 and FGF1 were also introduced as promising growth factors in *ex vivo* expansion and proliferation of HSC [87, 151]. Zhang *et al.* reported that using a new combination of growth factors that included SCF, TPO, IGF-2 and FGF-1(STIF) supplemented with Angiopoietin-like proteins (Angptls) for 10 days resulted in a 24- to 30-fold expansion of murine LT-HSCs [78, 95]. Furthermore, IGF-binding protein 2 (IGFBP2) and Angptl5 (A5) were introduced as additional secreted proteins that support human HSC expansion [152]. Using SCF [153], TPO [154], and FGF-1 supplemented with IGFBP2 and Angptl5 the number of human SCID-repopulating cells (SRCs) increased ~20-fold [155]. However, the ideal mixture of growth factors or cytokines, and the optimum culture duration for the *ex vivo* expansion of HSCs has not yet been determined.

As a gene therapeutic modality

Gene therapy has been introduced as a new concept for the treatment of certain human diseases, which may replace or assist traditional drug therapies. In contrast to traditional drug therapy life manufactured chemicals, gene therapy aims to make use of the patients own cells after introducing the desired genetic material into the patient. Gene therapy can use several approaches such as inserting a wild type gene into a non-specific location

within the genome to compensate for nonfunctional gene, or using homologous recombination to exchange a mutated sequence with wild type DNA sequence which is more difficult.

Initial trials were focused on diseases that are caused by a single-gene defect, such as cystic fibrosis [156], hemophilia [157], and muscular dystrophy [158]. In future, the main aim would be toward curing diseases that are caused by multiple genes.

The most common strategy that has been applied in gene therapy is introducing the normal gene by viral vectors into a type of stem cell [159], lymphocyte [160] or fibroblast which is removed from patients' body. Human hematopoietic stem cells have been used in vast majority of cell-based gene therapy for blood disorders. This is due to the self-renewal and multiputent ability of HSCs which allows reduction or elimination of repeated administrations of the gene therapy. Moreover, HSCs migrate to strategic locations, such as BM, spleen, liver and lymph node that are strategic locations for liver and metabolic diseases as well as the hematopoietic disorders.

However, there are still several major concerns before gene therapy could be considered as a regular treatment approach in the clinic. One major issue is the risk of insertional mutagenesis caused by the gene therapy vector. This may cause over-expression of an oncogene(s) or inactivate a tumor suppressor gene(s). This is evaluated by following the *ex vivo* expansion of the modified stem cells. The probability of over-expression of oncogenes or inactivation of tumor suppressor genes can be dramatically decreased by such procedure.

Outlines of this thesis

Ex vivo expansion of HSCs is one of the best ways to extend the boundaries of HSCs applications in cell and gene therapy area. The technical interests of the *ex vivo* expansion of hematopoietic stem cells (HSCs) are numerous. The aims of *ex vivo* expansion of HSCs are to increase the number of mature cells, to produce specific cells for adoptive therapy, and to increase the number of primitive stem cells for SCT or gene therapy.

The culture conditions and combination of different growth factors can indeed be very detrimental for the final outcome of HSCs *ex vivo* expansion. In this respect, one of the major questions in the HSC expansion field is whether different combinations of hematopoietic growth factors (HGFs) promote proliferation of HSCs in such a way that their stemness is maintained and/ or whether it directs them toward different differentiation pathways.

It has been an enormous challenge to establish a well-defined *ex vivo* expansion condition that can proliferate HSCs and sustain their stemness. Based on the observation that HSCs undergo a huge expansion in the fetal liver during embryonic development, it was found that $SCF^{+}DLK^{+}$ stromal cells of fetal liver supports HSC expansion [79]. Microarray studies on this sub-population showed that insulin like growth factor 2 and the angiopoietin like protein family, notably Angptl2 and 3, are highly expressed in these cells. Furthermore, it was shown that cultures of mouse bone marrow (BM) side population (SP) $CD45^{+}Sca\text{-}1^{+}$ cells in serum-free medium containing SCF,TPO, IGF-2, and FGF-1 supplemented with one of several Angptls (Angptl2, Angptl3, Angptl5, Angptl7, or Mfap4) stimulated the expansion of mouse long-term (LT) HSC [78].

The first aim of this thesis was to find an optimal growth factor combination for *ex vivo* expansion of murine and human hematopoietic stem cells under serum free culture condition, with a specific focus on the effect of angiopoietin like protein 3. We compared the effect of two distinct combinations of growth factors on expansion and proliferation of murine and human progenitors, ST- and LT-HSCs *in vitro* and *in vivo*. These are the HSC established growth factor combination,SCF, TPO, Flt3L (STF), and a new combination of SCF, TPO, FGF-1, IGF-2 (STIF), which was introduced previously by Lodish *et al.* [78] at the time of starting the study presented in this thesis. We elucidated the effect of murine Angptl3 supplemented with STF or STIF on the number of progenitors, ST, and LT-HSCs of fresh, 4-,7-, and 10-day cultured murine BM-Lin$^-$/LSK cells by colony forming units, spleen colony forming and long term repopulation assays, respectively. Furthermore, the effect of over-expression of Angptl3 in murine-HSCs *in vitro* and *in vivo* was studied. Initially, we examined the effect of over-expression of Angptl3 on murine BM-HSCs *in vitro*. Next, we studied the influence of ectopicly expressed Angptl3 on ST- and LT-HSCs.

In human studies, the consequence of including Angptl3 into the most common growth factors mixtures to *ex vivo* expansion of human UCB-HSCs in serum-free culture condition was investigated. We used UCB-CD34$^+$ as a starting population and compared the effect of STF and STIF on human HSCs. The effect of Angptl3 supplemented with STF or STIF combinations of growth factors was examined on progenitors and ST-HSCs by the colony forming unit assay and by transplantation of the serially diluted cells into NOD-SCID mice.

The second aim of this thesis was to *ex vivo* expand stem cells using well-defined culture conditions, including an up-to-date growth factor cocktail, focusing on hypoxia. We determined the effect of hypoxia (5% O_2) on the expansion and proliferation of human UCB-HSCs or murine HSCs during 7 days *in vitro* culturing in the presence of the STIFA3 cocktail (SCF) [161, 162], thrombopoietin (TPO) [27, 63, 65], insulin like growth factor 2 (IGF-2) [151, 155], fibroblast growth factor 1 (FGF-1) [85, 163], and angiopoietin like protein 3 (A3) [78, 95]). We evaluated the effects of hypoxia on human cord blood HSCs, by performing *in vitro* colony assays, and bone marrow reconstitution assays to determine whether the oxygen tension alters the functioning of human cord blood HSCs in recipient immunodeficient mice. We also investigated the effects of hypoxic culture conditions on murine bone marrow HSCs. *In vitro* colony assays, colony forming unit spleen (CFU-S) assays, and transplantation of sublethally irradiated mice with graded numbers of fresh or cultured cells under hypoxic or normoxic conditions was used to compare the effect of the different oxygen levels on progenitor cells, ST- and LT-HSC.

Taken together, we wished to develop an optimal condition which makes it possible to obtain the best grafts that might be useful for therapeutic purposes in adults as well as in children in the future.

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Chapter 2

Angiopoietin-like protein 3 promotes the proliferation and expansion of hematopoietic stem cells

Manuscript in preparation

Abstract

Hematopoietic stem cells (HSCs) can be exploited in various cellular and gene therapy treatments, but their use is often hampered by the limited number of stem cells available. Therefore, HSC applications will become more feasible if stem cells can be successfully expanded *ex vivo*. Recently, the novel group of angiopoietin-like proteins, particularly angiopoietin-like protein 3 (Angptl3), was reported to increase the number of long-term (LT)-HSCs by ~30 fold. In the present study, we initially compared the effects of the SCF+TPO+FLT3 (STF) and SCF+TPO+IGF-2+FGF-1 (STIF) cocktails, the most common used cocktails for expanding HSCs on LSK cells, at the most effective time points. We found that although short term (ST)-HSC expansion was affected similarly by STF and STIF conditions, STIF was ~2.5-fold more effective than STF at increasing the number of long term (LT)-HSCs. The addition of purified Angptl3 promotes expansion of both ST- and LT-HSCs under *in vitro* and *in vivo* conditions, respectively. The highest increase in ST-HSCs (~50-fold) was achieved by culturing LSK cells for 10 days in the presence of Angptl3 together with STIF (STIFA3). The number of LT-HSCs after culturing murine BM Lin^- cells for 7 days in STF were increased over 3-fold. Culturing in STF together with Angptl3 (STFA3) led to an increase of >30 fold. Supplementing Angptl3 also resulted in a significant increase of the number of LT-HSCs after culturing murine BM LSK cells for 7 days in STF and STIF conditions. Thus the addition of these factors to standard growth cocktails for culturing HSCs may substantially enhance survival of patients receiving a bone marrow transplant.

Introduction

Hematopoietic stem cells (HSCs) have the ability to self-renew and differentiate into all blood lineages. These properties make HSCs valuable for treatment of patients with blood disorders with cell- or gene-based therapies [1-4]. However, the limited number of HSCs in bone marrow or umbilical cord blood (UCB), and the shortage of suitable donors can hamper the application of HSC[5, 6]. Therefore, *in vitro* expansion of HSCs would be beneficial to patients who require a bone marrow or UCB transplant [7, 8].

Earlier attempts to boost the number of HSCs were not successful, resulting in a preferential expansion of mature rather than immature HSCs. However, our knowledge of hematopoietic stem cell expansion has increased, resulting in new methods for promoting the expansion of progenitor cells without causing differentiation. For example, ectopic expression of the transcription factor homeobox B4 (HoxB4) resulted in 1000-fold and 40-fold expansion of murine HSCs *in vivo* and *in vitro*, respectively [9, 10]. Also, HSC numbers increased in H-2K Bcl-2 transgenic mice, in which Bcl-2 is expressed from the major histocompatibility complex H-2K promoter [11]. However, knowledge on the anti-apoptotic pathways triggered by specific factors like Bcl-2 or HoxB4, *in vitro* is lacking. In addition, the delivery of such genes to target cells is hampered by the lack of safe and effective techniques. In contrast, transient stimulation using optimized extrinsic factors is simpler and less risky.

Several growth factors have been identified to influence mouse HSC self-renewal, including Notch ligands [12], Wnt3a [13], and angiopoietin-like proteins (Angptls) [14]. Recently, a number of studies suggested that Angptl3 plays a role in the regulation of HSC activity [14]. HSCs in Angptl3$^{-/-}$ mice were decreased in number, quiescence, and repopulating activity. In addition, Angptl3, that is expressed by BM endothelial and other stromal cells, directly binds to cell surface integrin αVβ3 expressed on HSCs, and regulates HSC activity [15]. Recently, it has been shown that the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several ANGPTLs including Angptl3 [16]. It was shown that Angptl3 regulates HSCs activity by repressing the expression of the transcription factor Ikaros, whose over-expression diminished the repopulation activity of HSCs [15].

Thus Angptl3 is an extrinsic factor that directly acts on HSCs and supports expansion of repopulating HSCs in coculture experiments. As a consequence, the combination of saturated levels of SCF, TPO, IGF2, FGF1 and Angptl3 promoted expansion of LT-HSCs up to ~30-fold [14].

In the present study, we tested optimized combinations of various growth factors on progenitor, short term (ST)-, and long term (LT) -HSC *ex vivo* expansion. Moreover, we investigated the effect of Angptl3 to further promote the expansion of ST- and LT- murine HSCs under serum-free culture conditions. *In vitro* colony assays, colony forming unit spleen (CFU-S) assays, and transplantation of sublethally irradiated mice with serially diluted numbers of fresh or cultured cells under different combination of growth factors in presence or absence of Angptl3 were used to compare the effect of the different combination of growth factors and Angptl3 on progenitor cells, ST- and LT-HSC.

Material and Methods

Mice

Female *a*-thalassemic Balb/c mice, 8-12 weeks of age, were used as BM recipients and male normal littermates as donors. These mice were bred and housed under specific pathogen free (SPF) conditions at the Experimental Animal Facility of Erasmus University (Rotterdam, the Netherlands). All experiments were approved by the ethical committee of Erasmus MC, Rotterdam in accordance with legislation in the Netherlands.

Stem cell enrichment

Lineage negative (Lin$^-$) cells were purified from bone marrow (BM) cells using the BD IMag Mouse Hematopoietic Progenitor Cell Enrichment Set (BD Biosciences, Breda, The Netherlands) according to the manufacturer's instructions. Further HSC enrichment of the Lin$^-$ cells was accomplished by incubating the cells with c-kit–allophycocyanin (APC; BD Biosciences) and Sca-1–R-phycoerythrin (PE; BD Biosciences). The cells were washed once with H+H (Hank's + HEPES, 300 mOsm) and sorted into Lin$^-$/Sca-1$^+$/c-kit$^+$(LSK) cell populations using a BD FACSAria flow cytometer.

***In vitro* suspension culture**

The cells were cultured in 24-well plates (Costar tissue-culture treated polystyrene, Corning, Corning, NY, USA) at a starting density of 4-6×10^4/ml in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 1% (wt/vol) bovine serum albumin (BSA), 0.3 mg/L human transferrin, 0.1 µM sodium selenite, 1mg/L nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyaenosine, thymidine and 2'-deoxyguanosine; all from Sigma, St. Louis, MO, USA), 0.1 mM ß-mercaptoethanol, 15 µM linoleic acid,15 µM cholesterol, 100 U/ml penicillin and 100 µg/ml streptomycin as previously described[17, 18].The medium was supplemented with STIF ((murine SCF (50 ng/ml, R&D, Abingdon, UK), murine TPO (20 ng/ml, R&D), murine IGF2 (20 ng/ml, R&D), and human FGF-1 (10 ng/ml, R&D)) ± murine Angptl3 (A3) (200 ng/ml, R&D) plus heparin (10 µg/ml, Sigma) or STF ((murine SCF (50 ng/ml, R&D), murine TPO (20 ng/ml, R&D), human FLT3-L (50 ng/ml, R&D)) ± murine Angptl3 (A3) (200 ng/ml, R&D) [14]. All cells were incubated at 10% CO_2 at 37 °C.

In vitro clonogenic progenitor assays

The *in vitro* frequency analysis of HSCs and progenitor cells was performed with semi-solid colony assays. Fresh or cultured Lin^- (2×10^3), LSK (0.2×10^3) cells were plated (BD BioCoat™ Collagen IV 35 mm Culture Dishes, tissue-culture treated polystyrene) in 1 ml of semi-solid methylcellulose culture medium containing 0.8% (wt/vol) methylcellulose (Methocel A4M Premium Grade, Dow Chemical, Barendrecht, The Netherlands) in enriched DMEM as described previously [17, 19]. Colony-forming unit granulocyte-macrophage (CFU-GM) levels were determined by culturing Lin^-/LSK cells with 10 ng/ml mouse interleukin-3 (mIL-3), 100 ng/ml m-SCF and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF). Burst-forming erythroid units (BFU-E) were cultured in the presence of 100 ng/ml m-SCF and 4 U/ml human erythropoietin (H-EPO, Behringwerke, Marburg, Germany). The cells were maintained for 14 days and each experiment was performed in duplicate.

Colony forming unit spleen (CFU-S)

The appropriate number of cells from BALB/c donors were transplanted into lethally irradiated (8 Gy) BALB/c recipients (n=7 per group). A total of 1,000 or 3,000uncultured Lin^- cells or 100 or 1,000 of 4 to 7 days cultured cells (equivalent to day 0) in STF or STIF±Angptl-3 were transplanted into lethally irradiated mice (Figure 1C). Additionally, a total of 100 or 30 LSK cells were transplanted at day 0, and similarly, equivalents to 30 LSK cells of 7-day or 10 LSK cells of 10-day cultures were injected into mice. One thousand 50 Gy-irradiated BM cells were co-administered with the 30 LSK D0 or D7 cells to improve homing. Colonies were not observed in a control group receiving 1000 50 Gy-irradiated BM cells. Ten LSK cells which were cultured for 10 days at the same conditions could be transplanted without any additional cells, showing sufficient homing. The number of spleen colonies (CFU-S) was counted after 12 days.

Long-term repopulation ability assays (LTRA)

Male BALB/c cells were transplanted into sublethally irradiated (6 Gy) female α-thalassemic mice. Blood was collected monthly for six months, and red blood cell (RBC) chimerism and peripheral blood cell counts and parameters were measured using the Heska Vet Animal Blood Counter (Heska, Fribourg, Switzerland). Mouse peripheral blood was prepared in 1 ml 0.6% NaCl buffer, and microcytic thalassemic RBCs were separated from healthy cells by flow cytometry in a FACS Calibur system (BD Biosciences). The chimerism percentage was calculated using the following formula: [-0.6+SQUART((0.6^2-4×0.002)×(10.43-donor cells %))/0.004] [20].

Y-chromosome Q-PCR

Y-chromosome Q-PCR was performed to detect the percentage of donor leukocyte chimerism in the recipients. DNA was extracted from BM using a Biorad kit (Biorad, Veenendaal, The Netherlands). Specific primers for the *Sry* locus in the Y-chromosome were designed using Beacon software (New Orleans, LA, USA). The sequences were as follows (5'-3'): sense, TCA-TCG-GAG-GGC-TAA-AGT-GTC-AC; antisense, TGG-CAT-GTG-GGT-TCC-TGT-CC. As a control for total DNA, the following *GAPDH* primers were used: sense, ACG-GCA-AAT-TCA-ACG-GCA-CAG; antisense, ACA-CCA-GTA-GAC-TCC-ACG-ACA-TAC. Each reaction mixture contained 8 µl DNA template (70 ng), 8 µl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1.25 µl of each primer (5 ng/µl) in a final reaction volume of 25 µl. Q-PCR was performed in a Biorad MyiQ thermocycler as follows: 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 45 seconds.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the Mann-Whitney U test. Standard deviations of colony counts were calculated on the assumption that crude colony counts show a Poisson distribution.

Results

Angptl3 stimulates the *ex vivo* expansion of murine Lin$^-$ cells

To test the effect of Angptl3 on murine HSCs, Lin$^-$ cells were cultured *in vitro* in the presence of STF and STIF in the absence or presence of Angptl3 for 4, 7 and 10 days. STF and STIF conditions yielded similar expansion of HSC numbers. Figure 1A shows the expansion of Lin$^-$ cells. Cells that were cultured in STIF medium did not show any significant change in numbers upon the addition of Angptl3 to culture medium. However, the addition of Angptl3 to STF-containing medium significantly increased the number of cells from 50±3-fold to 59±7-fold, respectively, at day 10 (Figure 1A).

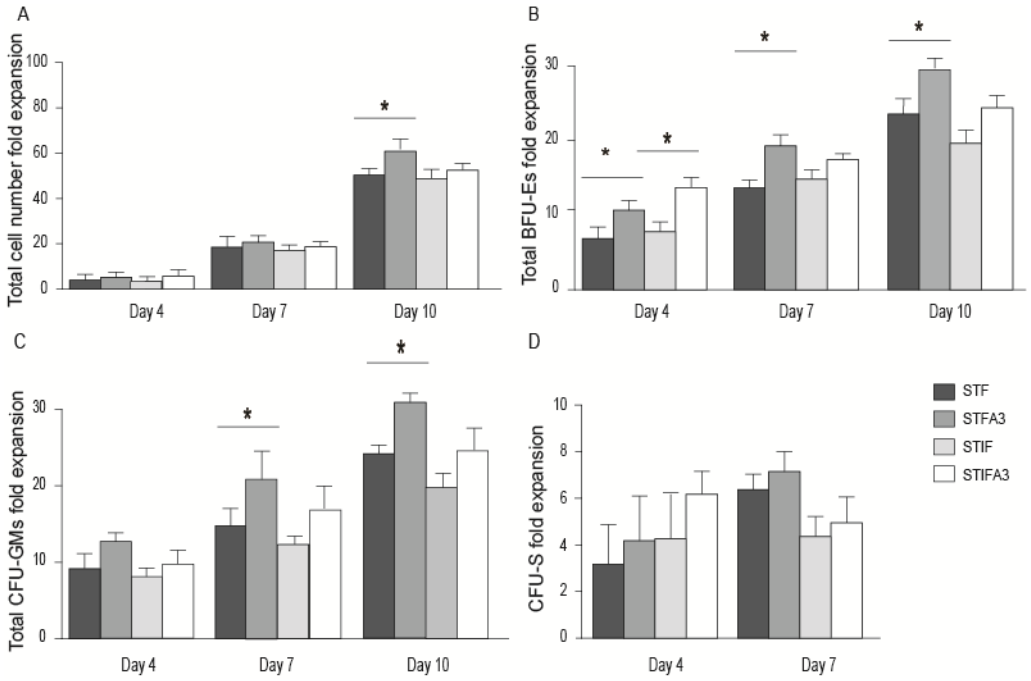

Figure 1. Angptl3 promotes the expansion of HSCs in Lin$^-$ cell populations.
(A) Lin$^-$ cells were cultured in STF, STFA3, STIF, orSTIFA3 medium for 7 days. The mean fold increase in total cell numbers was measured in comparison to day 0. The results of five independent experiments are shown. The error bars indicate the standard deviation (SD). * signifies P<0.05. **(B, C)** Colony forming-units (BFU-E and CFU-GM) of LSK cells cultured for 7 days relative to day 0. The results of 5 independent experiments are shown. **(D)** The CFU-S (12-day) expansion of LSK cells cultured for 7 days in the presence of 4 distinct combinations of growth factors relative to day 0. N=10 mice per group.

Angptl3 increases the expansion of BFU-E and CFU-GM of murine Lin$^-$ cells

Next, we performed colony-forming unit assays to test whether the increased proliferation caused by addition of Angptl3 during culture also resulted in expansion of hematopoietic progenitor cells. Culturing cells for four days resulted in a significant increase in Lin$^-$ BFU-Es when Angptl3 was added to STF- or STIF-containing medium (from 6±2- to 10±1-fold, and from 7±2- to 13±1-fold compared to day 0, respectively). This trend continued under STF conditions at days 7 and 10 of culture. The results show that under STF and STIF culture conditions, supplementing the cells with Angptl3 can further promote the expansion of immature erythroid progenitor cells. Granulocyte-macrophage progenitor cells from Lin$^-$ cells that were cultured for 7 days in STF were also increased significantly upon the addition Angptl3 (from 15±3 to 21±4). Angptl3 had a similar effect when the cells were cultured under STIF conditions, although this effect was less pronounced.

In summary, the number of progenitor cells (i.e., BFU-E and CFU-GM) after 10 days of *in vitro* culturing in STFA3- or STIFA3-containing medium increased 28±4 or 21.5±3-fold relative to day 0, respectively (Figure 1B-C). We conclude that the number of progenitor cells

was slightly higher when the cells were cultured in STF compared to STIF medium and that addition of Angptl3 further promoted expansion of progenitor cells under both STF and STIF conditions.

Angptl3 did not expand the number of short-term HSCs from murine Lin$^-$ cells

To assess the effect of Angptl3 on short-term HSCs (ST-HSCs), we cultured Lin$^-$ cells in either STF or STIF, and analyzed these using the CFU-S (12-day) assay (Figure 1D). We showed the effect of adding Angptl3 on the number of CFU-S after culturing Lin$^-$ cells for 4 days in STF medium (from 3±2 to 4±2-fold) as well as in STIF medium (from 4±2 to 6±1-fold) relative to day 0. Lin$^-$ cells that were cultured for 7 days in STF or STFA3 medium resulted in 6±1-fold or 7±1-fold expansion in the number of CFU-S compared to day 0. The similar trend was also observed under STIF conditions (from 4±1 to 5±1-fold).

As described above, we found no difference in the number of ST-HSCs when cells were cultured in medium that contained added STF or STIF. Under these conditions, also the addition of Angptl3 did not significantly affect the number of ST-HSCs.

Angptl3 causes the expansion of long-term HSCs from murine Lin$^-$ cells

Since we were interested to find out if Angptl3 would affect the expansion and proliferation of long term HSCs (LT-HSCs), we performed LTRA assays on Lin$^-$ cells cultured with Angptl3 in combination with STF. We estimated the effect of Angptl3 on the LT-HSC population of Lin$^-$ cells by serially diluted Lin$^-$ cell transplantation. Over a period of 6 months, transplantation of 3,000 or 1,000 fresh Lin$^-$ cells caused a 32±3 or 23±4% of RBC chimerism, respectively. The percent of RBC chimerism in the mice that had received the 1,000 or 300 Lin$^-$ cells that were cultured for 7 days in STFA3 increased from 45±3 (cultured in STF) to 82±6% (cultured in STFA3) or 32±4 (cultured in STF) to 63±5% (cultured in STFA3) (Figure 2A, B). We calculated the exponential rate of Lin$^-$ in STF and STFA3 conditions. The results show that culturing the cells in STF resulted in >3-fold increase in the number of LT-HSCs relative to day 0. Moreover, supplementing the culture conditions with Angptl3 caused a further ~10-fold increase compared to culturing in STF. In total therefore, culturing the cells for 7 days in STFA3 resulted in an over 30-fold increase in LT-HSC cell numbers over fresh cells (Figure 2A, B, and C).

Angptl3 does not affect the overall *ex vivo* expansion of LSK cells

To determine whether Angptl3 had a direct effect on the HSCs within the Lin$^-$ cells, or exerted this effect through action on other, supporting cells, we next tested the effect of Angptl3 on Lin$^-$ Sca-1$^+$ c-kit$^+$ (LSK) cells. LSK cells were cultured *in vitro* under 4 different conditions (i.e., STF with or without Angptl3, or STIF with or without Angptl3) for 7 days. We took this time period because it showed the optimum effect of adding Angptl3 in our Lin$^-$ experiments. There was no clear difference in LSK proliferation rates under STF or STIF conditions in 5 independent experiments. Moreover, we observed that Angptl3 did not have a

supportive or inhibitory effect on the *ex vivo* expansion of LSK cells, whether they were grown under STF or STIF conditions (Figure 3A).

However, we did find a significant difference between effectiveness of the STF and STIF culture conditions for maintaining the LSK phenotype (Figure 3B). Specifically, the percentage of LSK cells was 30±4% and 45±3% after 7 days *in vitro* culturing under STF and STIF conditions, respectively. The presence of A3, however, made no differences. This observation suggests that STIF is more effective at maintaining the stem cell's characteristics than STF.

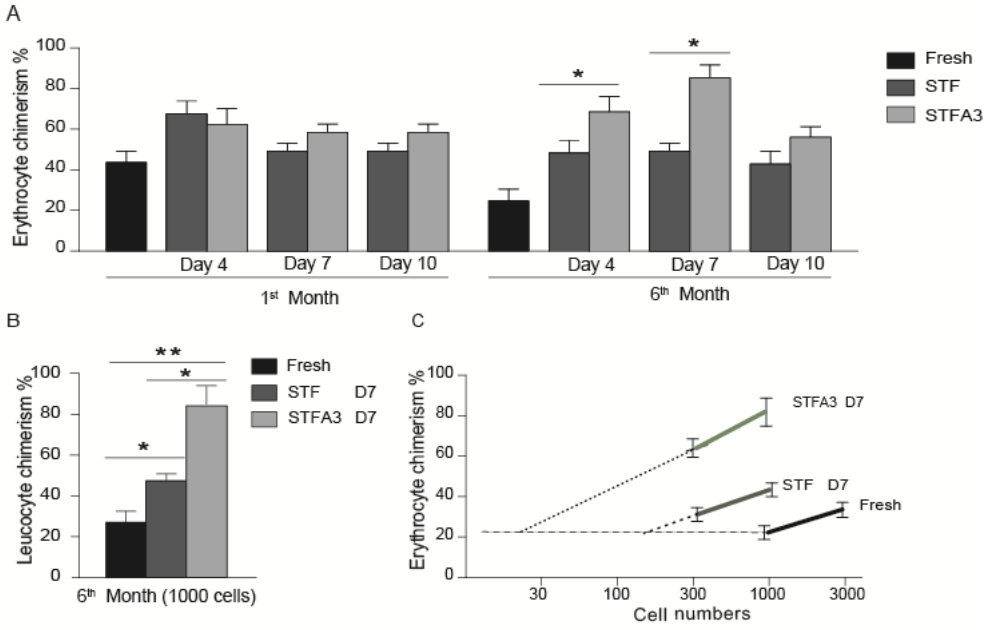

Figure 2. Angptl3 stimulates the expansion of LT-HSCs in Lin⁻ cell populations.
(A) One thousand Lin⁻ cells (equivalent to day 0), either fresh or cultured under STIF, STFA3, STIF, orSTIFA3 conditions were transplanted into sublethally irradiated recipients. The percent of RBC chimerism was determined 1 month and 6 months after transplantation. N=5 mice per group. The error bars indicate the standard deviation (SD). * signifies P<0.05. **(B)** The percentage of WBC chimerism was determined 6 months after retransplantation. N=5 mice per group. **(C)** Serial dilution of Lin⁻ cells (3,000 or 1,000) were transplanted into primary recipients. The data represents RBC chimerism of 3,000, 1,000, or 300 transplanted Lin⁻ cells after 6 months. N=5 mice per group. The error bars indicate the standard deviation (SD).

Angptl3 increases the number of colony-forming unit cells from the LSK cell fraction.

To study this effect further, we next determined the number of progenitor cells in cultured and uncultured LSK cells by performing a colony-forming unit assay. We found that after 7 days, Angptl3 enhanced the number of BFU-E by 1.45- and 1.3-fold in STF and STIF medium compared to day 0, respectively (Figure 3-D). The number of CFU-GM from the LSK cells that were cultured in STIF or STIFA3 for 7 days increased 7±3 or 14±2-fold compared to day 0, respectively (Figure 3-C).

In brief, Angptl3 significantly increased number of CFU-GMs and BFU-Es after 7 days *in vitro* culturing in presence of both STF and STIF conditions. However, there was no significant difference in the total number of BFU-Es and CFU-GMs between the STF and STIF culture conditions.

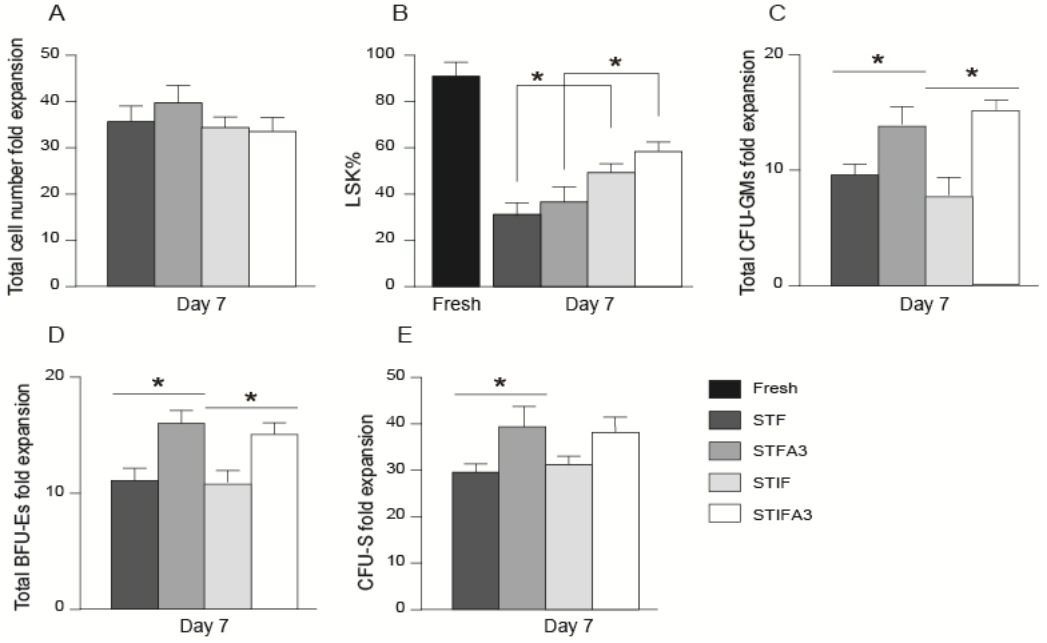

Figure 3.The effect of Angptl3 on progenitors and ST-HSCs
Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ (LSK) cells were cultured in STF, STFA3, STIF, or STIFA3 for 7 days. The mean fold increase in total cell numbers was measured in comparison to day 0. The results of five independent experiments are shown. The error bars indicate the standard deviation (SD). * signifies $P<0.05$, ** means $P<0.001$. **(B)** LSK cells were cultured for 7 days, labeled with Sca-1-PE and c-kit-APC, and analyzed by flow cytometry. **(C, D)** Colony forming-units (BFU-E and CFU-GM) of LSK cells cultured for 7 days relative to day 0. The results of five independent experiments are shown. The average number of colonies per 10^5 plated cells was calculated from duplicate experiments. **(E)** The CFU-S (12-day) fold expansion of LSK cells cultured for 7 days in the presence of 4 distinct growth factor combinations relative to day 0. N=10 mice per group.

Angptl3 increases the number of ST-HSCs from LSK cells

Our *in vitro* experiments suggested that Angptl3 has a potent stimulatory effect on the progenitor cells in the LSK cell fraction. Accordingly, we measured the number of ST-HSCs from LSK cells that were cultured in the presence or absence of Angptl3 in STF- or STIF-containing medium. Figure 3E shows that there's no difference in the number of ST-HSCs between STF and STIF, both expand ~30 fold in a week's time. However, addition of Angptl3 increased number of ST-HSCs from ~30 to ~40 in both STF and STIF.

Angptl3 increases the number of LT-HSCs from LSK cells

The effect of Angptl3 on the long-term expansion and proliferation of LSK cells was invested using the LTRA assay and estimating the number of LT-HSCs. Transplanting HSC from healthy male mice into a female α-thalassemic mice, provides the way to estimate the percentage of donor-derived populating cells based on the RBC size differences between healthy donor and α-thalassemic recipients in peripheral blood or the ratio of male or female cells in different blood lineages. Two hundred LSK cells, either fresh (non-cultured) or cultured in STF or STIF medium with or without Angptl3 were transplanted into primary recipients. After 7 months, there was almost full engraftment of donor cells in BM and PB of all groups, therefore we could not measure any differences between groups (Figure 4A). To overcome this, serial dilutions of BM cells (10^6, 3×10^5, 10^5 or 3×10^4 cells) from the primary recipients were then retransplanted into secondary recipients. After 6 months, all of the mice were healthy, and no tumors were observed. Six months after the secondary transplantation of 10^6 BM cells, the percentage of RBC chimerism of the initially fresh cells was determined to be 43%. The donor cells that were originally cultured in STF and STIF resulted in chimerism levels of 60 and 65%, respectively. For the cells that were originally cultured with additional Angptl3, these levels were increased to74±4 and 77±4 %, respectively, (Figure 4B). The percentage of leukocyte chimerism in recipient PB were measured by performing Y-chromosome PCR and found similar results (Figure 4B).

In a second experiment, primary recipients received 12 or 120 LSK cells that were either fresh or cultured for 7 daysin STF or STIF medium with or without Angptl3. The 12 or 120 freshly transplanted LSK cells were able to repopulate the peripheral blood cell compartment of the recipients after 6 months to 32±4% and 43±5%, respectively. However, when 12 LSK cells were transplanted that were first cultured for 7 days in STF or STIF medium, 37±5 or 40±4%, respectively, of the peripheral blood cells was reconstituted after 6 months. Similarly, 12 donor LSK cells that were cultured for 7 days in STFA3 or STIFA3 reconstituted approximately 45±4and 52±3%, respectively, of the peripheral blood compartment (Figure 4C-D). As an independent measure, we also performed Y-chromosome PCR and found virtually similar results in the percentage of leukocyte chimerism in recipient PB (Figure 4C). Based on these results, we concluded that Angptl3 stimulates the expansion of LT-HSCs under both STF- and STIF-containing culture conditions. We reached to >10-fold expansion in the number of LT-HSCs in the presence of STIFA3 during 7 days *in vitro* culturing (Figure 4D).

Discussion

Over the past two decades, many attempts have been made to increase the quantity or quality of LT-HSCs cultured *in vitro*. In this regard, Lodish *et al.* previously reported that the new combination of growth factors that contain SCF, TPO, IGF-2 and FGF-1 can expand LT-HSC populations. The addition of Angptl proteins, specifically, Angptl2 and Angptl3, promoted the *ex vivo* expansion of stem cells even further [14].

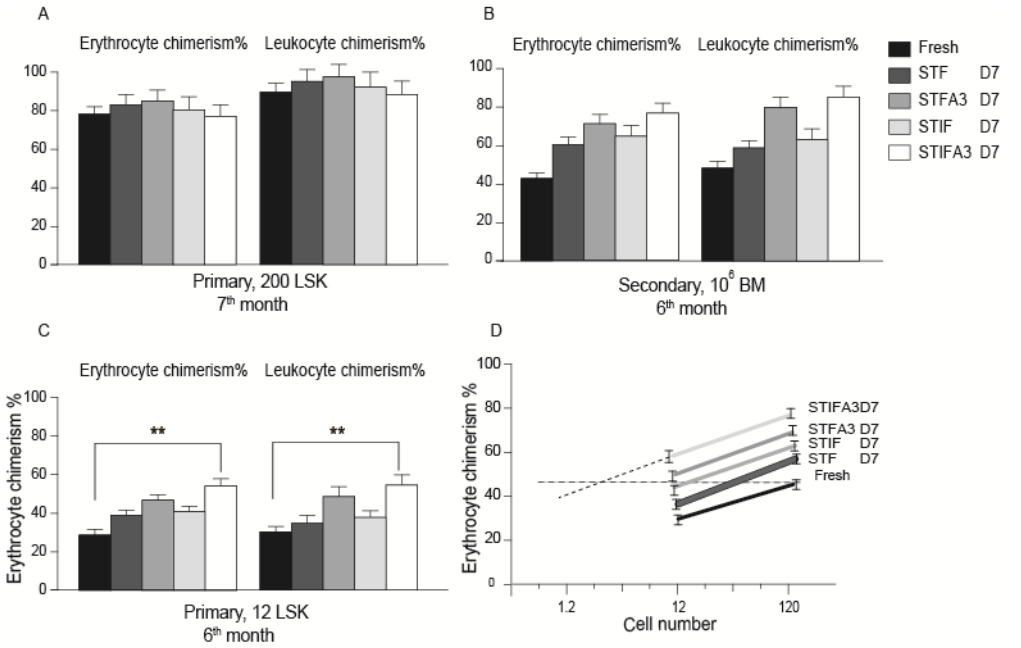

Figure 4. Angptl3 enhances the expansion of LT-HSCs in LSK cell populations
(A) Two hundred LSK cells (equivalent to day 0), either fresh or cultured under STIF, STFA3, STIF, or STIFA3 conditions, were transplanted into sublethally irradiated recipients. The percent of RBC and WBC chimerism in PB was determined 7 months after transplantation. N=5 mice per group. **(B)** Serial dilution of BM cells from primary recipients 7 months post-transplantation were retransplanted into secondary recipients. The data represents RBC and WBC chimerism in PB of transplanted 10^6 BM cells. The percent of RBC and WBC chimerism of secondary recipients was determined 6 months after retransplantation. N=5 mice per group. **(C)** The percentage of RBC and WBC chimerism in PB was determined 6 months post transplantation from primary recipients of only 12 LSK cells. N=5 mice per group. The error bars indicate the standard deviation (SD). **(D)** Serial dilution of LSK cells (120 or 12 cells) transplanted into primary recipients. The data represents RBC chimerism of 120 or 12 LSK transplanted cells after 6 months. N=5 mice per group. The error bars indicate the standard deviation (SD). * signifies $P<0.05$.

In this study we further investigated the effect of Angptl3 on expansion and proliferation of murine progenitors using a CFUs assay. Based on our study, Angptl3 did not affect the proliferation rate of LSK cells during 7 days *in vitro* culturing in STF or STIF medium. However, the relative frequency of LSK cells remained higher in cells cultured for 7 days in STIF medium (45±3%) compared to those cultured in STF (30±4%). We showed that addition of Angptl3 stimulated the expansion of progenitor cells when LSK cells were cultured for 7 days under either STF or STIF conditions. There was no significant difference in the total amount of progenitor cells cultured under STF or STIF medium.

We did not observe any alteration in the number of ST-HSCs between culturing LSK cells in STF or STIF medium, but the addition of Angptl3 increased the number of ST-HSCs under both conditions, as measured using the CFU-S assay. In addition, when we examined the effect of Angptl3 on LT-HSCs from Lin^- cells that were cultured *in vitro* in STF, we found

that the highest level of engraftment was achieved by transplanting cells that were cultured for 7 days in STF medium containing Angptl3. Culturing Lin^- cells under STF conditions resulted in a ~3-fold boost in the number of LT-HSCs compared to fresh cells. Also, after 7 days, Angptl3 further increased the number of LT-HSCs from Lin^- cells ~10-fold. Thus, by culturing the cells for 7 days in STFA3, we achieved an >30-fold expansion in the number of LT-HSCs compared to fresh cells. When investigating the effect of culturing Lin^- and LSK cells for 7 days in STFA3, we found that the stimulation of LT-HSC expansion by STFA3 was dramatically lower for LSK cells than for Lin^- cells (~10-fold and ~30-fold, respectively). We concluded that the Lin^- cell population contains additional cells that provide factors that, when combined with STFA3, are effective at expanding HSCs, and these cells are no longer present in the LSK cell population. However, culturing cells in STIFA3 has a more pronounced effect on LT-HSCs and maintains their self-renewal capacity for a longer period of time, as shown by our LTRA result that LSK cells that were cultured for 7 days in STIFA3 exhibited a >10-fold expansion relative to fresh cells, whereas culturing in STFA3 led an expansion of only 10-fold. In conclusion, STIFA3 is more effective to stimulate LT-HSCs in cultured LSK cells. Therefore, among the combinations that were tested, the combination of five growth factors (i.e., SCF, TPO, IGF-2, FGF-1 and A3) yielded the maximal expansion of mouse LT-HSCs [14, 21-23].

Angptl3 is a 455 amino acid protein encoded by the seven exons of the *Angptl3* gene that span approximately 11 kb in mouse chromosome 4. This polypeptide has the characteristic features of angiopoietins, including a signal peptide, an extended helical domain that forms dimeric or trimeric coiled coils, a short linker peptide and a globular fibrinogen homology domain (FHD). In addition, Angptl3, which is expressed by BM-endothelial and other stromal cells, binds directly to cell-surface on HSCs [15]. Recently, it has been shown that the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several ANGPTLs including Angptl3 [16]. Intracellular targets of Angptl3 in HSCs were identified by comparing the expression of several transcription factors and cell cycle regulators in HSCs isolated from Angptl3-null mutant and WT mice using real-time RT-PCR [15]. It was observed that Angptl3-null HSCs had 3-fold higher levels of Ikaros expression [15]. When the effect of Ikaros expression on HSC repopulation was measured using HSCs with enhanced Ikaros as donor cells, those recipients had dramatically decreased HSC repopulation compared with those that received control HSCs [15]. Moreover, it was shown that Angptl3 could repress the expression of Ikaros and up-regulate the expression of other genes, like Hes1 and Hoxa9, that are known to be important for HSC self-renewal and differentiation [15]. Therefore, Angptl3 is capable of supporting the stemness of HSCs through down-regulation of the expression of the transcription factor Ikaros [15].

These new growth factor combinations may promote superior engraftment using a minimal starting cell number, providing a platform for the effective treatment of a variety of diseases whose treatment are currently limited by a lack of sufficient numbers of stem cells. This method will likely improve the efficiency of other hematopoietic stem cell applications.

Acknowledgements

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Chapter 3

The effect of overexpression of angiopoietin-like protein 3 (Angptl3) on the proliferation and expansion of hematopoietic stem cells

Manuscript in preparation

Abstract

Hematopoietic stem cells (HSCs) have ability to self-renew as well as produce all types of blood cells. *Ex vivo* expansion of HSCs can improve the progress of HSC therapies, such as transplantation using umbilical cord blood. We have shown that Angiopoietin like protein 3 (Angptl3) when introduced as a hematopoietic stem cell (HSC) growth factor stimulates murine long term (LT)-HSC expansion over 30-fold. Moreover, it is shown that hematopoietic stem cells in Angptl3-null mice are decreased in number.

In this study, we show that ectopic expression of Angptl3 increased the overall proliferation rate of adult murine BM Lin^- cells during 7 days of *in vitro* culture. This was consistent with the increased number of progenitors and ST-HSCs measured using colony-forming unit and colony-forming spleen assays. 9 months post transplantation into sublethaly irradiated recipients, the percentage of transduced cells that express Angptl3 increased significantly and resulted in higher donor-derived chimerism percentage in peripheral blood (PB) and bone marrow (BM) of recipients compared to the control group. Moreover, over-expression of Angptl3 significantly increased the number of LSK cells in BM and PB of recipients, although no alteration was detected in other blood lineages. We did not detect any warning signs that could be attributed to leukemogenic or toxicity effects of Angptl3. Therefore, Angptl3 might be useful growth factor in combination with other well-known HSCs growth factors to expand HSC *ex vivo* for clinical applications.

Introduction

Hematopoietic stem cells (HSC) have the capacity to self-renew and differentiate into all blood lineages throughout life. These properties make them promising cell targets for cell and gene therapies to accomplish a permanent treatment. However, the progress of HSCs applications has been hampered by the limited number of available HSCs. One potential solution to overcome this problem is expansion of HSCs *in vivo* or *in vitro*.

To this end, the role of cytokines in regulation of HSCs fate is one of the most important aspects. Several of these cytokines, including stem cell factor (SCF) [1], thrombopoietin (TPO) [2, 3], fms-like tyrosine kinase-ligand (Flt3-L) [4], fibroblast growth factor (FGF-1) [5], insulin-like growth factor-2 (IGF-2) [6], and IGF binding protein 2 [7](IGFBP2), have been studied previously.

The Lodish group identified population of fetal liver stromal cells that supports *in vivo* expansion of HSC compartment and expresses high level of IGF-2 and angiopoietin like proteins in addition to SCF and delta like (DLK) [6, 8, 9]. It was shown that the growth factors IGF-2, Angiopoietin like 2 (Angptl2), and Angiopoietin like 3 (Angptl3) have a potential to *ex vivo* expand and proliferate HSCs [6, 9]. Recently, we confirmed the effect of Angptl3 on HSC potential by transplanting serially diluted numbers of freshly isolated or cultured cells into sublethally irradiated mice. Cells were cultured in the presence of saturated levels of HSC-growth factors supplemented with or without Angptl3. We showed that the presence of Angptl3 enhanced the expansion of murine BM LSK cells during 7 days *in vitro* culture in

medium containing the growth factors (SCF+TPO+IGF2+FGF (STIF)), and resulted in a >10-fold expansion in the number of LT-HSCs compared to freshly isolated cells.

Furthermore, the role of Angptl3 in the regulation of HSC activity has been assayed *in vivo* by studying Angptl3-null and WT mice. The HSCs in the Angptl3-null mice were decreased in number and showed reduced quiescence and repopulating activity [10]. Angptl3 regulates HSC activity by suppressing expression of the transcription factor Ikaros whose over-expression results in a reduction of the repopulation potential of HSCs [10].

Following this, we have now studied the effect of Angptl3 over-expression on the expansion and proliferation of HSCs, *in vivo*, and *in vitro*. We examined whether over-expression of Angptl3 gives leukemogenic or toxic effects in recipients. To model the effect of Angptl3 over-expression in HSCs, lentivirally-transduced adult murine bone marrow Lin^- cells were transplanted into sublethally irradiated mice. The percentage of transduced cells in the control and experimental groups were monitored at different time points up to 9 months.

Material and Methods

Mice

Female *a*-thalassemic Balb/c mice, 8-12 weeks of age, were used as BM recipients and male normal littermates as donors. These mice were bred and housed under specific pathogen free (SPF) conditions at the Experimental Animal Facility of Erasmus University (Rotterdam, the Netherlands). All experiments were approved by the ethical committee of Erasmus MC, Rotterdam in accordance with legislation in the Netherlands.

Stem cell enrichment

Lineage negative (Lin^-) cells were purified from bone marrow (BM) cells using the BD IMag Mouse Hematopoietic Progenitor Cell Enrichment Set (BD Biosciences, Breda, The Netherlands) according to the manufacturer's instructions.

Construction of lentiviral vector plasmids

The codon optimized m-Angptl3 cDNA was excised with SalI and XmaI from pUC57 plasmid and cloned in the pCCL.sin.cppt.SV40polyAeGFPminCMV.hPGK.WPRE lentiviral vector. The pCCL.sin.cppt.SV40polyAeGFPminCMV.hPGK-mAngptl-3.WPRE vector was as a consequence which can express both Angptl3 and GFP.

Production of lentiviral vectors

Third generation lentiviral (LV) vectors were produced by standard calcium phosphate transfection of HEK 293T cells. We used the packaging plasmids pMDL-g/pRRE, pMD2-VSVg and pRSV-Rev in combination with the third generation self inactivating LV transfer vectors. The pCCL.sin.cppt.SV40polyAeGFPminCMV.hPGK-mAngptl-3.WPRE vector (LV-Angptl3-GFP) contains the HIV central polypurine tract (cppt) and woodchuck posttranslational regulatory

element (WPRE) to enhance transduction efficiency. Angptl3 and GFP expression are driven by hPGK, and CMV promoter, respectively.

Titers of LV-Angptl3-GFP or LV-GFP were determined by end-point titration on Hela and 293T cells by flow cytometry. For further studies LV vector were concentrated by ultracentrifugation for 2 hours at 20,000 r.p.m and collected at 4 °C.

Lentiviral hematopoietic stem cell transduction

Lin^- cells, which were purified from the healthy BALB/c mice as described above, were transduced with the LV-Angptl3-GFP or LV-GFP vector overnight at a cell density of 10^6 cells/ml with a multiplicity of infection (MOI) of 1-2, in serum-free medium supplemented with growth factors [murine SCF (100ng/ml, R&D), murine TPO (20ng/ml, R&D), murine IGF-2 (20ng/ml, R&D)]. The next day, the cell concentration was reduced up to 5×10^4 cell/ml. After 2 days *in vitro* culturing, the GFP^+ population was sorted using the FACSAria. After sorting, the frequency of GFP^+ cells was > 90%. The sorted cells were used as the initial population in all experiments. A cocktail of the growth factors murine SCF (100ng/ml, R&D), murine TPO (20ng/ml, R&D), murine IGF-2 (20ng/ml, R&D), human FGF-1 (10ng/ml, invitrogen) (STIF) plus heparin (10 μg/ml) was prepared to promote murine stem cell expansion and proliferation. All of the cells were incubated in 10% CO2 at 37°C.

***In vitro* suspension culture**

The cells were cultured at a starting density of 2-6×104/ml in serum-free medium that contained enriched Dulbecco's modified Eagle's medium (DMEM) with 1% (wt/vol) bovine serum albumin (BSA), 0.3 mg/L human transferrin, 0.1 μmol/L sodium selenite, 1 mg/L nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyaenosine, thymidine and 2'-deoxyguanosine; all from Sigma), 0.1 mmol/L ß-mercaptoethanol, 15 μmol/L linoleic acid,15 μmol/L cholesterol, 100 U/ml penicillin and 100 μg/ml streptomycin as previously described [11, 12].

In vitro clonogenic progenitor assays

The in vitro frequency analysis of hematopoietic stem cells and progenitor cells was performed in semi-solid colony assays. Transduced with LV-GFP or LV-Angptl3-GFP or non-transduced Lin- cells were plated in 1 mL of semi-solid methylocellulose culture medium containing 0.8% (wt/vol) methylcellulose (Methocel A4M Premium grade Dow Chemical Co, Barendrecht, The Netherlands) in enriched DMEM as described previously [12, 13].

Colony-forming unit granulocyte-macrophage (CFU-GM) levels were determined by culturing Lin- cells with 10 ng/ml mouse interleukin-3 (mIL-3), 100 ng/ml m-SCF and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF). Burst-forming erythroid units (BFU-E) were cultured in the presence of 100 ng/ml m-SCF and 4 U/ml human erythropoietin (H-EPO; Behringwerke, Marburg, Germany). The cells were maintained for 14 days and each experiment was performed in duplicate.

Colony forming unit spleen (CFU-S)

The Lin- cells, obtained from the bone marrow of male BALB/c mice, were transduced with LV-GFP or LV-Angptl3-GFP. The GFP+ population was sorted 2 days after the transduction process. One thousand sorted Lin- GFP+ cells were transplanted intravenously into lethally irradiated (8 Gy) BALB/c recipients (n=7-10 mice per group). The number of spleen colonies (CFU-S) was counted after 12 days.

Long-term repopulation ability (LTRA) assays

The Lin- cells, obtained from the bone marrow of male BALB/c mice, were transduced with LV-GFP or LV-Angptl3-GFP. The GFP+ population was sorted 2 days after the transduction process. Ten thousand Lin- GFP+ cells were transplanted into sublethally (6Gy) irradiated female α-thalassemic mice.

Blood was collected monthly for nine months, and red blood cell (RBC) chimerism and peripheral blood cell counts and parameters were measured (Vet Animal Blood Counter).

Immunological phenotyping.

Flow cytometric analyses were performed on cells obtained from blood, bone marrow and spleen. Peripheral blood was collected monthly in EDTA tubes by retro-orbital puncture under isoflurane anesthesia. Complete blood cell counts were measured using a Vet ABC hematology analyzer (Scil animal care, Viernheim, Germany). Blood was lysed and leukocytes were washed three times with Hank's balanced salt solution (Invitrogen) containing 0.5% (wt/vol) bovine serum albumin and 0.05% (wt/vol) sodium azide (HBN). Cells were incubated for 30 minutes at 4 °C in HBN containing 2% heat-inactivated normal mouse serum and antibodies against CD4, CD8, B220, CD19, CD11b Sca-1, and C-kit directly conjugated to R-phycoerythrin, peridinin chlorophyll protein or allophycocyanin (all antibodies BD Biosciences). Subsequently, cells were washed and measured on a FACSCalibur (Becton Dickinson, Erembodegem, Belgium)[14]. BM and spleen cells were prepared similarly. Additionally, GFP expression and its frequencies in all different blood lineages were measured in mice treated with the LV- GFP or LV-Angptl3-GFP vectors.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the Mann-Whitney U test. Standard deviations of colony counts were calculated on the assumption that crude colony counts show a Poisson distribution.

Results

The over-expression of Angptl3 promotes the *ex vivo* expansion and proliferation of Lin$^-$ cells

Lin$^-$ cells were transduced with LV-GFP or LV-Angptl3-GFP. After 2 days, the GFP$^+$ cells in the LV-GFP or LV-Angptl3-GFP transduced populations were sorted using the FACSAria. Sorted cells from both LV-Angptl3-GFP, LV-GFP (negative control) were used to measure the expression of Angptl3 at the protein level using western blots (Figure 1D). The sorted cells were cultured immediately in the STIF growth factor cocktail, the day that cells were sorted is considered day 0. Cell numbers were counted on day 4, and 7. As shown in Figure 1, the cell numbers increased significantly in the LV-Angptl3-GFP transduced cells compared to those transduced with LV-GFP. In the period of 4 and 7 days, the number of LV-GFP cells increased ~5-fold and 7-fold, respectively, relative to day 0. At these same two time points, the number of LV-Angptl3-GFP cells increased ~11-fold and 20-fold, respectively. This result indicates that over-expressing Angptl3 results in a greater than 2-fold extra expansion of Lin$^-$ cells.

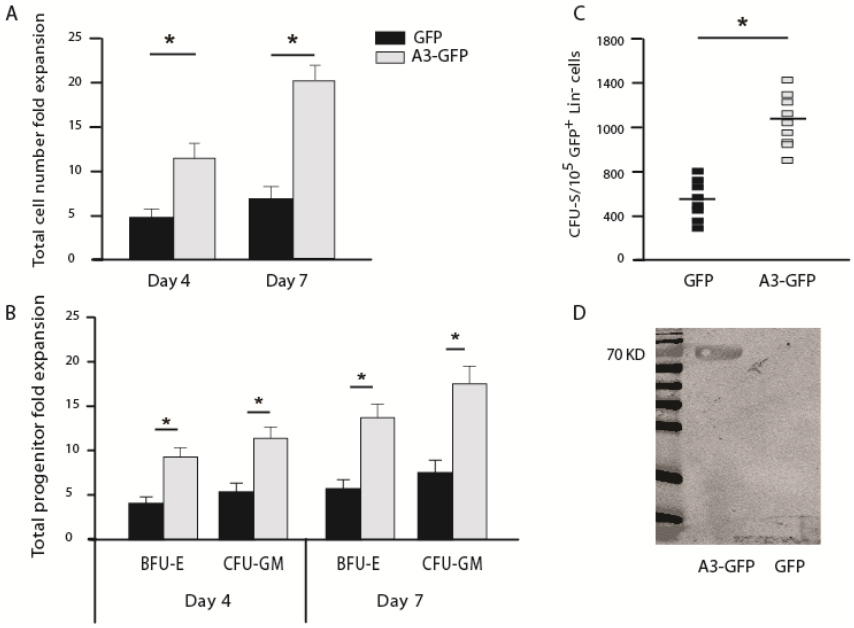

Figure 1: Angptl3 over-expression promotes the expansion of HSCs in Lin$^-$ cell populations.
(A) Lin$^-$ cells, were transduced with LV-GFP or LV-Angptl3-GFP (LV-A3-GFP), and cultured in STIF medium for 7 days. The mean fold increase in total cell numbers was measured in comparison to day 0. The results of five independent experiments are shown. The error bars indicate the standard deviation (SD). * signifies $P<0.05$.**(B)** Colony forming-units (BFU-E and CFU-GM) of Lin$^-$ cells cultured for 4, and 7 days relative to day 0. The results of 5 independent experiments are shown. **(C)** Sorted transduced Lin$^-$ GFP$^+$ were immediately transplanted into lethally irradiated mice. Number of CFU-S colonies in the spleen of recipients were counted, 12 days post transplantation. N=10 mice per group. **(D)** Western blotting analysis of Angptl3 in both sorted LV-Angptl3-GFP and LV-GFP cells, detected by Angptl3-specific monoclonal antibody.

Over-expression of Angptl3 increases the expansion of BFU-E and CFU-GM of murine Lin$^-$ cells

Next, we performed colony-forming unit assays to test whether over-expressing Angptl3 increased the number of hematopoietic progenitors (Figure 1B). Culturing cells for four and seven days resulted in a significant increase in Lin$^-$ BFU-Es when Angptl3 was over-expressed. The Lin$^-$BFU-Es increased by more than 2-fold (on day 4) in the LV-Angptl3-GF group relative to the LV-GFP group (from 3.5±1-fold to 10±2-fold, respectively, compared to day 0). This same trend of Lin$^-$BFU-Es expansion was observed at day 7 (the LV-GFP and LV-Angptl3-GFP groups were increased 5.7±2-fold and 13±3-fold, respectively, compared to day 0) (Figure 1B).

After 4 and 7 days of *in vitro* culture, the number of granulocyte-macrophage progenitor cells in the LV-GFP Lin$^-$ cell population was increased 4.6±1and 7.5±2-fold, respectively. The number of granulocyte-macrophage progenitor cells in the LV-Angptl3-GFP Lin$^-$ cell population increased up to 12±2-fold at day 4 and 16±3-fold at day 7, respectively compared to day 0. Taken together, the number of progenitor cells significantly increased by Angptl3 over-expression compared to the control group. These results show that over-expressing Angptl3 can indeed facilitate the expansion of immature progenitor cells.

The over-expression of Angptl3 increases the number of short-term HSCs from murine Lin$^-$ cells

Using the CFU-S (12-day) assay, we next examined the effect of over-expressing Angptl3 on short-term hematopoietic stem cells (ST-HSCs) from Lin$^-$ cells that were transduced with either LV-GFP or LV-Angptl3-GFP (Figure 1C).

One thousand sorted GFP$^+$ Lin$^-$ cells from Lin$^-$ cells that were transduced with LV-GFP or LV-Angptl3-GFP were directly transplanted into lethally irradiated BALB/c mice. We found that over-expression of Angptl3 significantly increased the number of CFU-S (1050±350 colonies/10^5 GFP$^+$ Lin$^-$ cells) compared to cells that were transduced with LV-GFP (586±217 colonies/10^5 GFP$^+$ Lin$^-$ cells).

The over-expression of Angptl3 increases the number of long-term HSCs from murine Lin$^-$ cells

We next performed LTRA assays to estimate the stimulation index of Angptl3 over-expression on the expansion and proliferation of LT-HSCs in Lin$^-$ cells. Two days after transducing Lin$^-$ cells with LV-GFP or LV-Angptl3-GFP, GFP$^+$ cell populations were sorted using the FACSAria, and 10,000 Lin$^-$ GFP$^+$ sorted cells were directly transplanted into sublethally irradiated mice.

During these 9-month experiments, all of the mice were healthy, and none of the mice exhibited tumors or an elevated WBC count.

At post-transplantation month 1, we detected <2% GFP$^+$ cells in the peripheral blood of the LV-Angptl3-GFP group (n=5) compared to ~8% in the LV-GFP group (n=5) (Figure 2A). During the 9 months of this experiment, the percentage of GFP$^+$ cells in the LV-GFP group

varied slightly (from 6.5 to 8%), whereas the percentage of GFP^+ cells in the LV-Angptl3-GFP group significantly increased (ultimately reaching 13±2% at month 9).

At post-transplantation month 9, these mice were sacrificed, and the percentages of GFP^+ cells were measured in the peripheral blood (PB), bone marrow (BM), spleen (Sp) (Figure 2B).

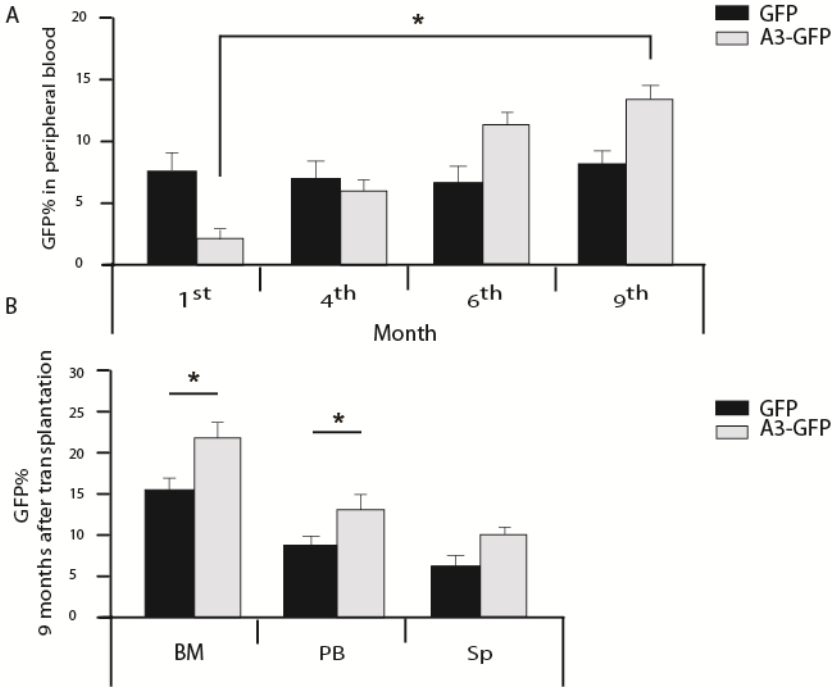

Figure 2: Angptl3 over-expression stimulates the expansion of LT-HSCs in Lin^- cell populations.
(A) Ten thousand sorted BM Lin^- Angptl3-GFP^+ or BM Lin^- GFP^+ cells were transplanted into sublethally irradiated recipients. The percentage of donor-derived cells (GFP^+) was determined 1, 4, 6 , and 9 month(s) after transplantation in PB. N=5 mice per group. The error bars indicate the standard deviation (SD). * signifies $P<0.05$. **(B)** The percentage of GFP chimerism was determined 9 months after retransplantation in PB, BM, and spleen. N=5 mice per group.

In the BM, we detected 23±2 percent GFP^+ cells in the LV-Angptl3-GFP group, which was significantly higher than in the LV-GFP group (~15 percent). In the spleen, the percentage of GFP^+ cells was slightly higher in the LV-Angptl3-GFP groups compared to the LV-GFP group (8% vs. 5%, respectively) (Figure 2B).

We measured the percentages of various blood lineages in the total GFP^+ cell populations from the PB, BM, and Sp (Figure 3). In the BM and PB, the percentages of HSPCs ($Sca\text{-}1^+$ and $Sca\text{-}1^+/c\text{-}kit^+$) significantly increased in the LV-Angptl3-GFP group compared to the LV-GFP group, which indicates that Angptl3 maintains HSCs in their immature state. We found no change in the percentages of T cells (either CD4 or CD8) or in the myeloid cells (CD11b) in any of the studied tissues. The percentage of B cells (CD19) was significantly

decreased in the LV-Angptl3-GFP group compared to the LV-GFP group in the PB, BM, and Sp (Figure 3).

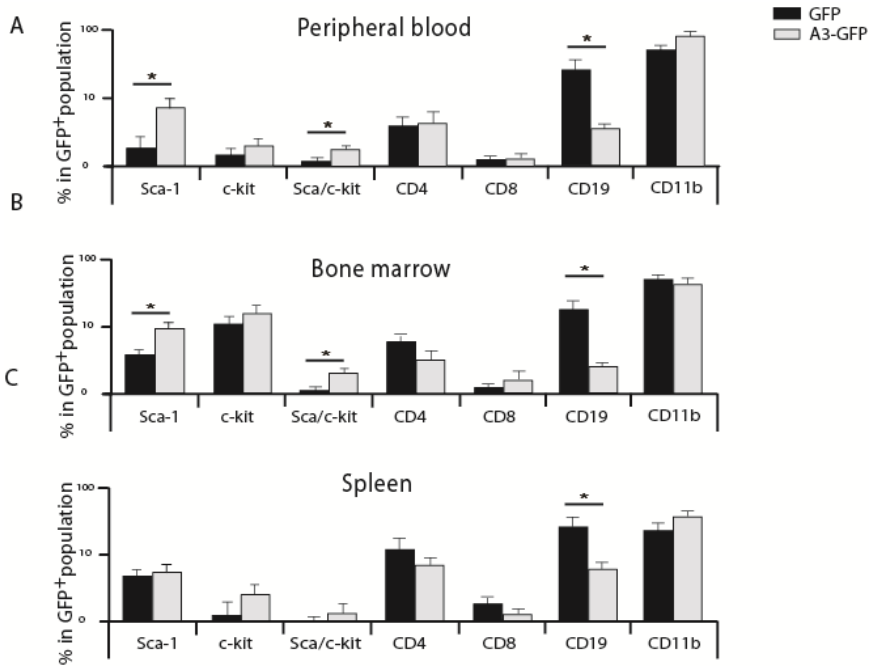

Figure 3: Angptl3 over-expression increases the percentage LSK population in PB and BM.
(A) The proportion of Sca-1, c-kit, Sca-1/c-kit, CD4, CD8, CD19, and CD11b cells within total GFP^+ population of PB was measured by flow cytometry (n=5). **(B)** Bone marrow **(C)** Spleen.

We next sorted GFP^+ Lin^- or Angptl3-GFP^+ Lin^- from the pooled BM cells of each group. Two thousands sorted GFP^+ Lin^- BM cells from the primary recipients of the LV-GFP or LV-Angptl3-GFP groups were plated in a semi-solid colony culture (n=4) to estimate the number of progenitors (Figure 4). There were no significant changes in the number of BM Lin^- GFP^+ BFU-Es in the LV-Angptl3-GFP group compared to the LV-GFP group (~450 vs. ~490 colonies/10^5 Lin^- GFP^+ cells, respectively) (Figure 4B). However, the number of BM Lin^- GFP^+ CFU-GMs was significantly increased in the LV-Angptl3-GFP compared to the LV-GFP group (3,100±165 vs. 2100±100 colonies/10^5 Lin^- GFP^+ cells, respectively) (Figure 4A).

The number of ST-HSCs in the BM Lin^- GFP^+ from the primary recipient was estimated using the CFU-S assay (Figure 4C). One thousand sorted BM Lin^- GFP^+ cells from the primary recipients were transplanted into lethally irradiated mice (n=7), and the number of colonies was counted after 12 days. The number of CFU-S was significantly higher in the LV-Angptl3-GFP group compared to the LV-GFP group (708±113 vs. 316±87 CFU-S/10^5 Lin^- GFP^+ cells, respectively). Thus, Angptl3 over-expression resulted in a >2.2-fold expansion of ST-HSCs.

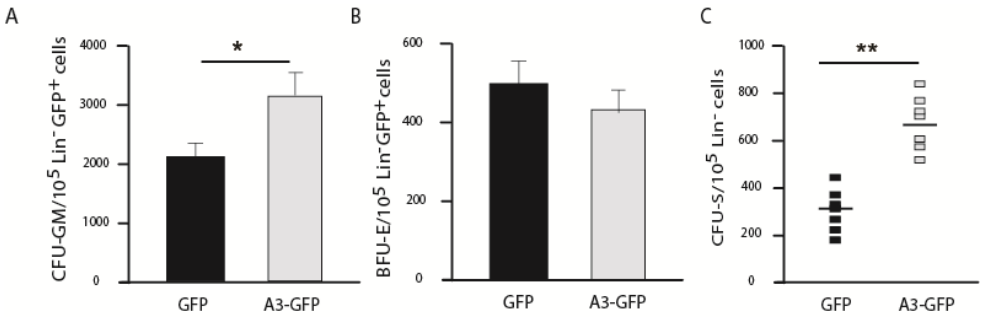

Figure 4: Angptl3 over-expression enhances the expansion of CFU-GM and ST-HSCs *in vivo*.
Two thousands sorted GFP^+ Lin^- BM cells from the primary recipients of the LV-GFP or LV-Angptl3-GFP groups were plated in a semi-solid colony culture (n=4), 9 months post transplantation. The number of the CFU-GM **(A)** and the BFU-E **(B)** was measured. The average numbers of 10^5 plated cells were calculated from quadruplicates. **(C)** A thousand BM Lin^- GFP^+ cells from recipient mice of LV-GFP or LV-Angptl3-GFP mice were transplanted into lethally irradiated mice (n=7). The CFU-S colony number was calculated per 10^5 BM Lin^- GFP^+ cells (*P<.01, **P<.001)

Discussion

We assayed the effect of Angptl3 over-expression on HSCs activity. Its over-expression increased the overall proliferation rate of adult murine BM Lin^- during 7 days of *in vitro* culturing. This was consistent with the increased number of progenitors and ST-HSCs measured using colony-forming unit and colony-forming spleen assays.

9 months post transplantation into sublethaly irradiated recipients, the percentage of transduced cells that express Angptl3 increased significantly and resulted in higher donor-derived chimerism percentage in peripheral blood (PB) and bone marrow (BM) of recipients compared to control group. Moreover, over-expression of Angptl3 significantly increased the number of LSK cells in BM and PB of recipients, though no alteration was detected in other blood lineages. We did not detect adverse effect that could be attributed to leukemogenicity or toxicity effects of Angptl3. Based on these studies, we suggest that Angptl3 can play a supportive role in the expansion and proliferation of HSCs *in vivo* and *in vitro*. Probably Angptl3 could be introduced as an effective growth factor in HSC expansion studies, though its effect should be further investigated on human HSC.

Using HSC growth factors is considered as one of the approaches to achieve expansion of HSC and make sources like umbilical cord blood more promising for clinical applications. It was reported that SCF^+DLK^+ stromal cells of the fetal liver support HSC expansion by expressing the angiopoietin like protein family, in particular Angptl3, and insulin like growth factor[8, 9]. Angptl3, a 455 amino acid protein, has the characteristic structure of Angiopoietin, i.e. a signal peptide, an extended helical domain to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen homology domain (FHD). However, unlike the other angiopoietins, Angptl3 does not bind to the tyrosine kinase receptor Tie-1 or Tie-2, and it has been suggested that Angptl3 functions differently from the other family members [15]. Several members of the Angptl family regulate lipid metabolism and angiogenesis [16]. Angptl3 in particular inhibits lipoprotein lipase activity *in vitro* and *in vivo*,

and mice deficient in Angptl3 display defects in lipid metabolism [16, 17]. Recently, several studies suggested that Angptl3 plays a role in the regulation of HSC activity [9]. The effect of Angptl3 was assayed in Angptl3 null-mice. This study showed that the number of LT-HSCs decreased in the bone marrow niche of Angptl3 null-mice compared to WT mice[10]. Following on from the Lodish *et al*. studies on Angptl3 as a hematopoietic stem cell growth factor, we examined the effect of Angptl3 and its over-expression on progenitors, ST-, and LT-HSCs, *in vivo* as well as *in vitro*. (Chapter 2 and 3)

The mechanism is not well characterized, although it is known that Angptl3 can bind directly to the cell surface receptor expressed on HSCs and repress the expression of the transcription factor Ikaros, when over-expressed it diminishes the repopulation activity of HSCs[10]. It was shown that mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several Angptls [18].

Thus Angptl3 appears to playing an important role in HSCs activity and probably does not have a leukemogenic or toxicity effect. Therefore, Angptl3 might be a useful growth factor in combination with other well-known HSCs growth factors to expand HSC *ex vivo* for clinical applications.

Acknowledgements

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Chapter 4

The effect of angiopoietin-like protein 3 (Angptl3) on the proliferation and expansion of umbilical cord blood cells

Manuscript in preparation

Abstract

Umbilical cord blood (UCB) cells are an attractive transplantation option when traditional methods are not feasible. However, the limited number of these cells greatly hampers their clinical applicability in adult patients. A superior method to produce sufficient numbers of UCBs for transplantation would be *ex vivo* expansion, facilitated by a combination of growth factors. In the previous chapters, we showed that proteins from the angiopoietin-like family support the expansion of murine long term (LT)-hematopoietic stem cells (HSCs). Here, we show the effects of human ANGPTL3 and ANGPTL5 on the expansion and proliferation of human UCB-$CD34^+$ cells under serum-free medium conditions, during a period of 7 days. ANGPTL3 significantly enhances the progenitor populations of UCB-$CD34^+$ cells during *in vitro* culturing in the presence of the growth factor cocktail SCF+TPO+FLT3L (STIF). STIF+ANGPTL3 significantly promoted the maintenance of the $CD34^+$ phenotype when compared to STIF, or STIF+ANGPTL5. Our *in vivo* experiments showed that adding ANGPTL3 to STIF-containing medium resulted in a 3-fold expansion of human cells in the bone marrow of NOD-SCID mice. In total, we achieved a greater than 6-fold expansion in human repopulating cells after seven days of *in vitro* culture in the presence of STIFA3 when compared to fresh cells. We propose that *ex vivo* expansion of UCBs increases their use for transplantation in adult and pediatric patients.

Introduction

Umbilical cord blood (UCB) contains large quantities of stem cells that could be used as a source of hematopoietic stem cells (HSC) [1, 2]. The use of UCB as a stem cell source has increased dramatically in recent years, particularly in children and young adults, but it is not without drawbacks. One major limitation of UCB-derived HSC therapy is delayed neutrophil and platelet engraftment and high rates of engraftment failure when compared to bone marrow (BM) or mobilized peripheral blood. This limited potential for rapid hematological recovery after transplantation of UCB cells is probably due to the limited number of cells available for transplantation and may therefore be improved by *ex vivo* generation of the required numbers of stem and progenitor cells [3, 4].

During the past two decades several methods have been used for *ex vivo* UCB cell expansion, such as liquid suspension culture or stromal cell co-culture [5] that expose the UCB stem cells to a cocktail of cytokines and growth factors for a specific period of time [6].

To establish an optimum growth factor cocktail for *in vitro* expansion of human HSCs, different combinations of growth factors have been studied. The analysis of 16 cytokines on stromal-free cultures of $CD34^+CD38^-$ bone marrow cells indicated that FMS-like tyrosine kinase 3 ligand (FLT3-L), Stem cell Factor (SCF) and IL3 gave the highest (30-fold) expansion of the input long-term culture-initiating cells (LTC-IC) [7]. In another study addition of Flt-3-L maintained the ability of human $CD34^+$ cells to sustain long-term HSCs[8]. TPO has also been reported to increase the multilineage growth of $CD34^+CD38^-$ bone marrow cells under stromal-free conditions in addition to the cytokine combination Flt3-L and SCF [9]. Levac *et al.* also reported that a combination of SCF, FLT3-L, and TPO (together called STF) can

successfully expand UCB-derived primitive HSCs [10]. Insulin-like growth factor 2 (IGF2) and fibroblast growth factor 1 (FGF1) were also introduced as promising growth factors for *ex vivo* expansion and proliferation of HSC [11, 12]. Zhang *et al.* reported that using a new combination of growth factors that included SCF, TPO, IGF-2, FGF-1, called STIF, supplemented with Angiopoietin-like proteins (Angptls) for 10 days resulted in a 24- to 30-fold expansion in the number of murine LT-HSCs [13, 14]. Further, IGF-binding protein 2 (IGFBP2) and ANGPTL5 (A5) were introduced as additional secreted proteins that support human HSC expansion [15]. The use of SCF [16], TPO [17], and FGF-1 supplemented with IGFBP2 and ANGPTL5 increased the number of human SCID-repopulating cells (SRCs) ~20-fold compared to freshly isolated cells [18]. However, the ideal mixture of growth factors or cytokines, and the optimum culture duration for the *ex vivo* expansion of HSCs has not yet been determined.

In this chapter, we compared the effect of the growth factor combination SCF+TPO+Flt3-L (STF) versus SCF+TPO+IGF2+FGF1 (STIF) or SCF+TPO+IGFBP2+FGF1 on the expansion and proliferation of UCB-CD34$^+$ cells in serum-free medium. In addition, since Angptl3 has been shown to be the most effective angiopoietin like family member to stimulate *ex vivo* expansion of murine LT-HSCs, we also investigated the effect of human ANGPTL3 (A3) on the expansion and proliferation of UCB-CD34$^+$ cells in the presence of the STIF growth factor cocktail and contrasted this with the previously described growth factor cocktail containing ANGPTL5.

Material and Methods

Mice

NOD/LtSz-SCID/SCID (NOD/SCID) mice, 8-12 weeks of age, were used in all experiments. These mice were bred and housed under Specific Pathogen Free (SPF) conditions at the Experimental Animal Facility of Erasmus University (Rotterdam, the Netherlands). All experiments were approved by the ethical committee of Erasmus MC, Rotterdam in accordance with legislation in the Netherlands.

Cells

Human umbilical cord blood cells (UCB)

Following full-term delivery, human UCB samples were collected from the umbilical cord by the nursing staff of the Department of Obstetrics and Gynecology at the Sint Franciscus Hospital (Rotterdam, The Netherlands). Informed consent to collect the samples was obtained from the donors according to legal regulations in The Netherlands. UCB cells were collected in sterile flasks containing 10 ml of 1% heparin in H+H at room temperature.

Low-density cells were isolated from the UCB samples using Ficoll Hypaque density centrifugation in 1.077g/cm^2 Lymphoprep (Nycomed Pharma, Oslo, Norway). The cell suspension was centrifuged at 600x*g* for 15 minutes, after which the mononuclear cell (MNC)

band at the interface was removed, and washed twice with Hank's balanced salt solution (HBSS, Gibco). The cells were used immediately in experiments.

$CD34^+$ cell purification

The $CD34^+$ cells were isolated from fresh UCB using the indirect $CD34^+$ Microbead kit (Miltenyi Biotec, Germany). Briefly, the $CD34^+$ hematopoietic progenitor cells were indirectly magnetically labeled using a hapten-conjugated primary monoclonal antibody and an anti-hapten antibody coupled to MACS MicroBeads (Miltenyi Biotec, Germany). The magnetically labeled cells were enriched in separation columns in the magnetic field of a manual MACS system. The resulting population of $CD34^+$ cells had a purity of >90%.

In vitro suspension culture

CD34+ cells were cultured at a starting density of 3-4×105/ml in serum free medium that contained enriched Dulbecco's modified Eagle's medium (DMEM) with 1% (wt/vol) bovine serum albumin (BSA), 0.3 mg/L human transferrin, 0.1 μM sodium selenite, 1 mg/L nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyadenosine, 2'-deoxy thymidine and 2'-deoxyguanosine; all from Sigma, St. Louis, MO, USA), 0.1 mM ß-mercaptoethanol, 15 μM linoleic acid, 15 μM cholesterol, 100 U/ml penicillin and 100 μg/ml streptomycin as described previously [19, 20]. The medium was supplemented with STIF (human SCF (50 ng/ml, R&D, Abingdon, UK), human TPO (20 ng/ml, R&D), murine Igf2 (20 ng/ml, R&D), and human FGF-1 (10 ng/ml, R&D) ± human ANGPTL3 (A3) (100 ng/ml, BioVendor, Heidelberg, Germany) or human ANGPTLl5 (A5) (500 ng/ml, Abnova, Taipei City, Taiwan) plus heparin (10 μg/ml, Sigma). ANGPTL3 dissolved completely by adding 0.1M acetate buffer pH4, in a final concentration of 10 μg/ml. Dissolved ANGPTL3 was converted to higher pH value by dilution in enriched DMEM. Human IGFBP2 (IB) (R&D) was added instead of IGF-2 in some experiments. Cells were incubated at 37 °C, in a humidified atmosphere containing 5% CO_2, and a normal concentration of O_2.

In vitro clonogenic progenitor assays

The in vitro frequency analysis of hematopoietic stem cells and progenitor cells was performed with semi-solid colony assays. Fresh or cultured UCB-CD34+ cells (1×10^3) were plated in 1 ml of semi-solid methylcellulose culture medium containing 0.8% (wt/vol) methylcellulose (Methocel A4M Premium Grade, Dow Chemical, Barendrecht, The Netherlands) in enriched DMEM as described previously[19, 21].

Gametocyte Macrophage colony formation (CFU-GM) was stimulated by 10 ng/mL hu-IL-3, 100 ng/mL hu-SCF, and 20 ng/mL GM-CSF. Burst-Forming Erythroid (BFU-E) colonies were grown in the presence of 100 ng/mL hu-SCF and 4 U/mL hu-EPO. The cells were maintained for 14 days and each experiment was performed in duplicate.

SCID repopulating cell (SRC) assay

Fresh or cultured human UCB-CD34$^+$ cells were transplanted into sublethally irradiated (3.5 Gy) SPF NOD/LtSz-scid (NOD/SCID) mice at the indicated days and density. The mice were sacrificed by CO_2 inhalation 6-8 weeks post-transplantation. After counting the number of cells isolated from the femurs, 1×10^5 cells were cultured in a colony assay and the rest was analyzed by flow cytometry to determine the percentage of human cells in the mouse bone marrow.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the Mann-Whitney U test. Standard deviations of colony counts were calculated on the assumption that crude colony counts show a Poisson distribution.

Results

STIF increases the percentage of repopulating cells obtained from UCB-CD34+ cells

To study the effect of ANGPTL3 on UCB-HSC, we initially tested whether ANGPTL3 alone can promote the expansion and/or proliferation of HSCs. We initially observed no expansion of HSCs when cultured *in vitro* either with or without ANGPTL3 (results not shown).We therefore switched to media supplemented with saturated growth factor levels. Two of the most frequently used combinations of growth factors are SCF+TPO+Flt3-L (STF) [10] and SCF+TPO+IGF2+FGF1 (STIF) [18, 22]. Insulin-like growth factor binding protein 2 (IBP) was also introduced to support HSC expansion [18]. We studied the effect of STF, STIF and SCF+TPO+IBP+FGF1 to determine the optimum saturated level of growth factors for our ANGPTL3 study. Culturing UCB-CD34$^+$ cells in these three distinct growth factor combinations for 7 or 14 days revealed a similar increase in total cell numbers under all three conditions (Figure 1A). Freshly isolated cells, or 7 or 14 day cultured UCB-CD34$^+$ cells in these 3 combinations of growth factors were transplanted into sublethally irradiated NOD-SCID mice (n=5). Six to 8 weeks post-transplantation, the percentage of human-CD45$^+$ chimerism was measured using flow cytometry. The cells that were cultured for 7 days in the three conditions yielded a remarkably higher chimerism percentage (chimerism %) when compared to the cells that were cultured for 14 days. For STF this was 39±3 versus 21±4, for STIF 45±5 versus 26±4, and for ST(IBP)F 42±2 versus 21±7 (Figure 1B). Because cells cultured for 7 days in STIF medium showed the highest chimerism%, we used the STIF cocktail as the optimum growth factor combination to determine the effect of ANGPTL3 on the expansion and proliferation of UCB-HSCs. Culturing for 7 days was used as the condition for the following experiments.

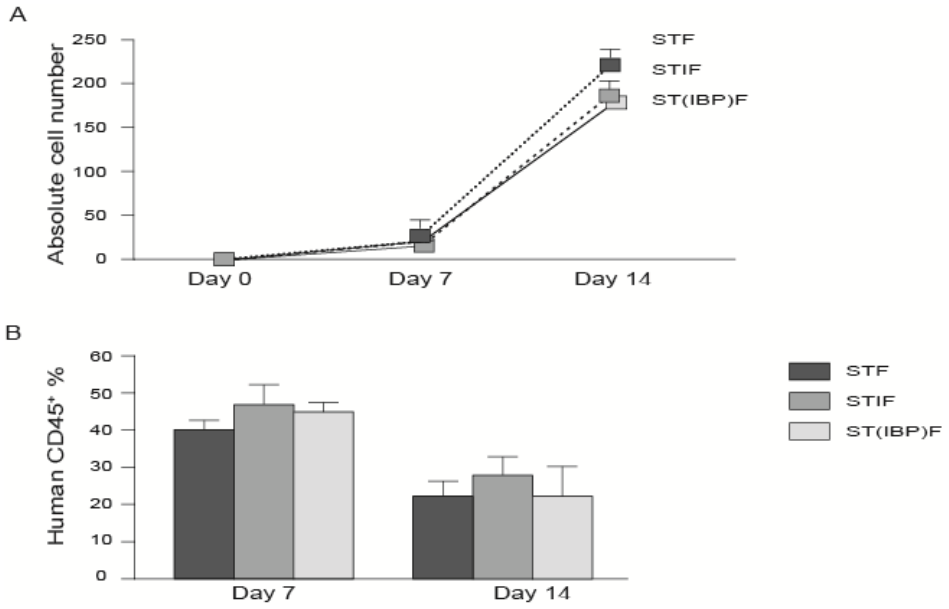

Figure 1: ***STF, STIF, and ST(IBP)F* support the expansion and the proliferation of UCB-CD34$^+$ cells.**
(A) UCB-CD34$^+$ cells were purified to >90% and cultured in medium supplemented with STF, STIF, or ST(IBP)F for up to 14 days. Total cell numbers were counted at days 7 and 14. The data represent the average results of 3 independent experiments. The error bars *indicate the standard deviation (SD).* **(B)** UCB-CD34$^+$ cells cultured for 7 or 14 days were transplanted into sublethally irradiated NOD-SCID mice (n=5). 6-8 weeks post-transplantation, the mice were sacrificed, BM cells were isolated, and the relative number of human CD45$^+$ cells was determined.

ANGPTL3 does not affect the overall *ex vivo* expansion of UCB-CD34$^+$ cells

Highly purified UCB-CD34$^+$ cells (>90% purity) were cultured *in vitro* under three distinct conditions (STIF, STIFA3, or STIFA5). After 7 days of in *vitro* culturing, the number of cells increased approximately 8-fold in all three conditions (Figure 2A). Thus addition of either ANGPTL3 or ANGPTL5 did not further increase the STIF-induced *in vitro* proliferation of UCB-CD34$^+$ cells.

After culturing the cells for 7 days *in vitro*, the percentage of CD34$^+$ cells was measured using flow cytometry. This showed a significant difference in CD34$^+$ phenotype maintenance between the STIF and STIFA3 conditions (Figure 2B). The percentage of CD34$^+$ cells of 90±5 in freshly isolated cells (at day 0) was reduced to 20±5% during 7 days *in vitro* culturing in STIF medium, however, the percentage was greater than 33±4% in STIFA3 culture conditions. This result indicates that ANGPTL3 helps to maintain stem cell characteristics.

ANGPTL3 increases the number of colony-forming unit cells from the UCB-CD34$^+$ cell fraction

A colony-forming unit assay was performed to estimate the number of progenitor cells in cultured and uncultured UCB-CD34$^+$ cells following 7 days of *in vitro* culturing. The results show that including AGPTL3 in STIF medium enhanced the number of UCB-CD34$^+$ BFU-Es and CFU-GMs by 1.45- and 1.3-fold, respectively (Figure 2C). Adding ANGPTL5 to STIF medium also resulted in an ~1.5-fold increase in the number of BFU-E and CFU-GM. After 7 days of *in vitro* culturing, the number of progenitor cells was increased by approximately 4-fold in the presence of STIF and adding either ANGPTL3 or ANGPTL5 (STIFA3 or STIFA5) further increased the number of progenitor cells to about 6-fold compared with day 0.

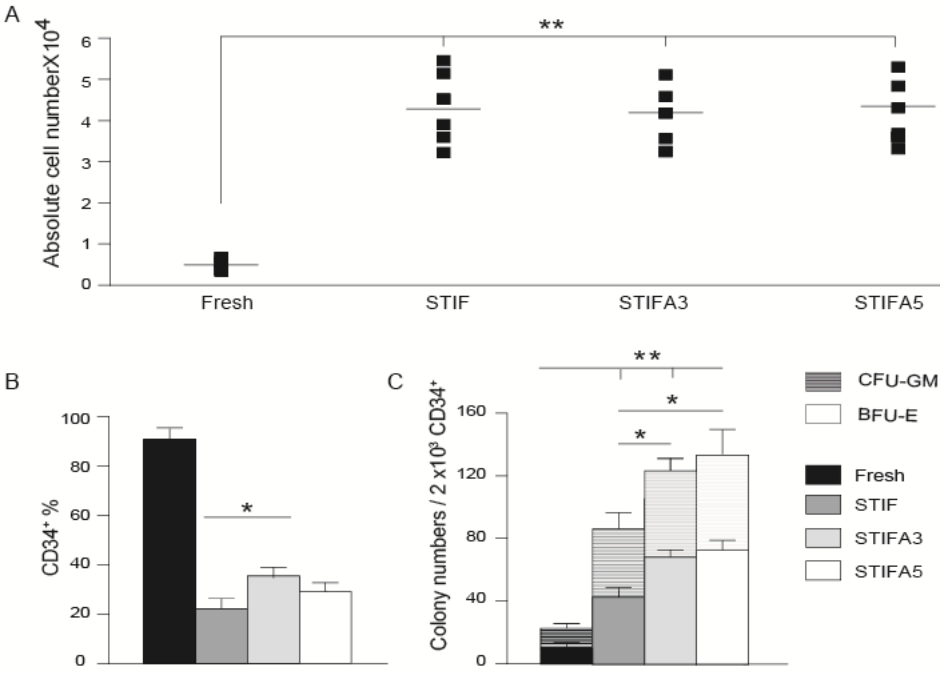

Figure 2: ANGPTL3 enhances the proliferation of progenitor cells of UCB-CD34$^+$ cells.
(A) UCB-CD34$^+$ cells were purified to >90% and cultured in STIF, STIFA3, or STIFA5 medium for a period of 7 days. Total cell numbers were counted at day 7. The data represent the average results of 5 independent experiments. **(B)** 7-day cultured UCB-CD34$^+$ cells were labeled with CD34-PE and measured by flow cytometry. **(C)** Colony-forming units (BFU-E and CFU-GM) of cultured UCB-CD34$^+$ cells for 7 days relative to day 0 cells. The average results of five independent experiments are shown. The average number of plated cells was calculated from duplicate experiments. The Error bars indicate the standard deviation (SD). *P< 0.01, **P< 0.001.

ANGPTL3 increases the number of repopulating cells of UCB-CD34$^+$ cell fraction

Human HSCs have the capacity for extensive proliferation and multi-lineage repopulation in NOD-SCID mice and are thus also called NOD-SCID-repopulating cells (SRCs). We examined the effect of ANGPTL3 and ANGPTL5 on the expansion and proliferation of the repopulating cells in UCB-CD34$^+$ cells. 6×10^5 fresh UCB-CD34$^+$ cells, or equivalent amounts of UCB-CD34$^+$ cells cultured for 7 days in STIF, STIFA3, or STIFA5 medium were transplanted into NOD-SCID mice (n=6). Six to 8 weeks post- transplantation, the percentage human leukocytes (CD45$^+$) in the BM was measured using flow cytometry.

Transplanting 6×10^5 fresh UCB-CD34$^+$ resulted in a 20±3% repopulation of human CD45$^+$ cells in the BM of recipient mice. The percentage of human CD45$^+$ cells increased to up to 43±4% when cells were transplanted into NOD-SCID mice after culturing for 7 days in STIF medium. Supplementing the STIF-containing medium with ANGPTL3 or ANGPTL5 also significantly increased the percentage of CD45$^+$ cells in the BM of the recipient mice. Mice that received cells that were cultured for 7 days in STIFA3 or STIFA5 medium exhibited 62±5% or 57±3% CD45$^+$ cells in their BM, respectively (Figure 3A, 4). Figure 3A also shows that the human CD45$^+$ percentage in STIFA3 and STIFA5 conditions increased 6-fold relative to day 0.

We next performed transplantations of serially diluted CD34$^+$ cells (6×10^5, 3×10^5, or 1×10^5 cells) to calculate the exponential rate of repopulating cells. Again, cells were cultured for 7 days in the presence of STIF, STIFA3 or STIFA5. The results show that culturing UCB-CD34$^+$ cells for 7 days in STIF increased SRC more than 2-fold compared to fresh cells. Culturing UCB-CD34$^+$ cells in STIFA3 medium resulted in a 3-fold increase in repopulating cells compared to cells that were cultured in STIF medium. Therefore, the number of repopulating cells increased 6-fold in cells that were cultured for 7 days in STIFA3 medium when compared to fresh cells (Figure 3D).

We next determined the percentage of the various subpopulations of human cells in the BM of recipient mice that received 6×10^5 fresh CD34$^+$ cells or 6×10^5 CD34$^+$ cells that were cultured in STIF, STIFA3, or STIFA5 medium. Transplanting fresh cells resulted in 15±4% CD34$^+$ cells in the total human CD45$^+$ population that repopulated the BM of the NOD-SCID mice, and this percentage increased to 20±3, 30±4 and 26±3% in mice that received cells that were cultured in STIF, STIFA3, or STIFA5 medium, respectively (second series in Figure 3A).

To estimate the number of myeloid cells, we calculated the percentage of CD15$^+$ cells in the CD45$^+$ cell population but found no difference between the various culture conditions (Figure 3B). Moreover, the percentage of B cells in the total CD45$^+$ population was estimated using the CD19 marker, and also there, no significant differences were detected for the various culture conditions (Figure 3B).

We also used equal numbers of recipient BM cells in the colony-forming unit assay to obtain an estimation of the number of progenitor cells. We observed in total 90±5 BFU-E and CFU-GM colonies from 10^5 BM cells from primary NOD-SCID mice that received 6×10^5 fresh cells. In contrast, 47±4 BFU-E and 60±4 CFU-GM colonies arose from 10^5 BM cells from

primary NOD-SCID mice that received 6×10^5 cells that were cultured for 7 days in STIF medium, whereas 55±3.5 BFU-E and 74±5 CFU-GM colonies arose from cells that were cultured in STIFA3 medium. Thus, the total progenitor number in the BM of primary NOD-SCID mice transplanted with cells cultured for 7 days in STIFA3 medium was significantly higher than in the BM of mice that received fresh cells (Figure 3C).

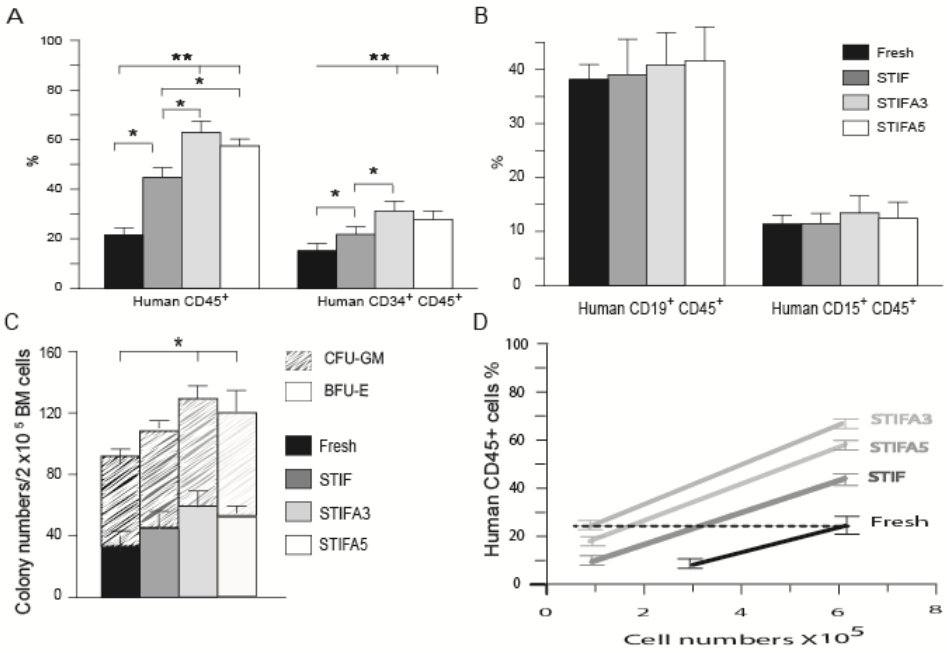

Figure 3: ANGPTL3 increases the proportion of UCB-HSCs *in vitro*.
(A) UCB-CD34+ cells were cultured in the presence of STIF, STIFA3, or STIFA5 for 7 days. Fresh or 7-day cultured UCB-$CD34^+$ cells (6×10^5) were transplanted into sublethally irradiated NOD-SCID mice. Six to 8 weeks after transplantation, the percentage of human leukocytes ($CD45^+$) was measured by flow cytometry. In addition, the proportion of $CD34^+$ cells was measured as a percentage of the total $CD45^+$ cell population. **(B)** Similarly, the proportion of myeloid cells (CD15) and B cells (CD19) was measured. N=12 mice per group. **(C)** Total colony-forming units (BFU-E and CFU-GM) from 10^5 recipient BM cells. The average number of plated cells was calculated from duplicate experiments, and was $1x10^5$. The error bars indicate the standard deviation (SD). *P< 0.01, **P< 0.001. **(D)** The percentage of repopulating human leukocytes ($CD45^+$) was determined 6-8 weeks after transplantation into NOD-SCID mice using serially diluted fresh or cultured $CD34^+$ cells. Transplanting 6×10^5, or 3×10^5 fresh $CD34^+$ cells (D0) resulted in 20±3 or 8±2% repopulation, respectively. Transplanting 6×10^5, 3×10^5, or 1×10^5 cultured $CD34^+$ cells in STIF medium resulted in 43±4, 20±2, or 10±3% repopulation, respectively. Furthermore, culturing 6×10^5, 3×10^5, or 1×10^5 $CD34^+$ cells for 7 days in STIFA3 medium raised the repopulation percentage to up to 62±5, 34±3, or 24±4%, respectively. ANGPTL3 caused a 3-fold expansion in the repopulation rate of $CD45^+$ cells compared to the STIF condition. The $CD45^+$ cell repopulation from cells cultured for 7 days in STIFA3 medium increased ~6-fold compared to fresh cells.

Discussion

In this study, we show that adding ANGPTL3 to the STIF combination of growth factors for 7 days can promote *ex vivo* expansion of UCB derived HSCs approximately 6-fold. ANGPTL3 alone is not sufficient to survive UCB-$CD34^+$ and we therefore used media supplemented with saturated growth factor levels to study the effect of ANGPTL3 on UCB-$CD34^+$. Analysis of the effect of 3 combination of growth factor growth STF (SCF,TPO, Flt3-L), STIF (SCF, TPO, IGF-2, and FGF-1), and ST(IBP)F (SCF,TPO, IGFBP2, FGF-1) indicated that STIF gave the highest *ex vivo* expansion of human repopulating cells. We did not detect any advantages of using IGFBP2 instead of IGF-2 in combination with SCF, TPO, and FGF-1. Because incubation time is an important factor in the expansion and proliferation of HSCs, we varied the culture time. We were able to show that incubating UCB-$CD34^+$ cells for 7 days in the STIF growth factor cocktail is better to stimulate *ex vivo* expansion of repopulating cells when compared to 14 days. We therefore used this condition to test the effect of ANGPTL3 on UCB-$CD34^+$ cells. In parallel, we also studied the effect of ANGPTL5 as a novel growth factor [18] and compared its effect with ANGPTL3. We showed that in the three conditions tested (STIF, STIFA3, and STIFA5), UCB-$CD34^+$ cell numbers increased greater than 5-fold during culturing for 7 days *in vitro*. ANGPTL3 has no influence on expansion of total number of UCB-$CD34^+$ cells although it helps to maintain the $CD34^+$ phenotype of UCB cells. Next, we showed adding ANGPTL3 significantly increased progenitor cell numbers, and that ANGPTL5 had a similar effect. Further, UCB-$CD34^+$ cells cultured in these three conditions were transplanted into sublethally irradiated NOD-SCID mice. Using transplantations of serially diluted cells, we calculated that cells cultured in STIF stimulated repopulating cells over 2-fold and supplementing it with ANGPTL3 caused an additional 3-fold expansion on repopulating cell number. As reported previously, we also showed that adding ANGPTL5 to STIF promotes *ex vivo* expansion of UCB-derived HSCs and increases the number of repopulating cells over 5-fold. ANGPTL3 as well as ANGPTL5, enhanced the proliferation of the immature population in the $CD34^+$ cells in BM of NOD/SCID mice.

Supplementing STIF with ANGPTL3 or ANGPTL5 had no special effect on the repopulation of B-cells or myeloid cell. Next, we compared the presence of progenitor cells in the BM of NOD-SCID recipient mice. We showed that the BM from primary recipients who received cells cultured in STIFA3 or STIFA5 medium give rise to higher numbers of CFU-GMs and BFU-Es. This result shows that supplementing ANGPTL3 and ANGPTL5 supports the expansion and proliferation of repopulating cells from UCB-$CD34^+$ cells. Our findings confirm what those of Zhang *et al*. concerning the effect of ANGPTL5 on *ex vivo* expansion of human HSCs [18]. The data presented in the current study show that both ANGPTL3 and ANGPTL5 facilitate the expansion of human repopulating cells in *ex vivo* cultures of UCB-derived $CD34^+$ cells. Also Himburg *et al*. reported that in a period of 7 days of liquid culture, the frequency of repopulating cells and engraftment in NOD-SCID mice could be increased using liquid suspension culture with thrombopoietin, stem cell factor (SCF), Flt-3 ligand and pleiotrophin (TSFP) [23]. Boitano *et al* showed that using a 3 week period of liquid culture treatment in the presence of TPO, SCF, FLt3L, IL-6, and StemRegenin1 (SR1), resulted in a 17 fold increase in

repopulating cells as measured by NOD SCID mice models [24]. Due to having different combination of growth factors and incubation period, it would be difficult to compare this study with our observations, although there is a possibility of the short-term SRCs expansion compared to long-term-SRCs due to a long period of incubation. Probably applying this new combination of growth factors or components for *ex vivo* expansion of HSCs can aid in using UCBs as an HSC source in clinical trials in both children and adult patients.

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Chapter 5

Effects of hypoxia on the ex vivo expansion of murine and human repopulating hematopoietic stem and progenitor cells

Manuscript in preparation

Abstract

Angiopoietin-like proteins (Angplt) and Insulin-like growth factors (IGF) stimulate cytokine-driven *ex vivo* expansion of hematopoietic stem and progenitor cells (HSPC). Previous studies investigated the effect of hypoxia on the *ex vivo* expansion of murine and human HSCs and progenitors and suggested that hypoxia induces expansion of HSCs. In this study, we examined whether hypoxia further enhanced the *ex vivo* expansion of murine bone marrow and human umbilical cord (UCB) HSPC, stimulated with SCF, TPO, IGF2, Flt3L and Angptl3 (STIFA3). During 7 days of *in vitro* culturing under hypoxia conditions, Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ (LSK) cells expanded 80±5-fold as opposed to 35±4-fold under normoxic conditions. Mouse CFU-GM and BFU-E and short-term repopulating HSCs (CFU-S) expanded 15±3-fold and 34±6-fold in normoxia and 42±7-fold and 76±9-fold, respectively, in hypoxia. Primary and secondary transplantation experiments of expanded LSK cells demonstrated >10-fold expansion of long-term repopulating HSCs compared to freshly isolated cells, irrespective of normoxic or hypoxic culture conditions. Six months post transplantation of the input-equivalent of only 12 LSK cells under normoxic or hypoxic conditions resulted in 50±5% and 48±4% donor chimerism, respectively. These data suggest that *ex vivo* mild hypoxia does not alter *ex vivo* expansion of murine long-term repopulating HSCs but promotes the expansion of short-term repopulating HSPC and more committed progenitor cells.

Next, we evaluated the effect of hypoxia on STIFA3-driven *ex vivo* expansion of human UCB-derived $CD34^+$ cells. Human UCB $CD34^+$ cells stimulated with STIFA3 for 7 days under normoxic or hypoxic conditions showed similar, approximately 12-fold, expansion of total nucleated cells. Transplantation of 4×10^5 fresh, $CD34^+$ cells or the input equivalents of 4×10^5 normoxic- or hypoxic-cultured $CD34^+$ cells into NOD/SCID mice resulted in approximately 13, 44, and 70% human chimerism in bone marrow, respectively, 6-8 weeks following transplantation. Hypoxia promoted the expansion of short-term SCID-repopulating cells (SRC) approximately >2 -fold. These findings may serve as a basis for the clinical implementation of *ex vivo* stem cell expansion for both allogeneic cord blood and autologous gene modified stem cell transplantation.

Introduction

Ex vivo expansion of hematopoietic stem cells (HSCs) is an attractive approach to increase the number of mature cells for adoptive therapy or raising primitive stem cell numbers for clinical HSC transplantation when limited numbers of HSCs are available, such as in umbilical cord blood (UCB) grafts and leukapheresis samples from intensively pretreated patients.

The *ex vivo* expansion of HSPCs depends on a variety of factors such as the O_2 level, combinations of hematopoietic growth factors (HGFs), the purity of starting population and culture duration[1-3]. The effect of the oxygen pressure on *ex vivo* expansion of HSCs has been studied since the late 1970s. Bradley *et al.* showed that culture of both mouse and human marrow cells under 6,8% oxygen significantly improved the clonal growth of

granulocyte-macrophage progenitor compared to atmospheric oxygen (20%) [4]. Eliasson *et al*. reported that hypoxic conditions reduced the yield of total mononuclear cells in *ex vivo* expansion cultures which was accompanied by an increased frequency of SCID repopulating cells (SRC) [2]. Using human HSC from BM or UCB resulted in similar observations; the preferential expansion of immature and not mature cells under hypoxia (1-1.5%) caused expansion/or maintenance of the number of repopulating cells (SRCs) [5, 6]. Moreover, Roy *et al*, reported that hypoxia (5%) enhanced the proliferation of UCB-derived HSC and progenitor cells and maintained the number of repopulating cells compared to atmospheric oxygen [7]. Taken together, the positive effect of hypoxia on maintenance of repopulating cells demonstrated that primitive progenitors and stem cells are particularly well adapted to proliferate in a low-O_2 environment [7].

The combination of growth factors during *in vitro* culturing is another factor that influences *ex vivo* expansion of HSPCs. Lodish and Zhang *et al*. introduced a cocktail of hematopoietic growth factors consisting of stem cell factor (SCF) [8, 9], thrombopoietin (TPO) [10-12], insulin like growth factor 2 (IGF-2) [13, 14], fibroblast growth factor 1 (FGF-1) [15, 16], and angiopoietin like proteins (Angptls) [3, 17] (STIFA) that can effectively stimulate *ex vivo* expansion of murine HSCs [3] and UCB-derived $CD34^+$ cells [14]. We further investigated how the combination of STIF+Angptl3 effects on the expansion of human and murine HSCs under serum free culture conditions at different time point. We reached to >10-fold expansion of LT-HSCs by culturing murine BM LSK for 7 days in presence of STIFA3 combination of growth factors (Chapter 2). We also showed that SCID repopulating cells from human UCB $CD34^+$ cells were expanded ~6-fold in similar culture conditions (Chapter 4).

The main purpose of this study was to investigate whether hypoxic conditions can further enhance the STIFA3-driven *ex vivo* expansion of mouse and human HSPC . We evaluated the effects of hypoxia on mouse-BM-derived and human UCB-derived HSPC, performed *in vitro* colony assays, and transplantations in immunodeficient mice to determine whether the oxygen tension during ex vivo expansion alters the characteristics of the expanded population of cells.

Material and Methods

Mice

Female *a*-thalassemic Balb/c mice, 8-12 weeks of age, were used as BM recipients and male normal littermates as donors. NOD/LtSz-SCID/SCID (NOD/SCID) mice were used as recipients of human HSPC. The mice were bred and housed under specific pathogen-free (SPF) conditions at the Experimental Animal Facility of Erasmus University (Rotterdam, the Netherlands). All experiments were approved by the independent animal ethical committee in accordance with legislation in the Netherlands.

Stem Cell enrichment

Mice:

Lineage negative (Lin^-) cells were purified from bone marrow (BM) cells using the BD IMag Mouse Hematopoietic Progenitor Cell Enrichment Set (BD Biosciences, Breda, The Netherlands) according to the manufacturer's instructions. Further HSC enrichment of the Lin^- cells was accomplished by incubating the cells with c-kit–allophycocyanin (APC; BD Biosciences) and Sca-1–R-phycoerythrin (PE; BD Biosciences). The cells were washed once with H+H (Hank's + HEPES, 300 mOsm) and purified Lin^- cells were sorted into $Sca\text{-}1^+/c\text{-}kit^+$ (LSK) cell populations using a BD FACSAria flow cytometer.

Human umbilical cord blood (UCB) cells

Following full-term delivery, human UCB samples were collected from the umbilical cord by the nursing staff of the Department of Obstetrics and Gynecology at the Sint Franciscus Hospital (Rotterdam, The Netherlands). Informed consent to collect the samples was obtained from the mothers according to legal regulations in The Netherlands. UCB was collected in sterile flasks containing 10 ml of 1% heparin in H+H at room temperature.

Low-density cells were isolated from the UCB samples using Ficoll Hypaque density centrifugation in 1.077g/cm^2 Lymphoprep (Nycomed Pharma, Oslo, Norway). The cell suspension was centrifuged at 600x*g* for 15 minutes, after which the mononuclear cell (MNC) band at the interface was harvested and, washed twice with Hank's balanced salt solution (HBSS, Gibco). The accumulated cells were used to select $CD34^+$ cells.

$CD34^+$ cell purification

$CD34^+$ cells were isolated from fresh UCB-MNC, using the indirect $CD34^+$ Microbead kit (Miltenyi Biotec, Germany). In brief, the $CD34^+$ hematopoietic progenitor cells were indirectly magnetically labeled using a hapten-conjugated primary monoclonal antibody and an anti-hapten antibody coupled to MACS MicroBeads (Miltenyi Biotec, Germany). The magnetically labeled cells were enriched in separation columns in the magnetic field of a manual MACS system. The purity of the selected population of $CD34^+$ cells generally exceeded 95%.

In vitro suspension culture

The cells were cultured in 24-well plate (Costar, tissue-culture treated polystyrene) at a starting density of $4\text{-}6\times10^4$/ml in the serum-free medium that contained enriched Dulbecco's modified Eagle's medium (DMEM) with 1% (wt/vol) bovine serum albumin (BSA), 0.3 mg/L human transferrin, 0.1 µM sodium selenite, 1 mg/L nucleosides (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyaenosine, thymidine and 2'-deoxyguanosine; all from Sigma, St. Louis, MO, USA), 0.1 mM ß-mercaptoethanol, 15 µM linoleic acid,15 µM cholesterol, 100 U/ml penicillin and 100 µg/ml streptomycin as previously described[18, 19]. Cells were incubated either in 20% O_2 and 5% CO_2 at 37°C innormoxia or hypoxia (5% O_2 and

5% CO_2) using an O_2/CO_2 multigas incubator (Sanyo). Murine STIFA3 cocktail (murine SCF (100 ng/ml, R&D, Abingdon, UK), murine Tpo (20 ng/ml, R&D), murine Igf-2 (20 ng/ml, R&D), human FGF-1 (10 ng/ml, Invitrogen, Breda, The Netherlands) and murine Angptl3 (200 ng/ml, R&D) plus heparin (10 µg/ml, Sigma) was used to promote murine stem cell expansion and proliferation.

CD34+ cells were cultured at density of 3-4×105/ml in serum free medium. Medium for human cells was supplemented with human STIFA3 cocktail (human SCF (50 ng/ml, R&D), human TPO (20 ng/ml, R&D), murine Igf2 (20 ng/ml, R&D), human FGF-1(10 ng/ml, R&D), and human ANGPTL3 (100 ng/ml, BioVendor, Heidelberg, Germany) plus heparin (10 µg/ml, Sigma)). Half of the medium was refreshed every 3-4 days.

In vitro clonogenic progenitor assays

The *in vitro* frequency analysis of hematopoietic stem cells and progenitor cells was assayed by semi-solid colony assays. Fresh or cultured Lin^- (2×10^3), LSK (2×10^2) or UCB-$CD34^+$ (1×10^3) cells were plated (BD BioCoat™ Collagen IV 35 mm Culture Dishes, tissue-culture treated polystyrene) in 1 ml of semi-solid methylcellulose culture medium containing 0.8% (wt/vol) methylcellulose (Methocel A4M Premium Grade, Dow Chemical, Barendrecht, The Netherlands) in enriched DMEM as described previously [18, 20].

Colony-forming unit granulocyte-macrophage (CFU-GM) levels were determined by culturing Lin^-/LSK cells with 10 ng/ml mouse interleukin-3 (mIL-3), 100 ng/ml m-SCF and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF). Burst-forming erythroid units (BFU-E) were cultured in the presence of 100 ng/ml m-SCF and 4 U/ml human erythropoietin (H-EPO, Behringwerke, Marburg, Germany). Granulocyte Macrophage colony formation unit (CFU-GM) of expanded UCB-derived $CD34^+$ cells were quantified by stimulation with 10 ng/mL hu-IL-3, 100 ng/mL hu-SCF, and 20 ng/mL GM-CSF. Burst-Forming Erythroid (BFU-E) were grown in presence of 100 ng/mL hu-SCF and 4 U/mL hu-EPO. The cells were cultured for 14 days and each experiment was performed in duplicate.

Colony forming unit spleen (CFU-S)

Bone marrow cells obtained from BALB/c donors were transplanted into lethally irradiated (8 Gy) BALB/c recipients (n=7-10 mice per group). A total of 1×10^3 fresh Lin^- cells or 3×10^2 input equivalents of 7-day cultured cells in STIFA3 were transplanted into lethally irradiated mice. Additionally, a total of 200 LSK cells were transplanted at day 0, and the equivalent of 20 or 10 LSK cells from 7-day cultures were injected into the lethally irradiated (8 Gy) BALB/c mice. One thousand BM cells that were irradiated with 50 Gy were co-administered with the LSK day 0 cells to prevent loss of cells during the injection and homing steps. No colonies were observed in a control group of mice that received only the irradiated BM cell. The number of spleen colonies (CFU-S) was counted after 12 days.

Long-term repopulation ability assays (LTRA)

Male BALB/c cells were transplanted into sublethally (6 Gy) irradiated female α-thalassemic mice. Blood was collected monthly for six months, and red blood cell (RBC) chimerism and peripheral blood cell counts and parameters were measured using the Heska Vet Animal Blood Counter (Heska, Fribourg, Switzerland). Mouse peripheral blood was prepared in 1 ml 0.6% NaCl buffer, and microcytic thalassemic RBCs were separated from healthy cells by flow cytometry in a FACS Calibur system (BD Biosciences). The chimerism percentage was calculated using the following formula: [-0.6+square root ($(0.6^2$-$4\times0.002\times$(10.43-donor cells %)))/0.004][21].

Y-chromosome Q-PCR

Y-chromosome Q-PCR was performed to detect the percentage of donor leukocyte chimerism in the recipients. DNA was extracted from BM using the Biorad kit (Biorad, Veenendaal, The Netherlands).

Specific primers for the *Sry* locus in the Y-chromosome were designed using Beacon software (New Orleans, LA, USA). The sequences were as follows (5'-3'): sense, TCA-TCG-GAG-GGC-TAA-AGT-GTC-AC; antisense, TGG-CAT-GTG-GGT-TCC-TGT-CC. As a control for total DNA, the following *GAPDH* primers were used: sense, ACG-GCA-AAT-TCA-ACG-GCA-CAG; antisense, ACA-CCA-GTA-GAC-TCC-ACG-ACA-TAC. Each reaction mixture contained 8 µl DNA template (70 ng), 8 µl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1.25 µl of each primer (5 ng/µl) in a final reaction volume of 25 µl. Q-PCR of the *Sry* locus and *GAPDH* was performed in a Biorad MyiQ thermocycler as follows: 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 seconds and 60 ºC for 45 seconds.

SRC assay

Fresh or cultured human UCB-$CD34^+$ cells were transplanted into sublethally irradiated (3.5 Gy) SPF NOD/LtSz-scid (NOD/SCID) mice at the indicated days and density. The mice were sacrificed by CO_2 inhalation 6-8 weeks post-transplantation. After counting the number of cells isolated from the femurs, 1×10^5 cells were cultured in a colony assay and the rest was analyzed by flow cytometry to determine the percentage of human cells in the mouse bone marrow.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the Mann-Whitney U test. Standard deviations of colony counts were calculated on the assumption that crude colony counts are a Poisson distributed.

Results

Hypoxia increase the total cell yield in murine bm-derived HSPC expansion cultures

To investigate whether hypoxic conditions can further enhance the *ex vivo* expansion of murine HSPC (hematopoietic pluripotent stem cells) stimulated with STIFA3, the HPSCs from different stages in the HSC lineage (e.g., Lin^- or LSK cells) were cultured *in vitro* under normoxic (atmospheric 20% O_2) or hypoxic (5% O_2) conditions. After 7 days of *in vitro* culture in the presence of the STIFA3 growth factor cocktail, the Lin^- cells that were cultured under normoxic conditions expanded 15±2-fold; whereas the Lin^- cells that were cultured under hypoxic conditions expanded 35±4-fold (Figure 1A).

Under the same conditions, LSK cells expanded 35±4-fold under normoxic conditions and 80±5-fold under hypoxic conditions. The frequency of LSK cells after 7 days of culture under normoxic or hypoxic conditions was ~45% and ~30%, respectively. Thus, the LSK cells expanded approximately 1.5-fold better under hypoxic conditions compared to normoxic conditions (Figure 1A, B).

Hypoxia increases STIFA3-driven expansion of CFU-GM and BFU-E from murine Lin^- and LSK HSPCs

After showing that hypoxic conditions increased the yield of LSK cells ~1.5-fold, we next analysed whether hypoxia also increases the yield of more committed progenitor cells. Therefore, Lin^- and LSK cells, cultured in the STIFA3 cocktail under normoxic or hypoxic conditions, were examined using the BFU-E and CFU-GM colony forming assays. The frequency of CFU-GM and BFU-E cells was increased significantly after Lin^- and LSK cell populations were cultured under hypoxic conditions relative to normoxic conditions. The expansion of progenitor cells in the Lin^- population was increased from 17.5±4-fold in normoxia to 25±5-fold in hypoxia after 7 days of *in vitro* culturing in the STIFA3 cocktail (Figure 1C, supplementary table 1A). Expectedly, hypoxic culture conditions also increased the expansion of CFU-GM and BFU-e when cultures were initiated with LSK cells (i.e., 15±2-fold expansion in normoxia *vs*. a 42±7-fold expansion in hypoxic circumstances). Therefore, the CFU-GM and BFU-e expanded 2-fold more in hypoxia as compared to normoxia (Figure 1C, supplementary table 1B).

Hypoxic conditions increase the expansion of short term-HSPC (Day 12 CFU-S) from murine Lin^- and LSK HSPCs

We next examined the effect of hypoxia on ST (short term)-HSCs using the CFU-S assay 12 days after transplantation. Lin^- or LSK cells cultured *in vitro* for seven days were transplanted into lethally irradiated healthy BALB/C mice, and twelve days after transplantion, the number of ST-HSCs was determined by counting the colonies that arose in the spleens of the recipient mice. After 7 days of *in vitro* culture of lin^- cells, the number of ST-HSC

increased 5±2-fold under normoxic conditions, whereas ST-HSC increased 15±4-fold under hypoxic conditions (Figure 1D, supplementary table 2). The ST-HSCs of cultured LSK cells expanded 34±6-fold under normoxic conditions, and 76±9-fold under hypoxic conditions. Based on these results, we conclude that hypoxia promotes the expansion of ST-HSCs and results in a significant increase in the number of ST-HSCs (Figure 1D, supplementary table 2).

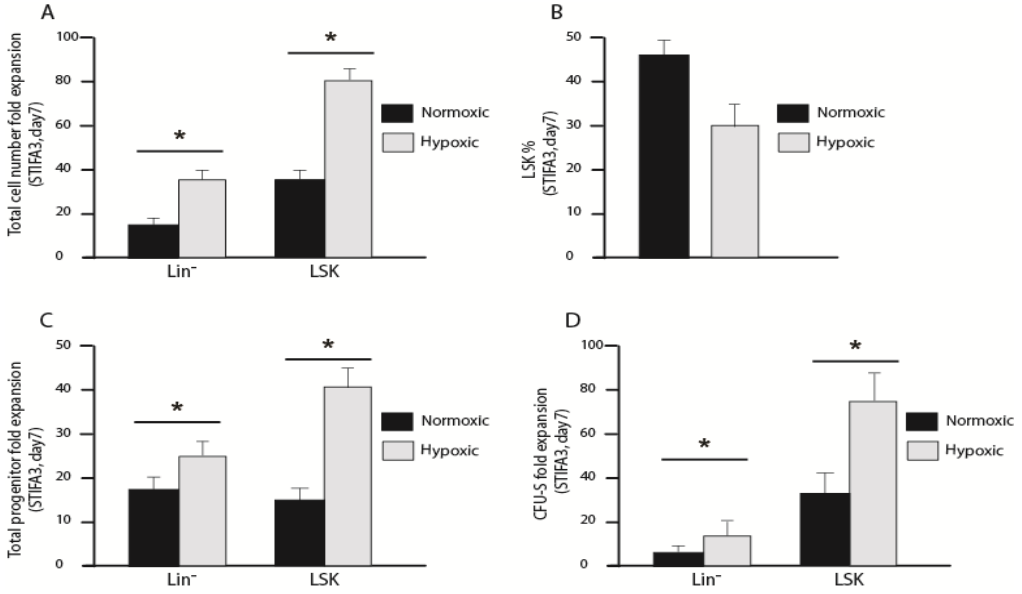

Figure 1: Hypoxia enhances the proliferation of hematopoietic stem cells and progenitor cells. (A) Lin^- or Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ (LSK) cells were cultured in 20% O_2 (normoxia) or 5% O_2 (hypoxia) for 7 days in serum-free medium supplemented with STIFA3 cocktail (SCF, TPO, IGF-2,FGF-1, and Angptl3). The mean fold increase in total cell numbers was measured in comparison to day 0. The results of five independent experiments are shown. The error bars indicate the standard deviation (SD). *P<0.05, **(B)** LSK cells were cultured for 7 days, labeled with Sca-1-PE and c-kit-APC, and then analyzed by flow cytometry **(C)** Expansion of colony forming-units (BFU-E and CFU-GM) of Lin^- or LSK cells cultured for 7 days relative to day 0 from 5 independent experiments. The average number of colonies per 10^5 plated cells was calculated from duplicate experiments. Results were shown in fold expansion relative to day 0 **(D)** The CFU-S (12-day) fold expansion of Lin^- or LSK cells cultured for 7 days in the presence of STIFA3 relative to day 0. N=10 mice per group.

Hypoxia has no effect on the number of long-term murine hematopoietic stem cells

To determine the effect of hypoxia on the number of LT (long term)-HSCs, 200 LSK cells or the input equivalent of 200 LSK cells that were cultured for 7 days in STIFA3 cocktail under normoxic or hypoxic conditions were transplanted into sublethally irradiated recipients. After 7 months, the red blood cell chimerism in peripheral blood (PB) was measured in alpha-

thalassemic mice by flow cytometry, leukocyte chimerism in PB and BM was measured using Y-chromosome Q-PCR. High levels of donor-RBC chimerism (>80%) were measured in all groups. We observed engraftment of donor cells in BM of all recipients. This was likely due to the high number of transplanted cells. To overcome this, serial dilutions of BM cells (10^6, 3×10^5, and 10^5) from the primary recipients were transplanted into secondary recipients. After 6 months, we found that the chimerism percentages in secondary recipients, upon (transplantation of 10^6 BM cells from primary recipients that were transplanted *ex vivo* expanded LSK cells gave rise to 77±4 and 70±5% for normoxic and hypoxic, respectively) was higher than that in secondary recipients, upon transplantation of 10^6 BM cells from primary recipients that were transplanted with freshly isolated LSK cells (35±4%) (Figure 2A, B). Based on the outcome of the transplantation of serially diluted BM cells of primary recipients to secondary recipients, there were no significant differences between normoxia and hypoxia conditions, which may be due to the use of saturated amounts of HSPC.

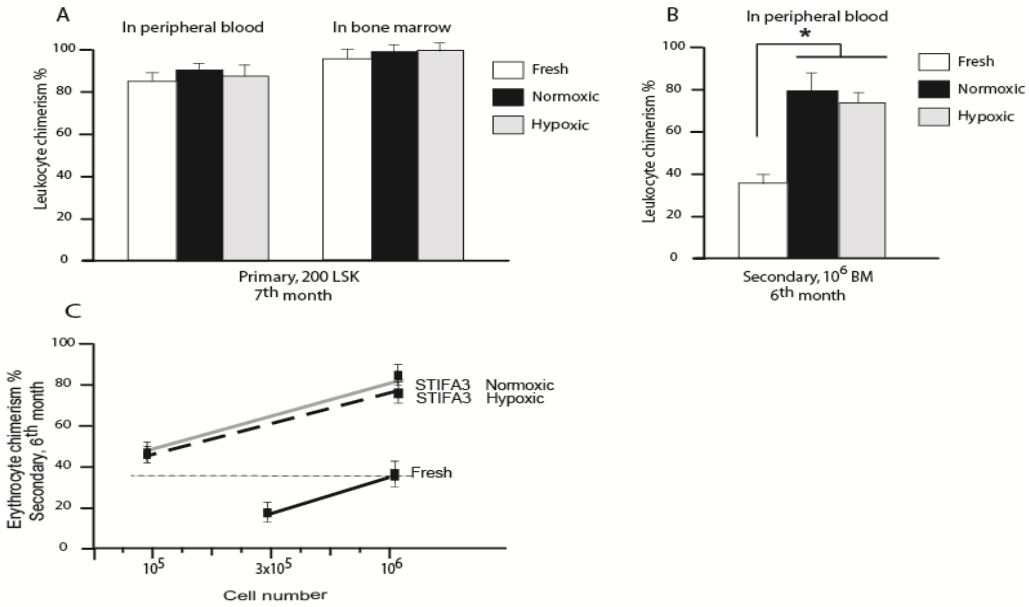

Figure 2: Hypoxia preserves the number of murine hematopoietic stem cells. (A) 200 fresh LSK cells or the input equivalent of 200 LSK cells after 7-days culture in STIFA3 medium under hypoxic and normoxic conditions were transplanted into sublethally irradiated recipients. The percentage of donor leukocyte chimerism was determined 7 months after transplantation in PB and BM. N=5 mice per group. **(B)** The percentage of WBC chimerism in peripheral blood of secondary recipients which received 10^6 BM cells from primary recipients was determined 6 months after retransplantation in PB. N=5 mice per group. **(C)** Serial dilution of BM cells from primary recipients 7 months post-transplantation were retransplanted into secondary recipients. The data represent donor RBC chimerism in peripheral blood of 10^6, 3×10^5, 10^5 transplanted BM cells after 6 months. N=5 mice per group. The error bars indicate the standard deviation (SD). *P<0.05

In a second experiment, primary recipients received 120 or 12 freshly isolated LSK cells or the input equivalent thereof after 7 days of culture under normoxic or hypoxic conditions. One month after transplantation of only 12 LSK cells, the percentage of donor cells (RBC), which is a reflection of ST-HSCs, was measured in blood. For both freshly isolated and hypoxic cultured cells this was 36±4%, and for normoxic cultured cells this was higher; 60±4% (data not shown). However, 6 months after transplantation, this percentage was essentially unchanged for fresh cells (32±4%), but was increased for the hypoxic cultured cells (48±4%), and approached the same level as cells cultured under normoxic conditions (50±5%) (Figure 3A, B). Based on the serially diluted LSK cells (120, 12) in primary recipients, LT-HSCs after culturing in STIFA3 for 7 days expanded approximately >10-fold either under normoxia conditions or hypoxic conditions. Thus, we did not detect any effect of hypoxia on the expansion of murine LT-HSCs (Figure 2, 3).

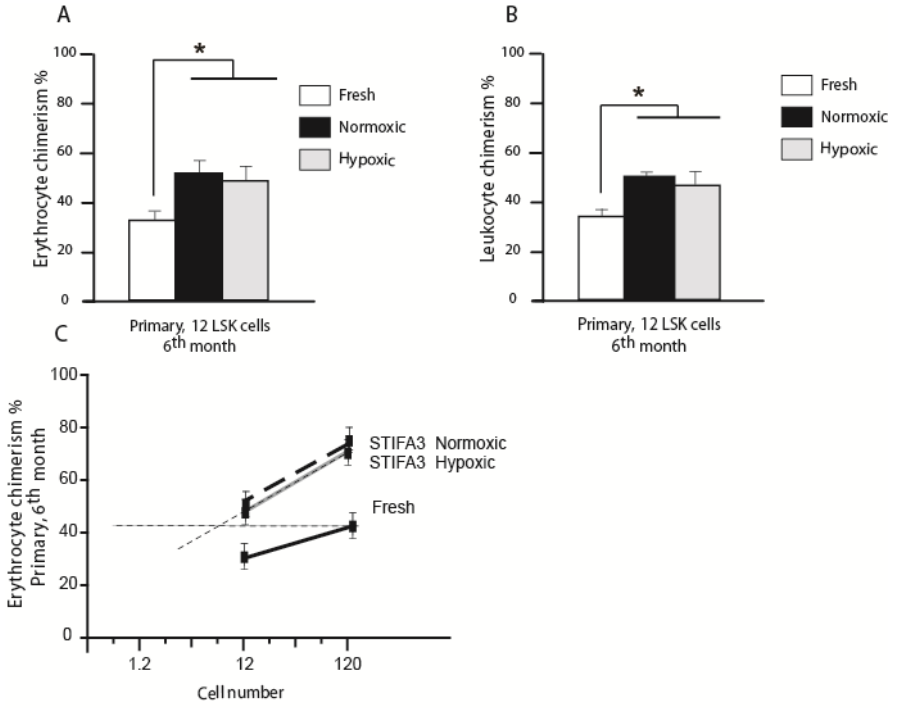

Figure 3: Effect of hypoxia on the engraftment potential of expanded murine-LSK cells. (A) The percentage of donor erythrocyte chimerism was determined 6 months post transplantation with 12 LSK cells **(B)** leukocyte chimerism% **(C)** The percentage of donor erythrocytes was determined 6 months post transplantation with 12o and 12 LSK cells. The error bars indicate the standard deviation (SD).

Hypoxia increases the yield of CD34$^+$ cells in UCB-derived CD34$^+$ HSPC expansion cultures

Next, we evaluated, whether hypoxia enhances *ex vivo* expansion of human UCB CD34$^+$ cells, stimulated for 7 days with STIFA3. Therefore, CD34$^+$ cells from UCB were cultured for 7 days in the STIFA3 growth factor cocktail under normoxic or hypoxic conditions. After 7 days in culture, the total number of cells increased 5±1-fold in normoxia and ~4.5±1-fold in hypoxia (Figure 4A). However, the percentage of cells that remained CD34$^+$ after 7 days in culture differed between the normoxic and hypoxic conditions. After culturing enriched UCB-CD34$^+$ cells (>95% purity) for 7 days, the frequency of CD34$^+$ cells dropped to ~35-40% under normoxic conditions, whereas the frequency of CD34$^+$ cells was still ~50-60% under hypoxic conditions (Figure 4B). These data suggest that hypoxic culture conditions may inhibit the differentiation of CD34$^+$ cells into more committed CD34$^+$ cells.

To study the effect of hypoxia on the expansion of more committed progenitor within the UCB-derived CD34$^+$ cells fractions, we used the CFU-GMs and BFU-E assays. No differences were found in the number of BFU-Es and CFU-GMs between normoxic and hypoxic conditions (Figure 4C). Thus, hypoxic conditions did not affect the expansion of CFU-GM and BFU-E.

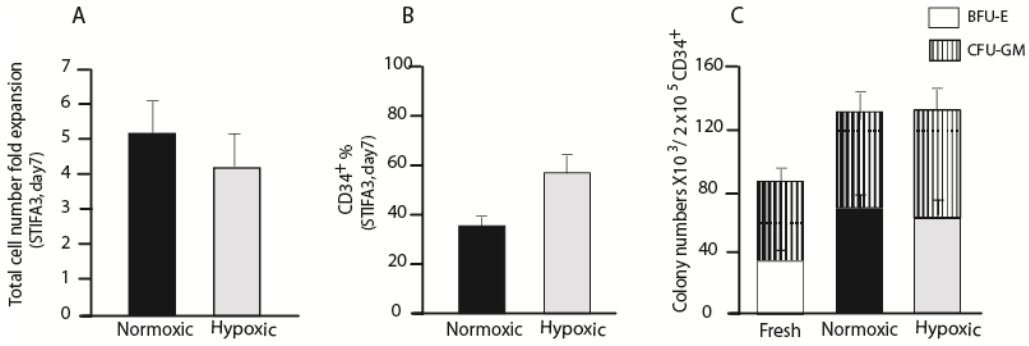

Figure 4: Hypoxia has no effect on the proliferation of UCB-CD34$^+$ cells *in vitro*. (A) UCB-CD34$^+$cells were cultured in 20% (normoxia) or 5% O_2 (hypoxia) for 7 days in serum-free culture medium supplemented with STIFA3 cocktail. The average fold increase in total cell numbers is shown relative to day 0. The error bars indicate the standard deviation (SD). **(B)** UCB-derived CD34$^+$cells were cultured for 7 days, the frequency of CD34$^+$ cells after culture was determined using flow cytometry. **(C)** Absolute number of colony-forming units (BFU-E and CFU-GM) of 7-day cultured UCB-CD34$^+$ cells relative to day 0. All data represent the results of 5 independent experiments.

Hypoxia increases the STIFA3-driven expansion of short-term SRC from UCB-derived CD34$^+$

Human HSCs are capable of extensive proliferation and multilineage repopulation in NOD/SCID mice and are then called SCID-repopulating cells (SRCs). SRCs are highly enriched

in the BM ($Lin^-CD34^+CD38^-$) fraction and are present in higher proportions in umbilical cord blood than in adult BM[22, 23]. We and others have previously tested various culture conditions for their ability to maintain or even enhance SRC activity *in vitro* [5, 15, 16, 24-32]. To test the effect of hypoxia in this setting, UCB-$CD34^+$ cells (4×10^5 fresh $CD34^+$ cells or the input equivalent of 4×10^5 $CD34^+$ cells after culture under normoxic or hypoxic conditions for 7 days in STIFA3 cocktail) were transplanted into sublethally irradiated NOD-SCID mice. Six to 8 weeks after transplantation, the percentage of human $CD45^+$ leukocytes in bone marrow was determined using flow cytometry. FACS analysis revealed 13±4% human $CD45^+$ cells in the recipients of fresh $CD34^+$cells. This percentage was much higher in recipients who received cells cultured for 7 days under normoxic (44±5%) or hypoxic (70±5%) conditions (Figure 5A).

We next measured the percentage of the various subpopulations of human cells in the BM of recipient mice that received 4×10^5 fresh or $CD34^+$ cells or input equivalents of after 7 days of culture under normoxia or hypoxia. Transplanting freshly isolated cells resulted in 13±3% $CD34^+$ cells of the total human $CD45^+$ population that repopulated the NOD-SCID mice and this percentage increased to 22±5 or 33±4% in mice that received cells that were cultured under normoxic or hypoxic conditions, respectively (Figure 5A).

To calculate the number of immature $CD34^+CD38^-$ cells, we measured the percentage of $CD34^+CD38^-$ cells within the human $CD34^+$ cell population in bone marrow. The percentage of $CD34^+CD38^-$ was ~6% after transplantation of fresh CD34+ cells. The percentage of $CD34^+CD38^-$ was significantly higher after transplantation of cells cultured under normoxia (10%) or hypoxia (22%) (Figure 5A).

Next, we used CD15 to measure the number of $CD15^+$ myeloid cells, and we found no difference in the percentage of $CD15^+$ myeloid cells in the $CD45^+$ cell population between the normoxic and hypoxic conditions. Also the percentage of $CD19^+$ B cells within the human $CD45^+$ population did not differ between normoxic and hypoxic conditions (Figure 5B).

Further, we performed the transplantations of serially diluted $CD34^+$ cells (1×10^5, 4×10^5, and 6×10^5) to calculate the fold increase in short-term SCID repopulating cells under normoxia or hypoxia environments. Again, cells were cultured for 7 days in the presence of STIFA3 under normoxic or hypoxic conditions. The results show that culturing cells under hypoxic conditions resulted in a ~2-fold increase in short-term SRCs compared to cells that were cultured for 7 days under normoxic conditions. The number of short-term SRCs increased ~12-fold during 7-day culture in STIFA3 under hypoxia conditions compared to input cells (Figure 5D).

To study the effect of hypoxic conditions on CFU-GM and BFU-e expansion, we plated equal numbers of recipient BM cells ($1x10^5$ cells) in the CFU assays. Nearly twice as many BFU-E colonies were counted under hypoxic conditions (100) than under normoxic (55) conditions. Also the number of CFU-GM colonies was ~2-fold higher under hypoxic conditions compared to normoxic conditions (122 or 66 colonies per 10^5 BM cells, respectively) (Figure 5C). In addition, the colonies were larger after prior expansion under hypoxia compared to under normoxia (data not shown).

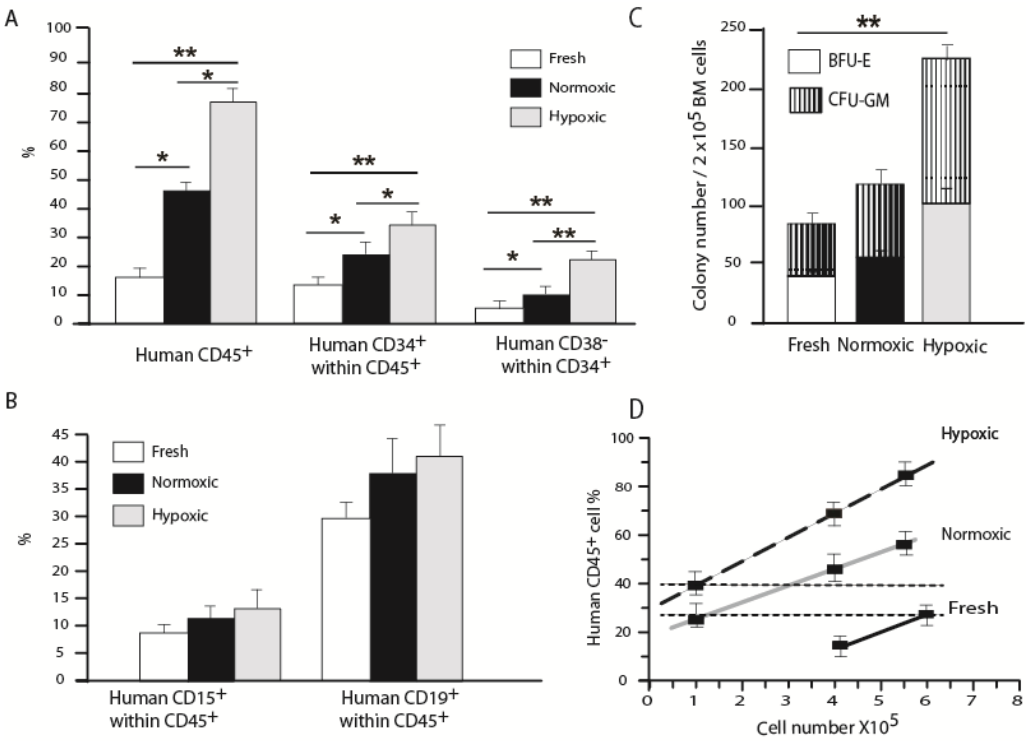

Figure 5: Hypoxia increases the proportion of UCB-HSCs *in vivo*. UCB-CD34$^+$ cells were cultured in 20% (normoxia) or 5% O_2 (hypoxia) for 7 days in serum-free culture medium supplemented with STIFA3. 4 x 10^5 fresh UCB-CD34$^+$ cells or the input equivalent of 4 x 10^5 UCB-CD34$^+$ cells cultured for 7 days in STIFA3 (4×10^5 cells) were transplanted into sublethally irradiated NOD-SCID mice (N=12 mice per group). **(A)** Six to 8 weeks after transplantation, the percentage of human leukocytes (CD45$^+$) or progenitor cells (CD34$^+$ or CD38$^-$ within CD34$^+$) in bone marrow was measured by flow cytometry as well as the proportion of **(B)** myeloid cells (CD15), and B cells (CD19) cells within the total CD45$^+$ cell population. **(C)** Absolute number of colony-forming units (BFU-E and CFU-GM) per 2 x 10^5 recipient BM cells. The average number of colonies was calculated from duplicate experiments. The error bars indicate the standard deviation (SD). *P<0.05, **P<0.01. **(D)** The percentage of human leukocytes (CD45$^+$) in BM was determined 6-8 weeks after transplantation with serially diluted fresh or cultured CD34$^+$ cells.

Discussion

We examined the effect of hypoxia (5% O_2) on STIFA-driven *ex vivo* expansion of murine and human HSPCs. Evaluation of hypoxia conditions on *ex vivo* expansion of murine bone marrow-derived HSPCs revealed that hypoxia, compared to normoxia (20% O_2), enhances the expansion of LSK cells, CFU-S progenitors, and ST-SRCs populations proliferation while the number of LT-SRCs were preserved. In contrast, hypoxic conditions enhanced *ex vivo* expansion of short-term SRC from UCB-derived $CD34^+$ HSPC, as measured by serially diluted transplantation into NOD-SCID mice, and preserved the number of CFU-GM and BFU-E progenitor cells compared to normoxic conditions. Taken together, our experiments showed that under 20% O_2, short-term repopulating cells from UCB-HSC and mouse BM-HSCs expands up to 6-, and 10-fold, respectively whereas repopulating cells from UCB and BM enhanced 12- and 10-fold under 5% O_2, respectively. This suggests that hypoxia (5% O_2) could be a more favorable condition for *ex vivo* expansion of UCB-$CD34^+$ derived short-term SRCs compared to normoxia. However, the reduction in O_2 concentration did not further enhance expansion of murine BM LT-SRC compared to normoxia. Because, it was assumed that under physiological conditions UCB-HSC are exposed to higher oxygen level (~5% O_2) compared to BM cells (<1-2% O_2), mild hypoxia conditions (5% O_2) might be not low enough to enhance expansion of enriched BM-derived HSCs compared to normoxic conditions.

Previously, Eliasson *et al.* reported that culturing murine BM-HSCs under hypoxia (1%) conditions suppressed the expansion of mouse BM-HSCs, maintained the number of progenitor cells but increased number of LT-HSCs as measured by reconstitution ability of donor cells in lethally irradiated mice [2]. The only element that varied between Eliasson *et al*. and our study was the O_2 level (5% and 1%). Comparison of Eliasson's and our data may suggest that murine BM-HSCs require O_2 levels below 5% to reach the quiescent stage.

The hypoxia (1% O_2) studies on human BM- and UCB-$CD34^+$ cells by Dnate and Shima et al. and mild hypoxia (5% O_2) by us showed that both strong hypoxia or mild hypoxia promotes expansion of the short-term SRC repopulation [5, 6]. In contrast to our results, Roy et al. reported that hypoxia (5%) enhanced *ex vivo* expansion of total/progenitor cells while preserving the SCID-repopulation efficiency of UCB-$CD133^+$ cells [7]. The differences between the result of Roy *et al*. and our study may be due to a different combination of growth factors (SCF+Flt3+TPO+**IL-6**+**IL-3**) which may induce cells to react differently to hypoxia. For example, Roy *et al.* used IL-3 which promotes the commitment of HSCs to the myeloid lineage and acts as a survival factor for myeloid progenitors [33].

We further showed that the number of short-term SRC of UCB-$CD34^+$ population that were cultured 7 days in the presence of STIFA3 under mild hypoxic conditions increased ~12 fold compared to fresh cells. The number of LT-HSC from murine BM was not enhanced under mild hypoxic compared to normoxic condition. We presume that an oxygen level of 5% may be an appropriate condition for *ex vivo* expansion of UCB-HSCs which are naturally exposed to higher levels of oxygen compared to BM. An oxygen level of 5% may led to stabilization of HIF-1α, which leads to the expression of several HIF-regulated genes, including erythropoietin

(EPO), erythropoietin receptor (EPO-R), transferrin and its receptor, VEGF and its receptor VEGF-R (Flt1), and endoglin, which are essential in both angiogenesis and hematopoiesis.

Besides extrinsic factors, such as oxygen level, cytokines, and adhesion molecules, several intrinsic factors such as signaling molecules and transcription factors can also influence *ex vivo* expansion of HSCs. For example, ectopic expression of the transcription factor homebox B4 (HoxB4) resulted in 1000-fold and 40-fold expansion of murine HSCs *in vivo* and *in vitro*, respectively [34, 35]. The number of HSCs also increased in transgenic mice, in which Bcl-2 is expressed from the major histocompatibility complex H-2K promoter[36]. However, the factors or signals that activate anti-apoptotic pathways (e.g. Bcl-2) or specific transcription factors (e.g. HoxB) *in vitro* are not completely known. Thus, delivery of such transcription factors or apoptosis regulator proteins is presently not possible but may become available in future. It should however be noted that transient stimulation using optimized extrinsic factors may be easier and safer.

In summary, we propose that culturing UCB-HSCs at an oxygen level of 5% provides a better condition to stimulate expansion of UCB-HSCs when compared to 20% O_2. However, it seems that the *ex vivo* expansion of mouse LT-HSCs was not affected under mild hypoxic compared to normoxic conditions. This leads us to suggest that either mouse BM cells require a lower level of oxygen than 5% during culturing to enhance expansion of LT-HSC (as in their original environment) or BM cells adapt to a higher O_2 level contrary to their natural environment. Combining hypoxic culture condition in combination with a suitable growth factor cocktail to expand HSC *ex vivo,* might help to apply UCB as a source of HSCs for both adult patients and children.

Acknowledgements

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Supplementary

A

Lin⁻ cells experiment No.	Fresh				Normoxia (D7, STIFA3)					Hypoxia (D7, STIFA3)				
	intial cell number	cells plated	BFU-E	CFU-C	cell number	Fold expansion	cells plated	BFU-E	CFU-C	cell number	Fold expansion	cells plated	BFU-E	CFU-C
1	40000	2000	16±1,4	24±2,8	580000	15	29000	190±14,4	340±12	1520000	38	76000	374±24	752±35
2	40000	2000	19±1,4	22±2,1	540000	14	27000	180±12	320±18	1350000	33,75	67500	356±15	620±28
3	40000	2000	8±2,1	9±1,4	700000	18	35000	240±17,3	460±28	1620000	40,5	81000	270±19	768±42
4	60000	2000	16±0,7	36±2,8	850000	14	28333	254±15	500±34	2100000	35	70000	310±22	603±25
5	60000	2000	14±2,8	32±1,8	900000	15	30000	410±34	601±30	1750000	29,2	58333	324±18	580±22
Mean± SD			15±4	25±10				255±92	444±116				327±41	665±88

B

LSK cells experiment No.	Fresh				Normoxia (D7, STIFA3)					Hypoxia (D7, STIFA3)				
	intial cell number	cells plated	BFU-E	CFU-C	cell number	Fold expansion	cells plated	BFU-E	CFU-C	cell number	Fold expansion	cells plated	BFU-E	CFU-C
1	4000	200	12±3	14±1,4	140000	35	7000	180±18	300±23	335000	84	16750	360±18	900±45
2	4000	200	9±1,4	13±4	130000	33	6500	150±14	200±17	305000	76	15250	300±32	700±28
3	7000	200	11±2,5	12±1,8	275000	39	7857	176±22	250±11	580000	83	16571	240±16	900±52
4	7000	200	15±3	18±3,4	210000	30	6000	130±16	320±21	520000	74	14857	320±12	840±38
5	6000	200	13±1,8	17±4	225000	38	7500	190±12	220±14	510000	85	17000	290±14	900±46
Mean± SD			12±2	15±3				166±25	258±37				302±44	848±87

Table 1: Primary results of colony assay (A) Lin⁻cells were grown for 7 days in serum free cultures supplemented with STIFA3 under normoxic or hypoxic conditions. The cultures were initiated with 40000-60000 cells/ml. Fresh (2×10^3) or cultured Lin⁻ (input equivalent of 2×10^3) were plated in 1 ml of semi-solid methylcellulose culture medium. Results are mean ± SD of n=5 experiments. **(B)** LSK cells were grown for 7 days in serum free cultures supplemented with STIFA3 under normoxic or hypoxic conditions. The cultures were initiated with 4000-7000 cells/ml. Fresh (0.2×10^3) or cultured LSK (input equivalent of 0.2×10^3) cells were plated in 1 ml of semi-solid methylcellulose culture medium. Results are mean ± SD of n=5 experiments.

A

Experiment No.	CFU-S colony/ injected Lin⁻ cell					
	Fresh		Normoxia (D7, STIFA3)		Hypoxia (D7, STIFA3)	
	initial cell No.	1000 injected Liñ cell	absolute cell No.	300 injected Liñ cell	absolute cell No.	300 injected Liñ cell
1	40000	5	580000	6	1520000	16
1	40000	3	580000	6	1520000	13
1	40000	3	580000	6	1520000	11
1	40000	2	580000	4	1520000	9
1	40000	5	580000	4	1520000	17
2	60000	5	850000	5	2100000	14
2	60000	4	850000	5	2100000	10
2	60000	3	850000	4	2100000	20
2	60000	3	850000	3	2100000	13
2	60000	2	850000	4	2100000	16
Mean±SD		3±2		5±1,6		14±3,4

B

Experiment No.	CFU-S colony/ injected LSK cell					
	Fresh		Normoxia (D7, STIFA3)		Hypoxia (D7, STIFA3)	
	initial cell No.	200 injected LSK cell	absolute cell No.	20 injected LSK cell	absolute cell No.	10 injected LSK cell
1	4000	6	140000	14	335000	20
1	4000	4	140000	18	335000	14
1	4000	7	140000	17	335000	25
1	4000	6	140000	19	335000	19
1	4000	4	140000	18	335000	19
2	7000	4	275000	14	580000	17
2	7000	7	275000	17	580000	23
2	7000	4	275000	19	580000	19
2	7000	6	275000	16	580000	18
2	7000	4	275000	14	580000	16
Mean±SD		5±1,3		17±2,1		19±3,2

Table 2: Primary results of CFU-S assay (A) Lin⁻ cells were grown for 7 days in serum free cultures supplemented with STIFA3 under normoxic or hypoxic conditions. The cultures were initiated with 40000-60000 cells/ml. Fresh (1000 Lin⁻ cells) or cultured Lin⁻ (input equivalent of 300 Lin⁻ cells) were transplanted into lethally irradiated mice. 12 days post transplantation, CFU-S colonies were counted. Results are mean ± SD of 2 experiments. N=5 mice per group.**(B)** A total of 200 LSK cells were transplanted at day 0, and the equivalent of 20 or 10 LSK cells from 7-day cultures were injected into the mice. 12 days post transplantation, CFU-S colonies were counted. Results are mean ± SD of 2 experiments. N=5 mice per group.

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Chapter 6

Discussion

Discussion

Hematopoietic disorders are in theory curable with hematopoietic stem cell transplantation (HSCT) [1-3]. However, bone marrow (BM) and peripheral blood (PB)-HSC transplantation applications for HSCT have several draw backs, including the limitation of suitable HLA-matched donors, complications of graft versus host diseases (GvHD) and tumorigenic potential of autologous bone marrow transplant (BMT). For these reasons alternative sources of hematopoietic stem cells (HSC) such as umbilical cord blood (CB) have been explored. UCB is easily accessible and has lower GvHD incidence regardless of HLA disparity. Laghlin *et al*. showed that UCB HLA mismatched transplants require longer time to engraft when compared to HLA-matched sibling or unrelated marrow transplants [4]. Since UCB allows more HLA disparity than BM or PBSC grafts, finding UCB donors for minority populations who are currently under-represented in donor registries is considerably easier[5-7].This makes UCB an attractive option when BM or PB-HSC donors are not the be best possible alternative [1]. Although the use of UCB as a stem cell source has increased significantly during recent years, especially for transplantation into children and early adulthood patients, it is not without downsides. One of the most important disadvantages of UCB as an HSC therapy is the limited available number of HSCs [8, 9]. This limitation of UCB raised considerable interest in the development of the *ex vivo* expansion of HSCs [8, 10, 11].

Several intrinsic factors, such as signaling molecules and transcription factors can influence the *ex vivo* expansion of HSCs. For example ectopic expression of the transcription factor homebox B4 (HoxB4) resulted in a 1000- and 40-fold expansion of murine HSCs *in vivo* and *in vitro*, respectively [12, 13]. In another study the number of HSCs increased in H-2K Bcl-2 transengic mice, in which Bcl-2 is expressed from the major histocompatibility complex H-2K promoter [14]. However, the factors or signals that activate anti-apoptotic pathways, such as Bcl-2 or specific transcription factors such as HoxB, *in vitro* are not completely known. Moreover, the delivery of these factors or proteins or their genes to target cells to the cell nucleus is hampered by the lack of suitable and effective techniques. In contrast, transient stimulation using optimized extrinsic factors is safer and easier.

One approach to *ex vivo* expansion of HSCs is liquid suspension culture, in which HSCs are exposed to a cocktail of cytokines, and growth factors for a specific period of time [8]. Different combinations of growth factors have been studied to come up with an optimum growth factor cocktail for expansion of HSCs. Analysis of 16 different cytokines on stroma-free cultures of $CD34^{+}CD38^{-}$ bone marrow cells indicated that the FMS-like tyrosine kinase 3 ligand (Flt3L), stem cell factor (SCF) and IL3 yield the highest (30-fold) expansion of starting long-term culture-initiating cells (LTC-IC) compared to fresh cells [15]. It was also shown that Flt3L maintains the long-term HSCs activity of human $CD34^{+}$ cells [16]. Thrombopoietin (TPO) has been also reported to increase the multilineage growth of $CD34^{+}CD38^{-}$ bone marrow cells in combination of Flt3L and SCF under stroma-free conditions [17]. Furthermore, Wognum *et al*. showed that serum free expansion cultures, stimulated with SCF, TPO and Flt3L (STF) result in expansion of progenitors, short term- and long term-HSCs [18]. This was followed by Levac *et al*. who reported that the combination of SCF, TPO and Flt3L (called STF), successfully

expand UCB-derived primitive HSCs [19]. A study by Himburg *et al*. reported that in a period of 7 days of liquid culture, the frequency of repopulating cells and engraftment level in NOD-SCID mice models could be also increased by using liquid suspension culture with SCF, TPO, Flt3L and pleiotrophin (STFP) [20]. Boitano *et al* showed that liquid culture supplemented with TPO, SCF, FLt3L, IL-6, and StemRegenin1 (SR1), results in 17 fold increase in repopulating cells relative to the control (TPO, SCF, FLt3L, IL-6) as measured by NOD SCID mice transplantation [21]. Insulin-like growth factor 2 (IGF2) and fibroblast growth factor (FGF1) were also introduced as promising growth factors in *ex vivo* expansion and proliferation of HSCs [22, 23]. IGF-2 in combination with other growth factors, promoted expansion of long term repopulation of fetal liver and adult bone marrow HSCs [24].

Zhang *et al*. reported that using a new combination of growth factors that included SCF, TPO, IGF-2 and FGF-1 (called STIF), supplemented with Angiopoitetin-like protein 2 or 3 (Angptl2, Angptl3) for 10 days resulted in a 24- to 30-fold expansion of murine LT-HSCs relative to uncultured cells [25, 26]. Furthermore, IGF-binding protein 2 (IGFBP2) and Angptl5 (A5) were introduced as additional secreted proteins that support human HSC expansion [27]. Using SCF [28], TPO [29], and FGF-1 supplemented with IGFBP2 and Angptl5 the number of human SCID-repopulating cells (SRCs) increased ~20-fold compared to uncultured cells [30]. However, the ideal mixture of growth factors or cytokines, and the optimum culture duration for the *ex vivo* expansion of HSCs has not been yet determined. In this thesis the condition that allows rapid *ex vivo* expansion of HSCs without losing their stemness ability has been explored. To this end, we examined the effect of different combination of growth factors (GFs), and varied oxygen tension (5 and 20% O2) during *in vitro* culturing of murine and human HSCs.

Initially we focused on the effect of Angptl3 on expansion and proliferation of HSCs. This protein has the characteristic structure of Angiopoietins; a signal peptide, an extended helical domain to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen homology domain (FHD). However, unlike the other angiopoietins, Angptl3 does not bind to tyrosine kinase receptor Tie-1 or Tie-2, and it has been suggested that Angptl3 function is different from the other family members [31].

Several members of the Angptl family play a role in regulating lipid metabolism and angiogenesis [32]. In particular, Angptl3 inhibits lipoprotein lipase activity *in vitro* and *in vivo*, and mice deficient in Angptl3 display defects in lipid metabolism [32, 33]. Furthermore, it was found that $SCF^{+}DLK^{+}$ stromal cells of the fetal liver, that supports HSC expansion, [34] express high level of insulin like growth factor 2 and the angiopoietin like protein family, notably Angptl2 and Angptl3 [25]. This finding suggested that Angptl3 might be involved in HSC biology. Angptl3 which is expressed by BM-endothelial and other stroma cells, binds directly to cell-surface integrin αvβ3 on HSCs. However it is not well-defined whether Angptl3 signals through integrin αVβ3 into HSCs or whether there is another specific receptor on HSCs for Angptl3. Recently, it has been shown that the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several ANGPTLs including Angptl3 [35]. Intracellular targets of Angptl3 in HSCs were identified by comparing the expression of

several transcription factors and cell cycle regulators in HSCs isolated from Angptl3-null mutant and WT mice. Angptl3-null HSCs had 3-fold higher levels of Ikaros expression. The effect of Ikaros expression on HSC repopulation was measured using transplantation of HSCs with enhanced Ikaros expression. Its over-expression dramatically decreased the HSC repopulation in recipients. Moreover, it was shown that Angptl3 could repress the expression of Ikaros and up-regulate the expression of other genes, such as Hes1 and Hoxa9, that are known to be important for HSC self-renewal and differentiation. It was therefore suggested that, Angptl3 is capable of supporting the stemness of HSCs through down-regulation of the transcription factor Ikaros which is an important transcription factor for differentiation of lymphoid, myloid, and erythroid cells [26]. Based on these studies, Angptl3 was introduced as an extrinsic factor that directly acts on HSCs and supports expansion of repopulating HSCs *in vitro*. Consequently, Zhang *et al.* showed that combination of saturated levels of SCF, TPO, IGF2, FGF1 and Angptl3 could promote expansion of LT-HSCs up to ~30-fold [25, 26].

We studied the effect of Angptl3 on murine progenitor, ST-, and LT-HSCs under serum-free culture conditions, using colony-forming unit assay (CFU), colony-forming unit-spleen assay (CFU-S) and long term repopulation assay (LTRA).

TPO is the only growth factor that can maintain LT-HSCs activity on its own during *in vitro* culturing [36]. In analogy we exposed murine HSCs to serum-free culture condition supplemented with only Angptl3 and found that it alone is not sufficient for HSCs expansion *in vitro*. Therefore, the use of saturated levels of other growth factor combinations seemed crucial to investigate the effect of Angptl3. Two of the most common hematopoietic growth factors (HGFs), STF and STIF were used in combination with Angptl3.

We examined the effect of STF combination of growth factors on murine BM Lin^- or Lin^- $Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells.. The reduced HSC activity in LSK cells cultured in STF, compare to Lin^- cells, and suggested that Lin^- cells provide additional factors that increase HSCs expansion. Hence, it seems that STF requires additional growth factors to further enhance the expansion of highly purified HSCs (e.g. LSK). FGF-1 was previously reported as the sole growth factor that preserves long-term repopulating-HSC of unfractionated mouse BM cells in serum free culture conditions. However, FGF-1 was unable to support highly purified HSCs in the absence of other growth factors [23]. Based on this knowledge, and using highly purified HSCs as the starting population we opted to find growth factors that can directly regulate the fate of HSCs rather than providing an indirect effect.

The proliferation rate of LSK cells during 7 days *in vitro* culture in medium supplemented with STF or STIF was similar, however the relative frequency of LSK cells remained higher in STIF medium compared to those cultured in STF. There was no significant differences in the total number of progenitor cells and ST-HSCs cultured supplemented either with STF or STIF medium, although the number of LT-HSCs grown in STIF condition for 7 days in our hand exhibited a higher expansion relative to fresh cells, compared to culturing in STF, leading less than a 10-fold raise in the number of LT-HSCs of LSK cells in STIF compared to fresh cells. Previously, Zhang *et al* reported that LT-HSC expanded in STIF about 8-fold compared to fresh cells [37], although they had 10 days incubation time compared to 7 days in our experiments and used BM SP instead of LSK cells as the starting population.

In human studies, analysis of three different growth factor combinations, STF (SCF, TPO, Flt3-L), STIF (SCF, TPO, IGF-2, and FGF-1), and ST(IBP)F (SCF, TPO, IGFBP2, FGF-1) after transplantation into sublethally irradiated NOD-SCID mice indicated that STIF yields the highest *ex vivo* expansion of human repopulating cells. The effect of IGFBP2 in combination with SCF, TPO, FGF-1 and Angptl3 has been reported, previously [38]. Although 21 days of culture, this combination of growth factors enhanced the number of long term repopulating mouse HSCs about 48-fold [38]. We did not detect any advantages of using IGFBP2 instead of IGF-2 in combination with SCF, TPO, and FGF-1 growth factors for the expansion of human repopulating cells. In the Huynh *et al.* study, the effect of STIF and ST(IBP)F is not shown side by side to show which combination is more effective to expand HSCs. Comparison of our observation with murine studies performed by Zhang *et al* and Huynh *et al.* showed that, culturing highly purified HSCs in STIF or ST(IBP)F yields to approximately the same repopulation ability. Finally it has recently been. reported that IGFBP2 and Neurotrophin-3 (NT-3) synergy promotes cord blood cell expansion through phospho activation of ERK1/2 and AKT [39].

Current doses of ANGPTLs are in the range of 100~500ng/ml [25, 30] may not be optimal but high concentrations would be very costly for clinical use. In order to determine the optimal dosage of ANGPTL3 and ANGPTL5, in our liquid culture system, we used 10, 30, 100, 300ng/ml of each growth factor supplied with the STF cytokine combination. CFU assays revealed that ANGPTL3 and ANGPTL5 can be highly effective at 100, and 30ng/ml concentration, respectively. Based on this result we used 100ng/ml ANGPTL3 in all experiments. However, despite to our results, ANGPTL5 was used at 500ng/ml concentration in all experiment to allow us to compare our result with a previous study [30].

In addition to the effect of growth factors, the duration of the culture is a very important factor in the expansion of HSCs and hence we tried to find the best culture duration time. In our experience incubating UCB-$CD34^+$ cells for 7 days in the STIF cocktail is the better culture condition to stimulate *ex vivo* expansion of repopulating cells, when compared to 14 days. Our results indicate that UCB-$CD34^+$ cells lose their stemness with longer incubation time. Therefore, we used STIF combination of growth factor for a period of 7 days to test the effect of ANGPTL3 on UCB-$CD34^+$ cells.

The effect of Angptl3 on expansion of murine HSCs

Adding Angptl3 did not affect the proliferation rate of LSK cells during 7 days *in vitro* culture in either STF or STIF conditions, although it increased the number of progenitor cells and ST-HSCs in both STF and STIF conditions. To our knowledge, the effect of supplementing Angptl3 in serum free culture condition was determined on LT-HSCs [25] but the effect of Angptl3 on progenitor cells or ST-HSCs is not known.

Culturing LSK cells for 7 days in STF and STIF supplemented medium resulted in a less than a 10-fold expansion of LT-HSCs compared to fresh cells whereas the number of HSCs increased to 10 and >10-fold in STFA3 and STIFA3, respectively. In addition, culturing Lin^- cells under STF and STFA3 conditions resulted in about <10-fold and >30-fold expansion in

the number of LT-HSCs compared to fresh cells. We conclude that Angptl3 significantly increased the number of LT-HSCs during 7 days of *in vitro* culturing.

Among the combinations of growth factors (STF or STF ±Angptl3) that were tested, the combination of five growth factors [SCF, TPO, IGF-2, FGF-1 and Angptl3 (STIFA3)] yielded maximal expansion of mouse LT-HSCs. Zhang *et al*, also reported that this new combination of growth factors may promote superior engraftment using a minimal cell number, and what this would provide a platform for the effective treatment of a variety of diseases whose treatment are currently limited by a lack of sufficient numbers of stem cells. In addition, this method will likely improve the efficacy of other hematopoietic stem cell applications.

Based on the potency of Angptl3 to stimulate *ex vivo* expansion of mouse HSCs, we studied the effect of Angptl3 over-expression on the maintenance, expansion, and proliferation of HSCs. We found that the number of Lin^- BM cells transduced with lentiviral carrying LV-Angptl3-GFP had more than 2-fold expansion after 7 days *in vitro* culture.

Four and seven days *in vitro* culturing resulted in a significant increase in the number of progenitor cells in the LV-A3-GFP Lin^- cell compared to the LV-GFP Lin^- control cell population. In the CFU-S (12-day) assay, revealed that Angptl3 over-expression significantly increased the number of short-term HSCs from murine Lin^- cells. LTRA assays showed that the percentage of transduced cells that express Angptl3 increased significantly and resulted in a higher donor-derived chimerism percentage in peripheral blood (PB) and bone marrow (BM), nine months post transplantation of Lin^- BM transdued with LV-A3-GFP or LV-GFP into sublethaly irradiated recipients. Moreover, over-expression of Angptl3 significantly increased the frequency of LSK cells in the BM and PB of recipients, though no change was detected in other blood lineages. After nine months post transplantation, we did not detect any sign of tumor development or toxicity effect that could be attributed to Angptl3.

The effect of Angptl3 on the proliferation and expansion of umbilical cord blood cells

Based on our and others studies [25, 26], it was suggested that Angptl3 can play a supportive role in the expansion and proliferation of HSCs *in vivo* and *in vitro*. Angptl3 could probably be introduced as an effective growth factor in HSC expansion studies, although its effect on Human-HSC should be further investigated. We therefore examined the effect of ANGPTL3 and ANGPTL5 [30] on the expansion and proliferation of UCB-$CD34^+$ cells.

Based on the all *in vitro* experiment, we showed that in all three conditions (STIF, STIFA3, and STIFA5), UCB-$CD34^+$ cell numbers increased greater than 8-fold in a period of 7 days of *in vitro* culturing. In contrast to our observation, Zhang *et al* showed that 11 days of *in vitro* culturing of $CD34^+$UCB cells in the presence of SCF+TPO+FGF-1 resulted in a 250-fold expansion in overall cell numbers [30]. ANGPTL3 had no influence on expansion of total number of $CD34^+$ UCB cells although it facilitated maintaining the $CD34^+$ phenotype of UCB in a period of 7 days. In contrast to Broxmeyer *et al.* that supplementing ANGPTL3 did not enhance the number of progenitor cells of $CD34^+$ UCB cells in a period of 7 days [40], they were significantly increased in our hands in the presence of either ANGPTL3 or ANGPTL5. The major difference between these two studies was the use of STIF in our study compared to STF

in the Broxmeyer *et al*. study. Further, serially diluted transplantation of cultured or uncultured $CD34^+$ UCB into sublethally irradiated NOD-SCID mice revealed that supplementing with ANGPTL3 caused a 3-fold expansion of the number of repopulating cells of $CD34^+$ UCB cells. Thus, culturing $CD34^+$ UCB cells for 7 days in presence of STIFA3 resulted in a 6-fold expansion of the number of short term (ST) - SCID repopulating cells and we conclude that supplementing with ANGPTL3 or ANGPTL5 supports the expansion and proliferation of repopulating cells from UCB-$CD34^+$ cells. Our findings confirm what Zhang *et al*. previously published, with respect to the effect of ANGPTL5 on the *ex vivo* expansion of human HSCs [30]. However, Zhang *et al* reported that the repopulating population of cultured $CD133^+$UCB cells increased 20-fold in SCF+TPO+Flt3L+ANGPTL5 that proliferated ~220-fold in the course of 11 days [30], whereas in our study $CD34^+$UCB cells cultured in the presence of SCF+TPO+IGF2+FGF1+ANGPTL5 that expanded 8-fold in overall cell numbers resulted in a 4-fold expansion of repopulating cells when compared to fresh cells. This result indicates that in our culture condition the expanded cells highly preserved their stemness ability. We presently do not know whether this is due to the shorter incubation time or using a different basal medium. Nevertheless, the data presented in the current study show that both ANGPTL3 and ANGPTL5 facilitate the expansion of human ST-SCID repopulating cells in *ex vivo* cultures of UCB-derived $CD34^+$ cells when compared to control cultures.

The effect of hypoxia on murine and human-HSCs

Although HGFs play an important role in the number and type of cells generated in serum-free static *ex vivo* cultures, other variables may also affect the outcome of stem and progenitor cell expansion. These include environmental conditions such as oxygen tension.

Hypoxia inducible factor-1α (HIF-1α) plays a key role in maintaining HSCs in the endosteal niche. HIF-1α, which is degraded rapidly under normoxic conditions, becomes stabilized in low O_2 levels (<5%) and forms a dimer with HIF-1β (ARNT) [41]. In contrast to HIF-1α, ARNT is not regulated by hypoxia. The HIF-1α/ARNT heterodimer is a transcriptional activator of a wide variety of genes that encode proteins such as erythropoietin, vascular endothelial growth factor (VEGF), glucose transporters and glycolytic enzymes [42]. Moreover, Rehn et al. reported that VEGFA, which is regulated by HIF, is essential for HSC survival [43-46].

We examined the effect of hypoxia (5% O_2) on the *ex vivo* expansion of murine and human HSCs. Hypoxia during *ex vivo* expansion of murine HSCs revealed that it, enhances BM-HSCs, progenitors, and ST-HSCs populations proliferation compared to normoxia (20% O_2), while the number of LT-HSCs were preserved. In contrast, Eliasson *et al.* reported that culturing murine BM-HSCs under hypoxia (1% O_2) conditions suppressed cell expansion of BM-HSCs and maintained the number of progenitor cells but promoted number of LT-HSCs [45]. The only element that varied between Eliasson *et al*. and our study was the O_2 level (5% and 1% O_2). Thus the combination of Eliasson and our studies suggests that murine BM-HSCs require O_2 levels below 5% to further enhance LT-HSC activity compared to normoxia.

The hypoxia (1% O2) studies on human BM- and UCB-CD34$^+$ cells by Dnate and Shima *et al.* also showed that hypoxia promotes expansion of the cells with ST-SCID repopulation potential [47, 48]. We extended these studies to include the promising combination of growth factors, STIFA3, to promote the *ex vivo* expansion of ST-SCID repopulation cells. The exponential rate of repopulating cells under normoxia or hyppoxia environments compared to fresh cells, were calculated using serially diluted transplantation. This showed that the repopulation of UCB-CD34$^+$ cells increased up to 6- or >12-fold after 7 days *in vitro* culturing in STIFA3 under hypoxia or normoxia compared to fresh cells. This result indicates that repopulating cells expanded more than 2-fold under hypoxia compared to normixia. Recently, the effect of hypoxia (5% O_2) has also been examined on human UCB-CD133$^+$. Contrary to our results, Roy *et al*. reported that hypoxia (5% O_2) enhanced *ex vivo* expansion of the total number of progenitor cells while preserving the SCID-repopulation efficiency of UCB-CD133$^+$ cells. The difference between the Roy *et al*. and our study were probably due to using a different combination of growth factors (SCF+Flt3+TPO+IL-6+ IL-3, STIFA3) and an altered period of the incubation (10 days, 7 days). This may have forced cells to react differently to the environmental situations. For instance, they used IL-3 which promotes the commitment of HSCs to the myeloid lineage and acts as a survival factor for myeloid progenitors [49].

We conclude that our experiments showed that under 20% O_2, repopulating cells from UCB-HSC and BM-HSCs expands up to 6-, and >10-fold whereas repopulating cells from UCB and BM enhanced 12- and >10-fold under 5% O_2, respectively.

Thus, we propose that culturing UCB-HSCs at a 5% oxygen level is a more favorable condition for the *ex vivo* expansion of repopulating cells as compared to normoxia. However, the *ex vivo* expansion of BM-HSCs appears not be affected under mild hypoxia compared to normoxia which suggests the idea that either BM cells require an even lower than 5% level of oxygen during culturing to enhance expansion of LT-HSC, or that BM cells manage to adapt to a higher percentage of O_2 which is inverse to their natural environment.

Conclusions and future directions

In conclusion we report that STIF is more effective than STF for the *ex vivo* expansion of murine HSCs. However in human studies we did not detect any significant differences between STIF, STF, and ST(IBP)F to enhance expansion of repopulating population, but STIF yielded to higher numbers of repopulating cells when compared to the two other combinations of growth factors.

Supplementing STF or STIF with Angptl3 significantly increased the number of LT-HSCs. Using STIFA3 for 7 days *in vitro* expanded the number of LT-HSC >10-fold relative to fresh cells. In addition ANGPTL3 has a significant effect on the expansion of UCB-HSCs, increasing the ST-SCID repopulating cells ~6-fold. We showed that ANGPTL5 also causes significant increase in the number of repopulating cells but less effective than ANGPTL3, which confirms the study by Zhang *et al*.

We also show that the expansion of murine BM-HSCs was not further enhanced by mild hypoxia (5% O_2). However, using hypoxia conditions during 7 days *in vitro* culturing of UCB-

HSCs significantly enhanced the expansion of repopulating cells by >12-fold. In future our *in vivo* human experiments may benefit from a replacement of the NOD-SCID mice by $Rag2^{-/-}\gamma c^{-/-}Kit^{W/Wv}$ mice as an universal recipient for human cells could be an alternative that enables human LTRA assays. The NOD/SCID mouse assay that has been used in all *in vivo* human experiments has provided a useful tool in the *in vivo* analysis of human hematopoietic stem cells, although only short-term repopulating ability of stem cells can be analyzed due to the development of lymphomas and leakiness of murine B and T cells in aging NOD/SCID mice. Another future direction of this study can be also combining the present state of growth factor stimulated HSC expansion with other variables, like SR1 [21], copper chelator tetraethylenepentamine [50], SALL4 [51] of which it has been shown they have potential to aid the expansion of HSCs.

Obtaining better conditions would be beneficial to the *ex vivo* expansion of UCB-HSCs and make UCB more valuable source of HSCs for both adult patients and children.

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Summary

Samenvatting

Summary

Hematopoietic stem cells (HSCs) can be exploited in various cellular and gene therapy treatments, but their use is often hampered by the limited available number of stem cells. Therefore HSCs applications, specifically umbilical cord blood cells, might be feasible if stem cells can be successfully expanded *ex vivo*.

The main aim of this thesis was to define a condition that allows rapid *ex vivo* expansion of HSCs without losing their stemness ability. To achieve this, we examined the effect of different combination of growth factors (GFs), and varied oxygen tension (5 and 20% O_2) during *in vitro* culturing of murine and human HSCs.

Initially, we compared the effect of commonly used HSC-GFs [SCF +TPO+Flt3L (STF) and combination of HSC-GFs (SCF+TPO+IGF2+FGF1 (STIF)] on the *ex vivo* expansion and proliferation of murine (Chapter 2) and human HSCs (Chapter 4). We focused on the effect of angiopoietin like protein 3 (Angptl3) in combination of STF or STIF culture conditions in progenitors, short term (ST)- and long term (LT)-HSCs during *in vitro* culturing at different time points (Chapter 2,4).

In murine studies, the number of progenitors, ST-, and LT-HSCs was measured by using colony-forming unit assay, colony-forming spleen assay, and transplantation of serially diluted number of cells into sublethally irradiated mice (LT-repopulation assay), respectively. In human studies, the number of progenitor cells was calculated by colony-forming unit assay and the number of repopulating cells was assayed by transplantation of serially diluted number of cells into NOD-SCID mice. Cell sorting in mice and human experiments were performed using FACSAria and manual MACS system. The monitoring of all blood cell lineages during *in vitro* and *in vivo* experiments were executed by FACSCalibur.

Murine bone marrow (BM) Lin^- or $Lin^-/Sca\text{-}1^+/c\text{-}kit^+$ (LSK) cells were cultured *in vitro* for 4-,7-, and 10-day in the presence of either STF or STIF ± murine Angptl3. Subsequently, the number of progenitor cells, ST- and LT-HSCs was measured. Briefly, the highest fold increase in ST-HSCs (~40-fold) was achieved by culturing LSK cells for 7 days in the presence of STFA3. Serial transplantation of fresh or cultured murine BM LSK into sublethally irradiated recipients revealed that culturing of the LSK cells in STIF increases the number of LT-HSCs compared to STF. Supplementing the STF or STIF culture conditions with Angptl3 resulted in a significant increase in the number of LT-HSC cells. These results prove that culturing LSK cells in STIFA3 condition for 7 days enhances the number of LT-HSCs an over 10-fold. Transplanting of as little as 12 LSK cells cultured for 7 days under STIFA3 culture condition resulted in about 50% donor-derived cells in recipient's peripheral blood (Chapter 2).

Moreover, by using lentiviral vectors, we introduced the *Angptl3* gene into murine BM Lin^- cells and studied the effect Angptl3 over-expression on progenitor cells, ST-, and LT-HSCs. Over-expressing Angptl3 significantly increased the number of progenitors and ST-HSCs in culture. Nine months post transplantation of murine BM Lin^- transduced with *Angptl3* into sublethaly irradiated recipients showed that *Angptl3* over-expression increases the number of long-term HSCs. During this nine-month period, all of the transplanted mice were

healthy and no sign of tumor development or an elevated WBC count was observed (Chapter 3).

In human studies, we primarily compared the effect of STF and STIF culture conditions on the *ex vivo* expansion of umbilical cord blood (UCB)-$CD34^+$ cells. Since the highest donor chimerism percentage was observed in cells cultured for 7 days in STIF medium, we used this condition as the optimum growth factor combination to determine the effect of ANGPTL3 or ANGPTL5 on the expansion and proliferation of UCB- CD34+HSCs. Colony forming unit assay revealed that supplementing STIF with ANGPTL3 for 7 days significantly increased number of progenitor cells. Using transplantations of serially diluted fresh and cultured UCB-$CD34^+$ cells into sublethally irradiated NOD-SCID mice, revealed that the repopulating cells were stimulated over 2-fold in STIF condition. Supplementing ANGPTL3 or ANGPTL5 caused an additional 3-fold expansion on the SRC number, resulting in a total of 6-fold expansion when compared to fresh cells (Chapter 4).

Thus these studies support the fact that Angptl3 facilitates the *ex vivo* expansion of murine LT-HSCs and human repopulating cells (Chapter 2,3,4).

We also the effect of hypoxia (5% O_2) on *ex vivo* expansion of murine and human HSCs. Hypoxia (5% O_2) enhances the proliferation of BM-HSCs, progenitors, and ST-HSCs populations, while the number of LT-HSCs was preserved when compared to normoxia (20% O_2). In contrast, similarly cultured human UCB-$CD34^+$ under hypoxia conditions (5% O_2), while enhancing *ex vivo* expansion of repopulating cells as measured by serially diluted transplantation into NOD-SCID mice, preserved the number of progenitor cells. Taken together, our experiments showed that under 20% O_2 repopulating cells from UCB-HSC and BM-HSCs expands up to 6-, and >10-fold but under 5% O_2 12- and >10-fold, respectively (Chapter 5).

In conclusion we propose that the culturing of UCB-HSCs at 5% O_2 provides a better condition to stimulate enriched-HSC of UCB expansion when compared to 20% O_2. However *ex vivo* expansion of BM-HSCs was not further enhanced under hypoxia. Since, it is assumed that under normal physiological conditions, the O_2 concentration in the BM endosteum is lower than in UCB, it is possible that BM-HSCs are very sensitive to high O_2 level, even as high as 5% O_2 or alternatively that BM-HSCs adopt to the oxygen level. Applying these new conditions in combination with the up-to-date growth factor cocktail to *ex vivo* expansion of HSC makes UCB an interesting source of HSCs for both adult patients and children.

Samenvatting

Hoewel hematopoietische stamcellen (HSCs) kunnen worden ingezet voor diverse cellulaire en gentherapeutische toepassingen, worden ze, door hun limiterende aantallen vaak niet gebruikt. Het is dus van belang dat HSCs, en met name de HSCs die aanwezig zijn in navelstrengbloed, *ex vivo* ge-succesvol expandeerd kunnen worden.

Het doel van het onderzoek dat beschreven is in dit proefschrift, was een conditie te definieren waarmee HSCs snel *ex vivo* te expanderen zijn zonder verlies van hun specifieke stamceleigenschappen. In dit onderzoek is het effect onderzocht van verschillende combinaties van groeifactoren (GF) en variatie in zuurstofspanning (5 en 20% O_2) tijdens *in vitro* celkweek van muis- en humane HSCs.

Initieel vergeleken we het effect op de *ex vivo* expansie en proliferatie van muis HSCs (Hoofdstuk 2) en mens HSCs (Hoofdstuk 4) door ze te kweken in aanwezigheid van de veelal gebruikte groeifactoren SCF + TPO +Flt3L (STF) en de combinatie van SCF+TPO_IGF2_FGF1 (STIF). We bekeken het effect van angiopoietin like protein 3 (Angptl3) in combinatie met STF en STIF kweekcondities in voorlopercellen (progenitorcellen) en lange termijn (LT)-HSCs op verschillende tijdstippen tijdens de kweek (Hoofdstuk 2, 4).

In de muisstudies werd het aantal voorlopercellen en korte-termijn (ST) HSCs gemeten door middel van de colony-forming unit assay (CFU) en colony-forming spleen assay (CFU-S). Verder werden deze cellen getransplanteerd (in een verdunningsreeks) in sublethaal bestraalde muizen waarmee het aantal lange-termijn repopulerende cellen (LT-repopulatie assay) werd geanalyseerd. Voor de hematopietische cellen van humane afkomst werd het aantal voorlopercellen bepaald middels CFU assays. Het aantal repopulerende cellen werd bepaald door het serieel transplanteren van cellen (in een verdunningsreeks) in NOD-SCID muizen. In zowel de muis- als humane experimenten werden de cellen gesorteerd in een celsorteermachine (FACSAria) of geselecteerd met behulp van het magnetische MACS systeem. In zowel de *in vitro* als de *in vivo* experimenten werden alle bloed cellijnen gemonitord met behulp van flow cytometry (FACSCalibur).

Muis beenmerg (BM) Lin^- of $Lin^-/Sca\text{-}1^+/c\text{-}kit^+$ (LSK) cellen werden gekweekt *in vitro* gedurende 4, 7 en 10 dagen in de aanwezigheid van STF of STIF ± muis Angpltl3. Daarna werden de aantallen voorlopercellen, ST- en LT-HSCs gemeten. In het kort, de meeste expansie van ST-HSCs (~40-voud) werd gevonden na kweek van LSK cellen gedurende 7 dagen in de aanwezigheid van STFA3. Seriele transplantatie van muis LSK cellen (vers of gekweekt) in sublethaal bestraalde ontvangers, gaf als resultaat dat het kweken van LSK cellen in STIF het aantal LT-HSCs verhoogt in vergelijking met kweek in STF. Toevoeging van Angptl3 aan STF of STIF kweekcondities resulteerde in een significante verhoging in het aantal LT-HSC cellen. Deze resultaten bewijzen dat het kweken van LSK cellen in STIFA3 gedurende 7 dagen, het aantal LT-HSCs 10-voudig verhoogt. Het transplanteren van slechts 12 LSK cellen, gekweekt gedurende 7 dagen, in de STIFA3 kweekconditie resulteerde in 50% cellen in het perifere bloed van de ontvanger die afkomstig zijn van de donor (Hoofdstuk 2).

Met behulp van lentivirale vectoren, werd het *Angptl3* gen in muis MB Lin^- geintroduceerd. Hiermee werd het effect van overexpressie van Anptl3 op voorlopercellen, ST-

en LT-HSCs bestudeerd. Het tot over-expressie brengen van Angptl3 verhoogt het aantal voorlopercellen en ST-HSC in kweek significant. Negen maanden na transplantatie van muis BM Lin^- cellen die getransduceerd werden met *Angptl3* in sublethaal bestraalde ontvangers, werden verhoogde aantallen LT-HSCs gevonden. Gedurende deze negen maanden bleven alle muizen gezond en werden er geen signalen van tumorontwikkeling gevonden. Ook werden geen verhoogde WBC aantallen gemeten (Hoofdstuk 3).

In de humane studies, werd het effect van STF en STIF kweekcondities op de *ex vivo* expansie van humaan navelstrengbloed (UCB) $CD34^+$ cellen gestudeerd. Omdat het hoogste percentage donor chimerisme werd gevonden nadat de cellen werden gekweekt in STIF medium gedurende 7 dagen, werd deze conditie gebruikt als optimale groeifactor combinatie om het effect te bestuderen van ANGPTL3 en ANGPTL5 op de expansie en proliferatie van UCB $CD34^+$. In CFU assays werd een significant hoger aantal progenitors gemeten na kweek in STIF met ANGPTL3 gedurende 7 dagen. In transplantatie assays met verse en gekweekte UCB-$CD34^+$ cellen in sublethaal bestraalde NOD-SCID muizen, werd een 2-voudige stimulatie gevonden in de STIF conditie. Het supplementeren van het STIF medium met ANGPTL3 of ANGPTL5 veroorzaakt een additionele 3-voudige expansie van de SCID-repopulerende cellen (SRC), resulterend in een 6-voudige expansie vergeleken met verse, ongekweekte cellen (Hoofdstuk 4).

Deze studies tonen aan dat Angptl3 de *ex vivo* expansie van muis LT-HSCs en humane repopulerende cellen faciliteert (Hoofdstuk 2, 3, 4).

We hebben ook het effect van hyppoxia (5% O_2) op de *ex vivo* expansie van muis en humane HSCs geanalyseerd. Hypoxia (5% O_2) verhoogt de proliferatie van BM-HSCs, voorlopercellen en ST-HSCs, en houdt het aantal LT-HSCs in stand bij vergelijking met normoxia (20% O_2). In contrast tot deze resultaten, laten humaan UCB-$CD34^+$ cellen, gekweekt onder hypoxia condities een iets ander beeld zien. SCID-repopulerende cellen zijn verhoogd na *ex vivo* expansie, maar het aantal voorlopercellen blijft gelijk. Samengevat, onze experimenten tonen aan dat bij 20% O_2, HSC geisoleerd uit UCB en uit BM, respectievelijk 6- en >10-voudig expanderen. Onder lage O2 concentraties, is dit 12- en >10-voudig, respectievelijk (Hoofdstuk 5).

In conclusie, wij tonen hiermee aan dat het kweken van UCB-HSCs bij 5% O_2 een betere conditie geeft om verrijkte HSC geisoleerd uit navelstrengbloed, te stimuleren in vergelijking met 20% O_2. Echter, *ex vivo* expansie van HSCs uit BM werd niet verhoogd onder hypoxia condities. Omdat, onder normale fysiologische condities, de O_2 concentratie in het BM endosteum lager is dan in UCB, is het mogelijk dat BM-HSCs zeer gevoelig zijn voor hoge O_2 concentraties, zelfs bij concentraties van 5% O_2. Een andere mogelijkheid zou kunnen zijn dat BM-HSCs zich makkelijker kunnen aanpassen aan het zuurstoflevel.

Door deze nieuwe condities toe te passen in combinatie met de up-to-date groeifactor cocktail in het *ex vivo* expanderen van HSCs, wordt navelstengbloed een interessante en bruikbare bron voor HSCs voor zowel kinderen als volwassenen.

List of abbreviation

Aldehyde dehydrogenase (ALDH)
Angiopoietin-1 (Ang1)
Angiopoietin like protein 3 (Angptl3)
Aorta gonad mesonephros (AGM)

Bone marrow (BM)
Bone morphologenetic protein (BMP)
Burst-forming erythroid units (BFU-E)

Ca^{2+} Receptor (CaR)
Colony-forming cells (CFCs)
Colony forming unit spleen (CFU-S)
Colony-forming unit granulocyte-macrophage (CFU-GM)

Erythropoietin (EPO)
Erythropoietin receptor (EPO-R)
Extracellular matrix (ECM)

Fibroblast growth factor-1 (FGF-1)
Fibroblast growth factor receptor (FGFR)
Fibrinogen homology domain (FHD)
Fluorescence-activated cell sorting (FACS)
Fms-related tyrosine kinase 3 (FLT3)

Graft-versus-Host Disease (GvHD)
Granulocyte macrophage colony-stimulating factor (GM-CSF)

Hematopoietic stem cells (HSCs)
Hematopoietic growth factors (HGFs)
Hematopoietic stem cell transplantation (HSCT)
Host-versus-Graft reaction (HvG)
Homeobox B4 (HoxB4)
Hypoxia-inducible factor-1 (HIF-1)
Human leukocyte antigen (HLA)

Insulin-like growth factor-2 (IGF-2)
Insulin-like growth factor binding protein 2 (IGFBP2)
Insulin receptor (IR)

Leukocyte immunoglobulin-like receptor B2 (LILRB2)
Lineage-committed hematopoietic cells (Lin^-)
$Lin^-/Sca\text{-}1^+/c\text{-}kit^+$(LSK)
Long-term repopulating (LTR)
Long-term culture initiating cell (LTC-IC)

NOD-SCID-repopulating cells (SRCs)

Osteopontin (OPN)

Paired immunoglobulin-like receptor (PIRB)

Parathyroid hormone-related peptide receptor (PPR)
Peripheral blood (PB)
Peripheral blood stem cells are (PBSCs)

Short-term (ST)
Specific pathogen-free (SPF)
Stem-cell antigen 1 (Sca-1)
Stem cell factor (SCF)
Stem Regenin1 (SR1)
Sympathetic nervous system (SNS)

Thrombopoietin (TPO)

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Curriculum Vitae

Personal Details

Name: Elnaz Farahbakhshian
Birth date: 24-07-1979
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Education and Research experience

1997-2001 B.Sc. Biology, major of Microbiology, Alzahra University, Tehran-Iran.

2003-2005 M.Sc. Cell and Molecular Biology, Faculty of Science, Khatam University, Tehran-Iran.

2006- 2011 PhD student, Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands (Prof. Dr. F. Grosveld).

2011-Present Postdoctoral fellow, Department of Pediatrics/Oncology, Erasmus University Medical Centre, Rotterdam, The Netherlands (Dr. J.P.P. Meijerink).

Name PhD student: Elnaz Farahbakhshian Erasmus MC Department: Hematology	PhD period:01-05-2006 until 01-05-2011 Promotor: F.G. Grosveld

1. PhD training

	Year
General academic skills	
Course on Laboratory Animal Science	2006
Molecular Medicine	2004
courses (e.g. Research school, Medical Training)	
Statistics summer program • Data analysis • Regression analysis	2006 2008
Partek Genomics site (Affymetrix University, London)	2008
Partek training (Erasmus MC, Rotterdam)	2008
Large scale data analysis in Ingenuity (Affymetrix University, London)	2007
Stem cell workshop (Mercury, France)	2006
Molecular medicine methods (Erasmus MC, Rotterdam)	2007
Biomedical research techniques (Erasmus MC, Rotterdam)	2007
Browsing genes and genomes with Ensemble (Erasmus MC, Rotterdam)	2007
The SNPs and human diseases (Erasmus MC, Rotterdam)	2010
Photoshop (Erasmus MC, Rotterdam)	2010
Illustrator (ErasmusMC, Rotterdam)	
Poster presentation:	
ISCT, Belgrade, Italy	2010
NIRM, Amsterdam, The Netherlands	2010
MOLMED, Rotterdam, The Netherlands	2007
MOLMED, Rotterdam, The Netherlands	2008
MOLMED, Rotterdam, The Netherlands	2009
Oral presentation:	
Leukerbad. Yearly reporting for Sixth Framework Programme: Concerted Safety & Efficiency	2008
Evaluation of Retroviral Transgenesis in Gene Therapy of Inherited Diseases (CONSERT)	
Leukerbad. Yearly reporting for Sixth Framework Programme: Concerted Safety & Efficiency	2009
Evaluation of Retroviral Transgenesis in Gene Therapy of Inherited Diseases (CONSERT)	
Teaching activities	
Tweede-jaars keuzeonderwijs Gentherapie" part of second year of MD training programme.	2009
Tweede-jaars keuzeonderwijs Gentherapie" part of second year of MD training programme.	2010
Supervising students	
Noveen Riaz	2009-2010
Aga Wabik	2010-2011