Mechanisms of survival and maintenance of Hematopoietic Stem Cells and Multipotent Progenitor Cells

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Molecular Mechanisms of Survival and Maintenance of Hematopoietic Stem Cells and Multipotent Progenitor Cells

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Abstract

Hematopoietic stem cells and progenitor cells are maintained in the bone marrow microenvironment, where factors including soluble and membrane-bound cytokines influence the processes of self-renewal, proliferation and differentiation. Two nonreduntant cytokines with effects on hematopoiesis, Kit Ligand (KL) and Flt3 Ligand (FL), signal via related tyrosine kinase receptors, c-kit and Flt3. In this thesis, we have studied which signaling pathways that are activated by these cytokines and are important for survival. We have shown that the serine-threonine kinase PKB (also known as Akt) is activated by both cytokines, but the upstream target PI-3 kinase is only necessary for the survival induced by KL and not FL. Also, KL could not prevent apoptosis in cells overexpressing a dominant negative form of PKB. The forkhead transcription factor FoxO3, which is phosphorylated and inactivated by PKB, was further shown to be of central significance for KL-mediated survival. The survival signals mediated by FL was shown to include antiapoptotic Bcl-2 family proteins. When overexpressed in progenitor cell lines, Bcl-2 was more effective in blocking apoptosis than PKB. Although both Bcl-2 and PKB could prevent a decrease in the mitochondrial membrane potential, Bcl-2 was superior with this respect. Some enhancement in survival was also seen when both proteins were overexpressed simultaneously. Finally, we studied the survival of bone marrow-derived multipotent hematopoietic progenitors in the presence of low levels of oxygen, hypoxia. We showed that survival was enhanced by hypoxia and improved the expansion of primitive colony forming cells.

Key words: Hematopoiesis, progenitor, cytokines, KL, c-kit, FL, Flt3, apoptosis, Bcl-2, Akt/PKB, forkhead, hypoxia.

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The Red Queen:

“You have to run faster than that
to stay in the same place”

from Through the Looking Glass

CONTENTS

ABBREVIATIONS

AGM	dorsal aorta, gonads and mesonephros
ASK	apoptosis signal-regulating kinase
ATF	activating transcription factor
AML	acute myeloid leukemia
ANT	adenine nucleotide translocater
Bax	Bcl-2 associated protein x
Bcl	B cell lymphoma
BCRP	breast cancer resistance protein
BH	Bcl-2 homology
BFU	burst forming unit
BM	bone marrow
CD	cluster designation
CFC	colony forming cell
CFU	colony forming unit
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSF	colony stimulating factor
E	erythrocyte
ED	embryonic day
ELTC-IC	extended long-term culture-initiating cell
epo	erythropoietin
FACS	fluorescence activated cell sorter
FL	Flt3 ligand
FRAP	FKBP-rapamycin-associated protein
G	granulocyte
GLUT	glucose transporter
GMP	granulocyte/monocyte progenitor
GSK	glycogen synthase kinase
HIF	hypoxia-inducible factor
HPP	high proliferative potential
HSC	hematopoietic stem cell
IAP	inhibitor of apoptosis
IL	interleukin

JNK	c-Jun N-terminal kinase
KL	c-kit ligand
LIF	leukemia inhibitory factor
LT-HSC	long-term HSC
LTC-IC	long-term culture initiating cell
M	macrophage
Mcl	myeloid cell leukemia
Meg	megakaryocyte
MEP	megakaryocytic/erythroid progenitor
MPP	multipotent progenitor
mTOR	mammalian target of rapamycin
NK	natural killer
PAS	para-aortic splanchnopleura
PDK	phosphatidylinositol-dependent kinase
Pgp	P-glycoprotein
PH	pleckstrin homology
PI	phosphatidylinositol
PKB	protein kinase B
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTP	permeability transition pore
S	spleen
SCID	severe combined immune deficiency
SCL	stem cell leukemia
SH	src homology
SP	side population
ST-HSC	short-term HSC
TNF	tumor necrosis factor
Tpo	thrombopoietin
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
wt	wild type

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals.

I **Phosphatidylinositol 3-kinase is essential for Kit ligand-mediated survival, whereas interleukin-3 and Flt3 ligand induce expression of antiapoptotic *Bcl-2* family genes**
Richard Karlsson, Maria Engström, Maria Jönsson, Peter Karlberg, Cornelis J. H. Pronk, Johan Richter, and Jan-Ingvar Jönsson
Journal of Leukocyte Biology 74(5), 2003

II **Inactivation of the forkhead transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by Kit ligand**
Maria Engström, Richard Karlsson, and Jan-Ingvar Jönsson
Experimental Hematology 31(4):316-323, 2003

III **Akt/PKB promotes antiapoptotic signaling in hematopoietic progenitor cells but does not account for Kit ligand-mediated survival in combination with Bcl-2**
Richard Karlsson, Maria Engström, Maria Jönsson, Camilla Persson, and Jan-Ingvar Jönsson
Manuscript

IV **Enhanced self-renewal and survival of primitive hematopoietic progenitor cells by early acting cytokines in combination with hypoxia**
Richard Karlsson, Håkan Axelson, and Jan-Ingvar Jönsson
Manuscript

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INTRODUCTION

Homeostasis, the maintenance of blood cells, is maintained throughout life by a rare cell type residing primarily in the bone marrow (BM), where it makes up less then 1 in 10^4 to 10^5 cells [1]. This cell type, the hematopoietic stem cell (HSC), gives rise to all blood cell progenitors through differentiation, but at the same time replenishes the pool of undifferentiated cells. While self-renewal and differentiation of HSCs and progenitor cells appear to be mostly stochastic processes within the cells, survival and proliferation are regulated by external factors [2,3]. Part of this regulation is due to a complex interaction between soluble and membrane-bound stimulatory and inhibitory cytokines and their corresponding receptors. Although a number of cytokines have extensive effects on HSCs and progenitor cells *in vitro* or *in vivo*, c-kit ligand (KL; also known as stem cell factor or mast cell growth factor) and Flt3 ligand (FL), appear to have unique and nonredundant activities on these cells [4].

The circumstances that regulate HSCs self-renewal and maintenance are not clear, but the HSC niche in the BM provides the environment to sustain long-term hematopoiesis [5,6]. Apart from cytokines, the extracellular matrix in the BM can present adhesion molecules, and it is thought that the interaction between signals induced by cytokines and adhesion molecules are important for hematopoiesis [7-9]. The BM is generally seen as well perfused with blood, but areas of lower oxygen levels, hypoxia, may also have a role in determining stem cell differentiation [10]. Indeed, several reports have shown that marrow repopulating ability is maintained by incubation in hypoxia [11-14].

The factors regulating the HSCs and progenitor cells induce survival signals that still are not elucidated. The B cell lymphoma (Bcl)-2 family of proteins (reviewed in [15]) and protein kinase B (PKB; also known as Akt, reviewed in [16]) have been inferred with these signals. However, further knowledge on the mechanisms that support survival of HSC and progenitor cells are needed. Therefore, in this thesis, we have investigated the relationships between KL and FL signaling and Bcl-2 and PKB. Also, the effects of hypoxia with early acting cytokines including KL on survival and self-renewal was studied.

BACKGROUND

The hematopoietic stem cell

The turnover of cells of the hematopoietic system in an individual weighing 70 kg are close to 1 trillion (10^{12}) per day, including 200 billion erythrocytes and 70 billion neutrophilic leukocytes [3]. The cells of the hematopoietic system originate from a common progenitor cell, the HSC, which is able to produce a steady level of erythroid, lymphoid and myeloid cells throughout a lifetime [17,18].

The HSC is defined by several characteristics. First, the HSC is the only cell that is capable of long-term reconstitution of the hematopoietic system of a BM ablated/ lethally irradiated recipient. By limiting dilution it has been shown that only a few cells are needed for this, which indicates the high proliferative potential of HSCs. Reconstitution of hematopoiesis can be further transferred into secondary and tertiary hosts. Second, in order not to exhaust the pool of HSCs, they must have the capacity to self-renew (i.e. to divide and produce daughter cells, which retain the properties of the original cell). Third, the HSC has the capacity to undergo differentiation and is able to form all different cell types in the hematopoietic system [19-23].

HSCs can be maintained in a non-cycling state (quiescence) for long periods of time. From this phase outside the cell cycle (G_0/G_1), a few HSCs at a given time re-enter the cell cycle and contribute to maintain the steady state of hematopoiesis. It has been calculated that approximately 8% of HSC asynchronously enter the cell cycle per day. Although approximately 75% of HSC are quiescent in G_0 at any one time, all HSCs are recruited into cycle regularly such that 99% of HSC divide on average every second month [24,25].

In recent years, the question whether stem cells are restricted to specific tissues or have the potential to differentiate into cell types normally not associated with the lineage they were derived from, has been challenged. This process of transdifferentiation (plasticity), has been shown in experiments with HSCs, where these cells could regenerate functional hepatocytes [26,27]. Neuronal stem cells have also been found to produce a variety of blood cell types, including myeloid and lymphoid cells as well as early hematopoietic cells. However, these and other transdifferentiation events are low in number and could be due to fusion between the different cell types involved [28-31].

Embryonic origin of hematopoietic stem cells

In the mouse embryo, there are three sites where hematopoiesis takes place; the yolk sac, the PAS-AGM (para-aortic splanchnopleura/dorsal aorta, gonads and mesonephros) region, and the fetal liver [32,33]. Hematopoiesis originally occurs by embryonic day (ED)7-7.5 in the yolk sac blood islands. These erythrocytes are primitive and can only form erythroid and myeloid progeny and lack the potential to produce lymphoid cells. Multipotent progenitors with erythroid/myeloid and lymphoid potential are generated first in the PAS region, that later develops into the AGM region. These cells appear to colonize the yolk sac beginning at ED8.5, when the circulation between these tissues is established. The first HSCs capable of full long-term, multilineage engraftment of lethally irradiated adult recipients are found in the AGM region at beginning of ED10. These cells are generated only in the AGM region and subsequently appear to colonize the fetal liver, which is the primary site for hematopoiesis during embryogenisis [34-36]. Shortly prior to birth, the BM becomes the primary site for hematopoietic generation. The BM is subsequently the primary organ where the multipotent HSCs can be found in normal adults [37].

In embryonic hematopoiesis, there is a key role for the basic helix-loop-helix transcription factor stem cell leukemia (SCL)/Tal. Expression of SCL/Tal is detected in embryonic and extraembryonic mesoderm at ED7.5, in blood islands of the yolk sac by ED8.5, and thereafter in adult hematopoietic tissues. Embryos lacking SCL/Tal lack embryonic red cells and die between ED9-10.5 due to anemia, and enforced expression of SCL/Tal can induce a stimulatory effect at the level of erythroid and megakaryocytic progenitor cells, while exerting a selective proliferative action on downstream erythropoiesis [38-40]. An early role for SCL/Tal in hematopoiesis is also suggested by the immature phenotype of leukemic cells seen in humans with deregulated SCL/Tal expression [41]. Interestingly, SCL/Tal is required for c-kit expression in hematopoietic cells [42,43].

Isolation of hematopoietic stem cells

The most common sources of BM in mice are the femurs (thigh-bones) and tibiae (shin-bones). In adult humans, the BM is the primary reservoir of HSCs used for transplantations. HSCs from peripheral blood are commonly used for autologous or allogeneic transplantation following high-dose chemotherapy. The introduction of hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) has greatly enhanced the mobilization of HSCs from the BM, although the mechanism

of HSC mobilization is not yet clarified [44,45]. KL and FL can also induce recruitment of BM cells to the circulation, although this is not used clinically [46-49]. Umbilical cord blood is also a rich source of HSCs, since the stem cells in the fetus home to the BM via the blood [50].

Two inventions facilitated the technology of purification of HSCs; the advent of monoclonal antibodies and the development of the fluorescence activated cell sorter (FACS) [51]. It has been found that HSCs lack expression of many antigens present on mature and lineage committed blood cells, including cluster designation (CD) 3 (lymphocytes), B220 (murine B lymphocytes), CD19 (murine and human B lymphocytes), CD4 and CD8 (T lymphocytes), NK1.1 (natural killer (NK) cells), Mac-1/Gr-1 (myeloid cells), and Ter119 (murine erythroid cells). Murine HSCs have been shown to have at most a low expression of CD34 and to be expressing CD38 [52,53]. Contrary, human HSCs have been indicated to express CD34 and lack the expression of CD38 [54,55]. However, it was demonstrated that the CD34- fraction of normal human BM contains cells capable of long-term engraftment and which can differentiate into CD34+ progenitors [56]. In agreement with the finding that HSCs are critically regulated by KL, c-kit is expressed on these cells [57]. Other markers, such as Sca-1 and Thy-1 are also used when isolating HSCs [26,58], although these antigens are mouse strain specific and cannot be used in all cases [59]. In the murine system, transplantation of single BM cells sorted as lineage negative, Sca-1 positive, c-kit positive and CD34 negative/low can reconstitute hematopoiesis for a lifetime [52].

Other strategies, including metabolic states, cell size and density gradients, can be used for the enrichment of HSCs. HSCs were early found to be more or less resistant to a number of cytostatic drugs, which exercise their effects primarily when DNA duplication is performed, and it was hypothesized that HSCs were mostly quiescent. In accordance with this, most HSCs in steady state can be found in the G_0/G_1 of the cell cycle [25,60]. Moreover, HSCs have been found to be able to exclude certain compounds, and by use of the dyes Hoechst 33342 and Rhodamine 123, HSCs can be isolated [24]. These cells have been denoted the side population (SP), and extensive research is currently investigating the properties of these cells. It was initially believed that the multidrug resistance protein P-glycoprotein (Pgp) was responsible for this efflux, and Pgp was later found to be a marker for hematopoietic progenitor cells [61-63]. Transduction of murine BM cells with Pgp enabled *ex vivo* stem cell expansion, although they caused a myeloproliferative syndrome, and enforced Pgp pump function in murine BM cells

resulted in expansion of side population stem cells *in vitro* and repopulating cells *in vivo* [64,65]. These results indicate that Pgp could be involved in hematopoiesis, perhaps by influencing cell cycle regulation or by extrusion of differentiating compounds, since down-regulating multidrug resistance triggers proliferation of noncycling hematopoietic progenitors, and Pgp has been shown to have affinity for several hormones, among them retinoic acid [66-68]. Recently, breast cancer resistance protein (BCRP; also known as ABCG2), a multidrug resistance transporter closely related to Pgp, was also found to be overexpressed in HSCs [69]. This pump has been found to be responsible for the efflux of Hoechst 33342 and is a new putative marker for HSCs [70].

Evaluation of hematopoietic stem cells

The best way to assess a HSC is its ability to reconstitute the hematopoiesis into a BM ablated recipient. For obvious reasons, this is not achievable with humans, although artificial systems exist (e.g., immuno-compromised mice or the *in uterus* transfer into sheep fetuses) [56,71,72]. In the early 60s, the colony forming unit-spleen (CFU-S) assay was developed, where transplanted cells formed characteristic colonies on the spleens of lethally irradiated recipient mice [73,74]. Cells from these colonies were shown to be serial-transplantable, indicating self-renewal, but it has later been shown that these cells were already committed to the myeloid compartment, and thus the CFU-S assay is currently not considered to be an assay for HSCs [75].

In the mid 80s, a method was developed that used random chromosomal integration sites of retrovirus vectors as unique clonal markers to analyze cell lineage relationships within the hematopoietic stem cell hierarchy. Anemic mutant mice were reconstituted with infected BM and further analysis indicated insertion into primitive pluripotent stem cells capable of producing both myeloid and lymphoid progeny, as well as into more committed stem cells apparently restricted to either the myeloid or lymphoid lineages [20,22,76,77]. In all, these experiments proved that there indeed exists a cell in the BM that has, at the single cell level, the potential to both self-renew, form multilineage offspring and is able to functionally restore hematopoiesis.

By use of a stroma feeder layer that imitates the BM environment for the HSCs, an assay has been developed, the long-term culture initiating cell (LTC-IC) assay, and its modification, extended LTC-IC (ELTC-IC) assay [78-80]. The preformed layer of BM stromal cells is imagined to produce growth factors and to provide cell-to-cell contact with adhesion molecules and membrane-bound growth factors. Following 5-8 weeks

in culture, the cells are subsequently evaluated for their potential to form colonies in semisolid media, such as methylcellulose or agar as a response to cytokine stimulation. It is nowadays modified to evaluate both myeloid and lymphoid progenitors, including natural killer (NK) cells [50].

There are also assays for evaluating hematopoietic progenitor cells without any supporting cells. After *in vitro* incubation with cytokines the cells are seeded in semisolid media, and since the cells are immobilized, the different cell types from one colony represent the offspring from one original progenitor cell. Multipotent progenitor cells can thus give rise to multi-lineage colonies (e.g., colony forming unit-granulocytes, erythroids, macrophages and megakaryocytes, CFU-GEMM; colony forming unit-mix, CFU-mix; pre-colony forming cells multilineage, pre-CFC$_{multi}$). One type of multi-lineage colony, the high proliferative potential colony forming cells (HPP-CFC), actually give rise to colonies that can be seen with the naked eye. More differentiated cells give rise to colonies with dual lineages, like granulocytes and macrophages (CFU-GM), and unipotent progenitors will give rise to colonies containing macrophages (CFU-M), granulocytes (CFU-G), megakaryocytes (CFU-Meg), and the burst forming unit-erythrocytes (BFU-E) [71,81-83].

REGULATION OF HEMATOPOIESIS

Hematopoietic hierarchy

The HSC is multipotent and is thus able to develop into the eight different hematopoietic lineages [3]. The exact mechanisms that underlie and direct these processes are still to a large degree unknown. However, due to recent years advances in cellular isolation and characterization techniques, a fairly descriptive picture of the earliest differentiation processes have evolved [84]. HSC can be divided into a long-term subset (LT-HSC), capable of indefinite self-renewal, as well as a short-term subset (ST-HSC) that self-renews for a defined interval. Each stage of differentiation of multipotent steps involves functionally irreversible maturation steps. The lineage of multipotent cells is thus LT-HSC that differentiates to ST-HSC, which in its turn differentiates to multipotent progenitors (MPP), that can give rise to the entire hematopoietic system, but has lost its capacity to self-renew [85]. Included in the progeny of mouse HSCs are two kinds of oligolineage-restricted cells: common lymphoid progenitors (CLPs), which at a clonal level are restricted to give rise to T lymphocytes, B lymphocytes,

and NK cells [86], and common myeloid progenitors (CMPs), which are progenitors for the myeloerythroid lineages. CMPs give rise to granulocyte/monocyte progenitors (GMPs) and megakaryocytic/erythroid progenitors (MEPs) [87]. All these populations (LT-HSCs, ST-HSCs, MPPs, CLPs, CMPs, GMPs, and MEPs) are separable as pure populations using cell surface markers (*Figure 1,* adapted from [84]).

How the differentiation process is controlled is not fully understood, and it is not clarified whether cell division is a prerequisite for differentiation to occur, or whether alterations in the genetic control can occur in a cell at cell cycle rest. The most immature lymphoid-committed progenitors (CLPs), have been shown to maintain a latent granulocyte/macrophage differentiation potential that can be initiated by signals emanating from interleukin-2 (IL-2) or granulocyte/macrophage- (GM-) CSF receptors. It was concluded that cytokine signaling can regulate cell fate decisions, and thus a critical step in lymphoid commitment is down-regulation of cytokine receptors that drive myeloid cell development. [88,89].

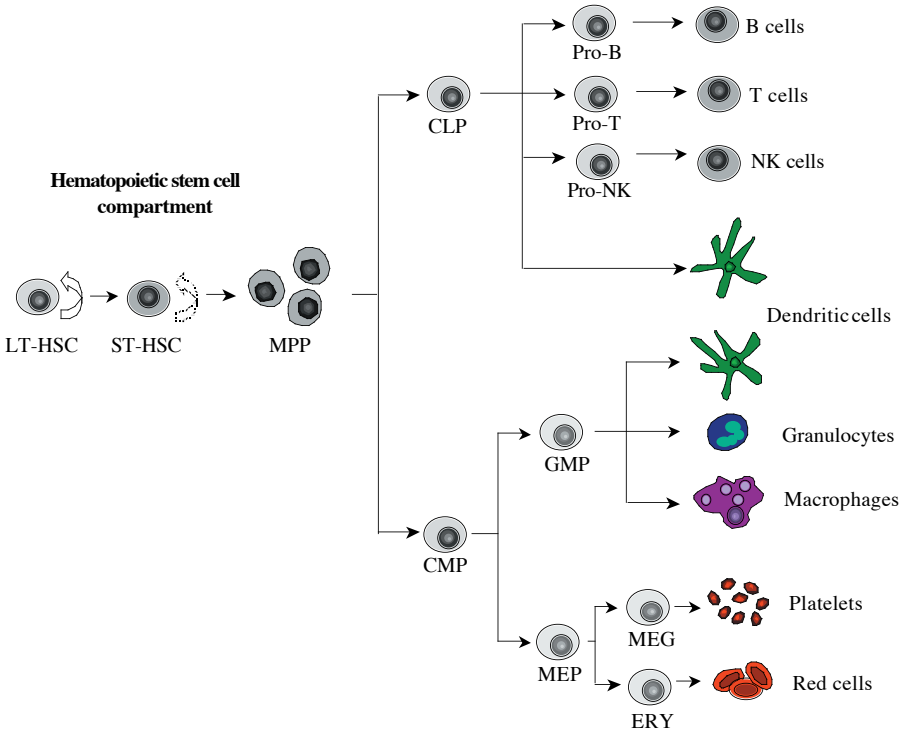

Figure 1: *The hematopoietic tree.*

Hematopoietic cytokines and self-renewal

The hematopoietic cytokines have been suggested to regulate proliferation, survival, differentiation and cell activation. Cytokines mostly act on the auto- and paracrin level during hematopoiesis, which can lead to high concentrations locally, and regulation of cytokines is exerted by rapid degradation both on the mRNA and on the protein level [90]. That cytokines regulate differentiation is debated, and two models have been proposed. According to a *stochastic model*, the choice of lineage is decided intrinsically, independently of cytokines, and by chance. The role of cytokines would be to regulate proliferation and survival, and not for commitment, and thus select for cells that are responsive to the cytokine [91]. Suppression of programmed cell death (apoptosis) allowed differentiation of the multipotent hematopoietic cell line FDCP-mix in the absence of added growth factors, thus supporting the hypothesis that differentiation is intrinsically determined and that the role of the hematopoietic growth factors is enabling rather than inductive [92]. Also, insertion of the erythropoietin (epo) or macrophage (M)-CSF receptor into multipotent precursors, did not stimulate erythroid or macrophage colony formation, respectively, but increased proliferation of all progenitors [93].

However, an *instructive model* exists, that suggests that cytokines can induce differentiation. In support of this, studies have shown that a mutant form of G-CSF receptor is unable to conduct signals for differentiation, though it is still capable to relay signals for proliferation [3,94,95]. Both models may be correct in that they could act at different levels of hematopoiesis. The intrinsic factors controlling differentiation and commitment, whether controlled by cytokine signaling or by chance, are specific combinations of transcription factors. The transcription factors are often activated in positive autoregulatory loops, which can explain the irreversible differentiation processes found in hematopoiesis. Two important transcription factors for the maintenance of LT-HSCs are SCL/Tal, mentioned above as important in the embryonic development of hematopoiesis, and Lmo2, which has been shown to be able to physically interact with SCL/Tal, and thus regulate transcription [96,97].

Most cytokine receptors consist of two or three subunits, of which one is cytokine specific and binds the ligand, while another subunit transmits the intracellular signal. The receptor subunit mediating signal transduction is often shared by different receptors. For example, the receptors for IL-3, IL-5 and GM-CSF have a common signal transducing β-chain [98], and the family of cytokines signaling through the common receptor subunit gp130 comprises of IL-6, IL-11, leukemia inhibitory factor (LIF),

oncostatin M, ciliary neurotrophic factor and cardiotrophin-1 [99]. This could explain that, although the receptors have affinity for different cytokines, there is a redundancy between some of the hematopoietic cytokines. Also, this could explain the similarity of the effects from different cytokines, like the size of a colony formed after the addition of certain cytokines.

In order not to empty the stem cell pool, the HSCs have the capacity for self-renewal [3]. Upon cycling, HSCs may undergo symmetric division (i.e. either self-renewal or commitment to differentiation of the progeny) or asymmetric divisions (i.e. generation of a self-replicated and a differentiated cell). In the embryonic mice, an expansion of cells with LT-HSC activity can be seen in the AGM region after ED10 [36]. Although extensive amplification of LT-HSC numbers can be observed in recipients of adult LT-HSC transplants [52], attempts to enhance normal LT-HSCs *in vitro* have generally been without any success. However, when adult mouse BM cells were incubated for 10 days in serum-free medium with IL-11, FL and KL, there was a 3-fold increase of cells able to reconstitute the lymphoid and myeloid systems of recipient mice [100].

Other signals than cytokines, such as adhesion factors and ligands expressed in the surroundings of the HSC pool in the BM, could also be important for HSC self-renewal. Pluripotent HSCs are immortalized by constitutive Notch1 signaling, which could be a candidate mechanism for LT-HSC self-renewal [101]. It has also been shown that the Wnt signaling pathway has an important role in self-renewal, and that activation of Wnt signaling in HSCs induces increased expression of Notch, and also HoxB4, which has been implicated in self-renewal of HSCs [102-104]. Telomerase seems also to be important for HSC self-renewal, since telomerase activity has been found in HSCs, and long-term self-renewal of HSCs is compromised upon telomere loss. Unlike tumor cells, HSCs are not immortal and show decreasing telomere length with increasing age. Thus, telomerase may regulate self-renewal capacity by reducing the rate at which telomeres shorten [105,106].

c-kit/KL and Flt3/FL

Absence of KL (the Steel mutation), or the cell surface receptor c-kit (the White mutation), results in death *in utero* or in the perinatal period with severe macrocytic anemia [107]. The gene corresponding to *c-kit* was cloned in the late 80s [108,109], and its ligand a few years later [110-116]. Mutations at either of these loci resulted in very similar phenotypes characterized by alterations of coat color ("white spotting"), anemia and lack of mast cells in the tissues, suggesting that the genes involved were important

for hematopoiesis and melanogenesis (reviwed by [4]). By use of reconstitution assays, it has been shown that LT-HSCs express c-kit [57], and progenitor cells for the lymphoid and myeloid compartments express c-kit as well [86,87]. Studies have indicated that c-kit is expressed at low levels on LT-HSCs, increases in density to reach a peak at the progenitor level, and then decreases with terminal maturation with the exception of basophiles/mast cells, who express high levels of c-kit [4]. That KL is important for the hematopoiesis has been shown in several studies, and it is interesting to note that it was the only cytokine, out of several studied, that was able to support *in vitro* proliferation and recruitment of HSCs by preventing apoptosis [117].

KL exists in both soluble and membrane-bound forms as a result of differential splicing and proteolytic cleavage. It has been shown that a more sustained signaling is mediated by membrane associated KL, and the phenotype of *Sl/S*^{ld} mice, which produce only soluble KL, have anemia, pigmentation defects and lack tissue mast cells [46]. KL has also been shown to promote the survival of HSCs and progenitor cells in the absence of cell division [118] and enhances hematopoiesis by synergistic interactions with early acting cytokines [83,119-121]. However, differentiation into B cells can occur in the absence of c-kit [122].

The Flt3 receptor has strong sequence similarities with other members of the class III tyrosine kinase receptors family, which includes c-kit, and it was cloned in 1991 [123-125]. Using the receptor as bait, the corresponding ligand was cloned some years later [126,127]. Flt3 has been reported to have a fairly limited expression pattern confined to candidate HSCs and more committed progenitors [4]. Mice lacking FL or its receptor Flt3 have deficiencies in hematopoiesis [128,129], but recent studies indicate that the effects are mostly on the lymphoid compartment [130]. It has recently been shown that LT-HSCs do not seem to express Flt3 receptor [131,132], and cell cycle analysis shows that a large number of cells expressing low levels of Flt3 receptor are in the G_0 phase of the cell cycle, whereas cells with high expression are predominantly in G_1. This is in accordance with the observation that FL can induce proliferation of hematopoietic progenitor cells [133].

Endothelial cells (EC) express a membrane-bound form of FL and produce large amounts of soluble FL, which are derived from the membrane-bound FL by proteolytic release. BM microvascular EC also produce FL, suggesting that EC are an important source of FL in the BM [134,135]. FL has been shown to stimulate the expansion of primitive murine BM progenitor cells *in vitro* and to have synergistic interactions

with hematopoietic growth factors [127,136-140]. FL also promotes the survival of primitive hematopoietic progenitor cells with B lymphoid as well as myeloid potential [141,142].

Although similar in structure, c-kit and Flt3 seem to convey different functions in the hematopoietic system [143]. A major difference between FL and KL is that FL has no growth promoting effects on progenitors committed to the erythrocyte, megakaryocyte, eosinophil, or mast cell lineages [137,144]. Instead, FL interacts with IL-7 to promote B-cell commitment and differentiation from uncommitted BM progenitor cells [145]. Though not studied in detail for the Flt3 receptor, c-kit and Flt3 seem to activate similar transduction components, which include the Src family members, the JAK/STAT pathway, the Ras-Raf-MAP kinase cascade and phosphatidylinositol-3 (PI-3) kinase [146]. KL and FL have also shown synergistic effects in supporting [79] and expanding [100] LT-HSCs and dendritic progenitor cells [147].

Bcl-2 family proteins and apoptosis

In order to maintain a steady state of hematopoiesis, HSCs can either self-renew or commit to differentiation. In the case of an overcapacity, apoptosis can also be induced. One reason why the culturing of HSCs fails, is that the cells undergo apoptosis, but blockage of apoptosis alone does not permit successful culture. Simply blocking apoptosis, for example by overexpressing the Bcl-2 protooncogene in the absence of the correct stimuli, leads to differentiation of the cells [92,148].

Bcl-2 was initially identified as a cellular gene located at the site of a frequent t(14; 18) chromosomal translocation in follicular B cell lymphomas [149,150] and systemic overexpression of Bcl-2 in the hematopoietic system protects from apoptosis [151-153]. In the adult, Bcl-2 is expressed in BM progenitor cells representing all lineages, suggesting a role in the survival of these immature cells [154]. Bcl-$2^{-/-}$ mice develop normally, but later exhibit marked lymphoid apoptosis [155]. Crosses of knockout mice revealed that the proapoptotic Bcl-2-associated protein x (Bax) is responsible for the death of lymphoid cells in Bcl-$2^{-/-}$ mice [156].

The Bcl-2 family proteins are divided into three groups depending on their functions. The *Bcl-2-like survival factors* (e.g., Bcl-2, Bcl-x_L, Mcl-1 and Bfl-1/A1) contain three to four so-called Bcl-2 homology domains (BH1-BH4), which are absolutely required for their survival functions. *Bax-like death factors* (e.g., Bax, Bak and Bok) lack the N-terminal BH4 domain, although this is probably not the cause for their death-inducing

capacity. Finally, the *BH-3-only death factors* (e.g., Bim, Bad and Bid), which are so-called because they share with each other, and with the other members of the Bcl-2 family of proteins, only the BH3 domain (reviewed in [15]).

In short, most of the pro- or antiapoptotic effects are seen when the Bcl-2 family proteins are integrated into membranes, especially the mitochondria [157]. It is believed that the Bcl-2-like survival factors only repress the function of the Bax-like death factors [158]. Alternatively, the Bcl-2-like survival factors and the Bax-like death factors either form pores in the mitochondria, thus regulating the function of the organelle, or stabilize or perturb a pre-existing channel (the "permeability transition pore"; PTP), that forms across sites of contact between the inner and outer membranes of the mitochondria, through which adenine nucleotides and other small molecules traffic [159]. The core components of this channel include the voltage-dependent anion channel (VDAC) in the outer membrane, adenine nucleotide translocater (ANT) in the inner one, and cyclophilin D in the matrix [160].

In any case, the BH3-only death factors are thought to regulate the function of the other two groups, either by inhibiting the antiapoptotic Bcl-2-like survival factors, or by enhancing the function of the proapoptotic Bax-like death factors. If the balance of the pro- and antiapoptotic Bcl-2 family proteins favors apoptosis, cytochrome c, which is a part of the electron transport chain, is released into the cytosol. There it forms an "apoptosome" together with Apaf-1 and procaspase-9, which is cleaved to form the activated caspase-9. Caspase-9 in its turn cleaves caspase-3 and other caspases, executing the programmed cell death (reviewed in [161,162]).

As mentioned above, Bcl-2 and Bax have been shown to have a role in the survival of hematopoietic cells. There seems to be a redundant function of Bax and Bak, and individual Bax and Bak knock-out mice have remarkably little immune phenotype. Bax deficient mice have mild hyperplasia and Bak-deficient mice have no discernable phenotype at all [163]. In contrast, $Bax^{-/-}/Bak^{-/-}$ mice have increased hematopoietic progenitor cells in the BM and white blood cells in the blood. The spleens and lymph nodes are also up to 30-fold enlarged [164]. The myeloid cell leukemia-1 gene (Mcl-1) was discovered because its expression increased early in the differentiation of a myeloid leukemia cell line [165]. Mcl-1 interacts with Bax in hematopoietic FDC-P1 cells and can prolong cell viability [166]. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage [167]. Bax and Bak have been shown to accelerate the opening of VDAC, allowing

cytochrome c to pass through, whereas the antiapoptotic protein Bcl-x_L closes VDAC by binding to it directly [168]. Bcl-x_L has been reported to be expressed in primitive hematopoietic progenitors [169], and null mutants die during embryogenesis with severe hematopoietic deficiencies [161,170,171].

Hematopoietic cytokines have been shown to regulate Bcl-2 family members. A1 is necessary for cytokine-dependant neutrophil survival [172,173], whereas Hrk has been shown to be induced in hematopoietic progenitors after growth factor deprivation [174]. FL has been shown to prevent upregulation of Bax [175], and KL has been shown to upregulate Bcl-2 in NK cells [176], and both Bcl-2 and Bcl-x_L in erythroid progenitors [177,178]. Also, Mcl-1 has been shown to be a target of KL and interleukin-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/PKB pathways [179].

Another, partly mitochondria-independent apoptotic pathway has also been inferred with hematopoiesis; the activation of the death-inducing Fas and tumor necrosis factor (TNF) receptors by their respective ligand. This pathway has been shown to be functional in hematopoietic progenitor cells [180-182], fetal HSCs [183] and LT-HSCs [184,185]. Activation of these receptors activates caspase-8, which in its turn activates caspase-3 without the involvement of the mitochondria. However, caspase-8 is also able to cleave cytosolic Bid, generating a smaller fragment that translocates to the mitochondria and enhances the release of cytochrome c [157].

PI-3 kinase, Akt/PKB, and forkhead

PI-3 kinase is a heterodimer consisting of two subunits, catalytic and regulatory, with molecular weights of 110 kD (p110) and 85 kD (p85), respectively. The regulatory p85 subunit consists of several motifs implicated in protein-protein interaction, which include two src homology 2 (SH2) domains, an SH3 domain, a proline-rich domain, and the inter SH2-sequence which is responsible for binding to p110 [186,187]. KL induces the association of p85 with c-kit through the SH2 domains and thus localizes p110 together with potential substrates at the plasma membrane, where it produces 3´-phosphorylated phospholipids including the PI–3,4-P_2 and PI–3,4,5-P_3 [188-190].

Knockout studies have shown an important role of PI-3 kinase in hematopoiesis, including both lymphoid and myeloid cells [191-193]. Both PI–3,4-P_2 and PI–3,4,5-P_3 bind with high affinity to the pleckstrin homology (PH) domain of several proteins, including PKB and PI-dependent kinase 1 (PDK1). In response to KL, PI–3,4-P_2 and PI–3,4,5-P_3 recruits PKB to the plasma membrane, where it can be phosphorylated and

activated by PDK1, which by itself is PI-activated [194]. PI–3,4-P_2 and PI–3,4,5-P_3 can be dephosphorylated on the 3′ of the inositol ring by PTEN (phosphatase and tensin homologue deleted on chromosome 10), thus inhibiting PKB activity [195,196].

The oncogenic PKB was originally cloned from a T cell lymphoma where it was truncated and fused to the viral sequence [197]. Mutations of c-kit activating the PI-3 kinase/PKB pathway have been implicated in a variety of malignancies, including acute myeloid leukemia (AML) [198]. Active PKB has been found to phosphorylate several substrates including Bad [199-201], IKK [202], glycogen synthase kinase-3 (GSK-3) [203], proteins involved in nitric oxide synthesis [204,205], caspase-9 [206], p70S6K [207] and forkhead transcription factors [208-210]. Although the antiapoptotic activity of PKB is well known, it also regulates other aspects of cellular functions, including migration, protein synthesis, and glycolysis [211].

PKB controls several processes involved in metabolism, which include effects on the activity of GSK-3 and p70S6K, the translocation of the glucose transporter (GLUT) 4 to the plasma membrane, the induction of GLUT 1 synthesis and the activation of hexokinase and other enzymes involved in glucose metabolism [212-218]. Although many of these processes are cell-type specific, PKB may generally regulate aspects of metabolic homeostasis and thereby promotes survival [16]. The reverse transcriptase subunit of telomerase has also been found to be a substrate of PKB, indicating that PKB may also inhibit senescence [219]. An overview of signals emanating from c-kit (and possibly from Flt3) are depicted in *Figure 2* (adapted from [220]), with emphasis on targets downstream of PI-3 kinase and PKB.

Bcl-2 family targets other than Bad have also been shown to be affected by PKB signaling. Mcl-1 has been shown to be a target of KL and interleukin-5 for apoptosis prevention activity via the MEK/MAPK and PI-3K/PKB pathways [179]. Also, PKB has been shown to exert antiapoptotic effects in hematopoietic cells by inhibiting Bax conformational change and its redistribution to the mitochondrial membranes [221]. Further, the pro-survival Bfl-1/A1 has been shown to be a transcriptional target of the transcription factor NF-κB [222], which is activated via the phosphorylation of IKK by PKB, as mentioned above. Activating transcription factor 2 (ATF-2) activities are also reduced by PKB phosphorylation, as is apoptosis signal-regulating kinase 1 (ASK1), which is a c-Jun N-terminal kinase (JNK) activator [223]. Finally, the transcriptoin factor CREB has been shown to be activated by PKB [224].

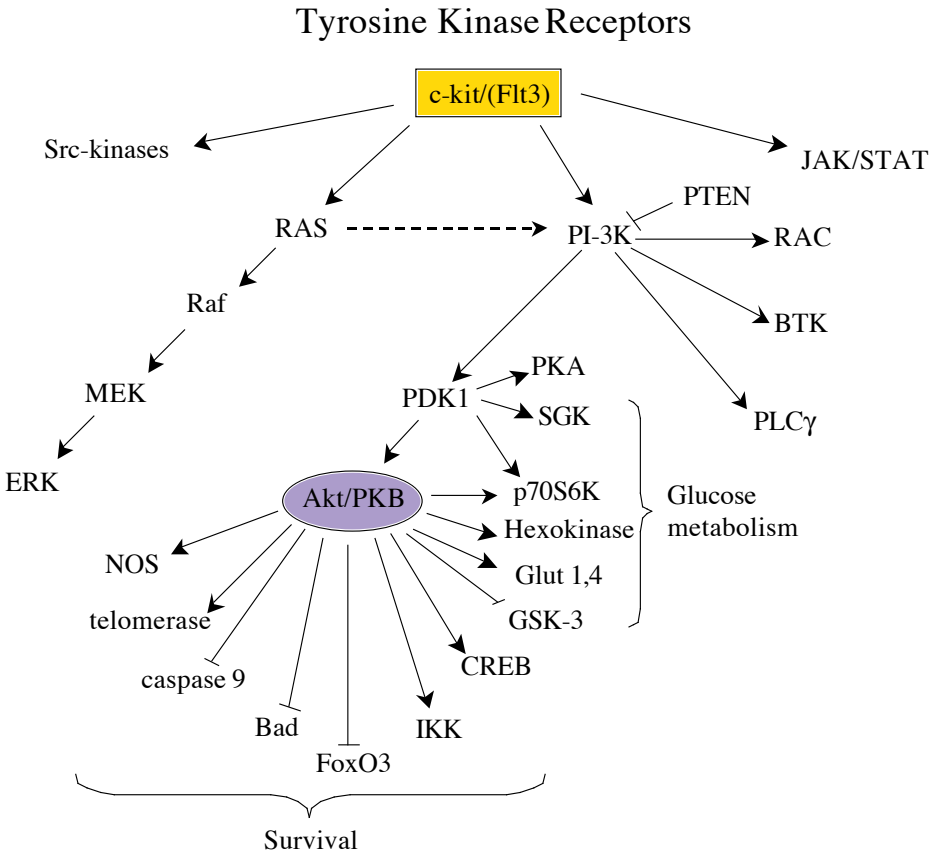

Figure 2: *Overview of signals downstream of c-kit, PI-3 kinase and Akt/PKB.*

After PKB activation, the 14-3-3 proteins have been shown to bind and inactivate, not only the proapoptotic Bcl-2 family protein Bad, but also the forkhead transcription factors [210]. In mammalian cells, three members of the forkhead family have been identified as FoxO1, FoxO3 and FoxO4 (also known as FKHR, FKHRL1, and AFX, respectively), all at sites of chromosomal rearrangements in certain human tumors [225-229]. The expression of a constitutively active version of PKB within cells is sufficient to induce the phosphorylation and inactivation of all three forkhead isoforms [208,210,230,231], and the phosphorylation of forkheads can be blocked with the PI-3 kinase inhibitors wortmannin and LY294002 [232]. A mutant of FoxO3 in which all three sites of PKB phosphorylation were converted to alanine was localized to the nucleus even in the presence of survival factors, indicating that the cytoplasmic retention of FoxO3 by 14-3-3 proteins is directly linked to its phosphorylation of PKB.

FoxO3 has also been found to bind to sites present in the promoter of the Fas ligand gene, and to induce expression of a reporter gene driven by the Fas ligand promoter [210]. Thus, when PKB is inactive, forkhead family members may induce endogenous Fas ligand gene transcription, which in its turn binds to the cell surface receptor Fas in an autocrine or paracrine fashion, and triggers a cascade of events leading to apoptosis [16]. FoxO3 has also been shown to induce the expression of the proapoptotic protein Bim [233,234] and the cell cycle regulator p27 [235].

Hypoxia and HIF

Although combinations of cytokines including KL, FL, thrombopoietin (Tpo), and IL-6 have been demonstrated to support the short-term growth of HSCs, extended studies of LT-HSCs have revealed that they may be eventually lost during *in vitro* culture [4,236,237]. Since hematopoiesis occurs in close proximity to the stromal microenvironment, adhesion molecules and environmental cues must be considered, and there is a need to define more precisely the culture conditions required to optimize expansion of HSCs and other primitive progenitor populations. It has been suggested that HSCs reside in hypoxic areas in close proximity to the bone surface [238], and thus hypoxia could offer a HSC niche environment which prevents differentiation and promotes self-renewal. Hypoxia is known to lead to increased epo production and survival of BFU-E, and to induce erythroid maturation [239,240]. Incubation of murine BM cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors [11-14]. More importantly, there has been a recent report of an 4-fold increase of human LT-HSCs with BM-repopulating activity in severe combined immune deficiency (SCID) mice under hypoxic conditions [241]. The mechanisms behind these effects have not yet been elucidated, but hypoxia has been shown to activate several pathways involved in apoptosis, such as the inhibitor of apoptosis 2 (IAP2) [242] and the inhibition of GSK-3 by hypoxia-induced activation of PKB [243].

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that functions as a master regulator of oxygen homeostasis [244]. HIF-1 is a heterodimer composed of an inducibly-expressed HIF-1α subunit and a constitutively-expressed ARNT (also known as HIF-1β) subunit [245]. ARNT can also form a heterodimer together with HIF-2α (also known as EPAS1). HIF-1α and HIF-2α are subject to rapid ubiquitination and proteosomal degradation under non-hypoxic conditions [246-248] and this process

is inhibited under hypoxic conditions [249]. Targeted disruption in mice of the HIF-1α and ARNT genes show a connection between hypoxia and maintenance of HSCs [250]. Mice rendered deficient of the HIF-1α show impaired proliferation of embryonic multilineage hematopoietic progenitors [251], and $ARNT^{-/-}$ embryos exhibit decreased numbers of progenitors in the yolk sac [252]. HIF-2α has also been shown to be required for normal hematopoiesis in mice, and its loss results in pancytopenia [253]. It was suggested that HIF-2α has a critical role in maintaining a functional microenvironment in the BM for effective hematopoiesis. Interestingly, HIF-2α has been shown to be strongly expressed within subsets of BM macrophages [10].

HIF-1 is a substrate for various kinase pathways including PI-3K and the MAP kinases ERK and p38 [254], and it has been suggested that activation of PKB promotes stabilization and accumulation of HIF-1α [255]. ARNT has also been shown to contain a PKB consensus phosphorylation site [16]. Hypoxia–induced overexpression of vascular endothelial growth factor (VEGF) is inhibited in dominant negative mutants of PI-3 kinase and PKB, and it has been suggested that this is dependent on HIF-1 regulation [256]. It is interesting to note in this context that VEGF in hematopoiesis is likely to maintain survival of hematopoietic progenitors through the activation of antiapoptotic pathways [257,258]. PKB might act directly on HIF-1, but it has also been suggested that downstream targets of PKB like the downstream kinase FRAP/mTOR (FKBP-rapamycin-associated protein/mammalian target of rapamycin) is involved [259]. Also, the forkhead transcription factor FoxO4 has been shown to induce the down-regulation of HIF-1α [260].

PRESENT INVESTIGATION

Comparison of induction of Bcl-2 family proteins by IL-3, KL and FL (Paper I)

In the first paper, we wanted to study early hematopoietic cytokines and their effects on cell survival. Since the receptor for KL is expressed on and has been shown to be a major survival factor for the earliest HSCs, our main focus was on this cytokine, but IL-3 and FL have also been shown to have effects on HSCs and progenitor cells. By alternating the growth conditions for the IL-3 dependent multipotent cell line FDCP-mix (mouse BM-derived cells) between IL-3 at 200 U/ml and IL-3 at 1 U/ml together with KL (100 ng/ml), we established a KL-responsive variant cell line (93.1). To be able to compare the effects of IL-3, KL, and FL, we further infected another progenitor cell line, FDC-P1, with a retroviral construct carrying the coding sequence for the receptor of FL. This cell line was denoted FDC-P1/flt3.

Initially, we found by Western blot analysis that all three cytokines induced phosphorylation of the PI-3 kinase downstream target PKB in FDC-P1/flt3 cells. This phosphorylation of PKB by FL had not been shown previously. By adding LY294002, an inhibitor for PI-3 kinase, this phosphorylation was abrogated, indicating that the PI-3 kinase was the upstream target for all three cytokines. By use of Annexin V staining and flow cytometry to determine the proportions of dead cells, it was revealed that all three cytokines could maintain the cells for a limited time period, but after blockage of the PI-3 signaling, the cells incubated with KL became apoptotic. Survival of the cells incubated with IL-3 and FL was also inhibited by LY294002, but not to the same extent as with KL. At lower concentrations of LY294002, with continued strong inhibition of KL-mediated survival, no significant effect was seen on cells incubated with IL-3 or FL. Thus, despite similar effects on PKB phosphorylation, IL-3 and FL are able to induce alternative survival pathway not linked to the antiapoptotic effects mediated via PKB. When we repeated these experiments with inhibitors for the MAPK pathway, PD98059 and U0126, no major effects could be seen on survival. Phosphorylation of the MAPK target ERK was inhibited, indicating that the inhibitors were functional.

We continued by looking at the Bcl-2 family proteins. By using Western blot, we found that the antiapoptotic mechanism induced by KL did not involve Bcl-2, Bcl-x_L or Mcl-1, and this was confirmed at mRNA level for Bcl-2 and Bcl-x_L. In contrast, all three proteins were upregulated after IL-3 stimulation and we showed that the regulation was at the transcriptional level. The mode of action of FL was similar to IL-3, although

with slower kinetics for Bcl-2 mRNA, and not with as high induction of Bcl-x_L mRNA as seen with IL-3. The major difference was that FL could not induce Mcl-1 protein expression. The expression of Bcl-2 family proteins was shown not to be mediated by PI-3 kinase. We also obtained similar results for IL-3 and KL in the FDCP-mix 93.1 cell line and murine Lin^- BM-derived cells. Thus, it was confirmed that KL does not induce Bcl-2 family proteins, and its pro-survival signaling is susceptible to PI-3 kinase inhibition.

The conclusion from this paper was that FL has similar roles in the maintenance of the progenitor cell lines as IL-3 by upregulating Bcl-2 family proteins, while the antiapoptotic properties of KL would have to be further investigated.

Activation of Akt/PKB and inactivation of FoxO3 by KL (Paper II)

In this paper we wanted to elucidate the downstream targets important for the survival signals elicited by c-kit activation and whether this was linked to PI-3 kinase and PKB. PKB has in its turn been shown to inactivate the forkhead transcription factors, FoxO1, FoxO3, and FoxO4, known to regulate apoptosis. Initially, by Western blot analysis we showed that PKB is phosphorylated in FDCP-mix 93.1 cells after IL-3 and KL stimulation, and the effects of KL on survival was shown to be blocked by the PI-3 kinase inhibitor LY294002. We then showed that KL phosphorylated FoxO3 and to some extent FoxO1, but not FoxO4, and this phosphorylation was PI-3 kinase-dependent. By use of immunofluorescence and subcellular fractionation, we found that FoxO3 is translocated out of the nucleus after KL-stimulation, and this was shown to be PI-3 kinase dependent.

We further infected FDC-P1 cells by retroviral gene transfer to overexpress an inducible FoxO3. After addition of tamoxifen, which induces the activity of the triple-mutated form of FoxO3 by translocating it to the nucleus, apoptosis was induced, and these results were obtained despite the presence of KL. Also, triple-mutated FoxO3 was able to inhibit the colony formation of BM-derived Lin^- progenitors, either in the presence of KL alone for myeloid colony formation, or epo and KL in combination for erythroid colony formation. In these cells only FoxO3 was phosphorylated after addition of KL, whereas FoxO1 and FoxO4 were not.

The conclusion from this paper was that FoxO3 is involved in KL-mediated survival of hematopoietic progenitors.

Antiapoptotic effects of Bcl-2 and Akt/PKB in combination with KL (Paper III)

In this study, we compared the effects of PKB and Bcl-2 in the FDC-P1 cell line, either alone or in combination. To begin with, FDC-P1 cells were transfected with wild type PKB (wtPKB) or a kinase dead variant of PKB (K179M), which is mutated in its ATP binding site. This kinase dead form has previously been shown to inhibit endogenous PKB [261]. After selection for successfully transfected cells and subsequent Annexin V staining and flow cytometry, we found that more cells expressing K179M were apoptotic compared to cells transfected with wtPKB. Thus PKB seemed to mediate antiapoptotic signals in FDC-P1 cells. Overexpression of Bcl-2 and to a lower extent PKB, was shown to improve survival in selected clones, and this could be further enhanced by addition of KL. These effects were not due to increased proliferation, as measured by MTT-assay, but rather due to sustained mitochondrial potential. The mitochondrial integrity, measured by rhodamine-1,2,3 staining, was clearly increased in Bcl-2 expressing cells, whereas introduction of PKB only led to partial enhancement. Similar results were obtained when we overexpressed wtPKB or a mutated form of PKB (E40K), which has an amino acid substitution at its PH domain. This mutation increases the affinity for phospholipids, which has been shown to lead to increased activity [261]. When PKB was introduced in Bcl-2 expressing cells, no further protection from apoptosis could be seen, although the mitochondrial integrity was slightly enhanced. However, the combination of PKB and Bcl-2 did not suffice to reach the mitochondrial potential of Bcl-2 expressing cells incubated with KL.

The data from this study suggest that a combination of KL and Bcl-2 enhances survival of hematopoietic progenitors, but enforced expression of active PKB together with Bcl-2 seemed not to be a sufficient substitution. In future studies it would be of importance to further investigate other downstream signals of PI-3 kinase that might be responsible for the survival effects seen with KL.

Antiapoptotic effects of hypoxia in combination with KL and early acting cytokines (Paper IV)

Hypoxia has recently been inferred to be involved in the maintenance of hematopoietic stem and progenitor cells. Survival of FDCP-mix 93.1 was shown by Annexin V staining to be increased in low oxygen tension, when incubated with KL or suboptimal concentrations of IL-3 (0.2 U/ml), whereas survival in the presence of high concentrations of IL-3 (200 U/ml) remained the same independently of oxygen level (1%, 5% or 20%). FDCP-mix differentiation induced by G-, M- and GM-CSF was similar when the cells were incubated in 1% or 20% oxygen, as shown by Gr-1 and Mac-1 expression in FACS analysis. Surprisingly, when kept in the presence of IL-3 (normally needed for FDCP-mix maintenance), and hypoxia, the number of differentiated cells increased. Thus, hypoxia induced differentiation in the progenitor cell line.

In a clonal assay using the mononuclear fraction from mouse BM incubated in the presence of IL-1, IL-3 and KL for 4 days, and subsequently seeded in IL-3 and epo, the number of pre-CFC$_{multi}$ were increased more than twice in hypoxia compared to 20% oxygen, whereas the number of CFU-C were higher in the normoxic cultures. When we used the $Lin^{-}Sca^{+}Kit^{+}$ fraction from mouse BM and incubated for 4 days in KL, FL, IL-3, IL-6, epo, and G-CSF, which has been shown to support partial rescue of LT-HSC [131], the level of expansion of cells was greater in hypoxia than under normoxic conditions (7.1-fold compared to 3.2-fold). Moreover, the frequency of HPP-CFCs increased four-fold under hypoxia compared to the original numbers of HPP-CFC present in the $Lin^{-}Sca^{+}Kit^{+}$ fraction without any preincubation, whereas the frequency decreased under normoxia. Thus, this indicates that self-renewal of the HPP-CFCs had occurred under hypoxic conditions.

In conclusion, we show in this paper that maintenance of multipotent BM progenitor cells and HSCs is dependent on combination of factors (e.g., KL and low oxygen tension) and that self-renewal of early HSCs is favored by hypoxia.

DISCUSSION

In this thesis, we have focused on the signaling pathways important for the maintenance and survival of HSCs and progenitor cells. In a clinical aspect, it would be beneficial to elucidate how self-renewal and expansion could occur *in vitro*, since BM transplantation techniques would be improved, and life-long gene therapy would be feasible. Several studies have indicated that certain cytokines in combination are essential to inhibit apoptosis and differentiation, including KL, FL, Tpo, IL-3, IL-6, and IL-11 [4,118,262-266]. When added as single factors, however, these cytokines have little or no effect, although KL has been found to increase the survival of hematopoietic progenitor cells [118,262,267,268]. The ability of cells to escape the apoptotic machinery can be mediated by activation of antiapoptotic members of the Bcl-2 family or by inhibition of the proapoptotic proteins of the same family. Also, the activation of PI-3 kinase is a major intracellular signal transduction pathway by which many cytokines can prevent apoptosis.

There is also the possibility that regulation of the cell cycle and/or general metabolism are important for the maintenance of HSC [269,270]. Further, death-inducing receptors could play a role [183,184]. More importantly, the factors that prevent differentiation are still obscure. For example, suppression of apoptosis by overexpressing Bcl-2 still allows differentiation of a multipotent hematopoietic cell line in the absence of added growth factors [92]. The limited increase in HSCs in the BM of Bcl-2 transgenic mice indicates that HSCs, despite their resistance to apoptosis, are not simply accumulating in ever larger numbers, but are still subject to regulation [153]. The limits on the increase of HSC numbers probably reflect a limit in the HSC supporting microenvironment, the HSC niche.

A “two signal model” was recently presented by Domen and Weissman [117], which suggests that two separate signals are necessary to prevent apoptosis in HSCs. They found that high levels of Bcl-2 expression did not prevent rapid death under serum-free conditions, although it did so in the presence of serum. A large number of cytokines were tested for their ability to support survival of the Bcl-2 overexpressing cells in the absence of serum, including IL-1, IL-2, IL-4, IL-6, IL-7; IL-11, FL, KL, G-CSF, GM-CSF, TNFα, TGF-β, MIP-1α, BMP-4, LIF, OSM, epo, Tpo, bFGF, EGF, and VEGF. Apart from KL, the only effect seen was a very limited response to Tpo, and cell numbers never exceeded input numbers. When incubated with KL, the cells

were increased in number. However, the cells did not maintain their phenotype but differentiated, primarily into early myeloid cells.

That KL-induced protection against apoptosis employs a separate biochemical pathway other than Bcl-2 has been reported earlier [271]. It was thus suggested that the two pathways synergized in expansion of differentiating progenitors, without the expansion of HSCs by self-renewal. Still, the question remained what signals were activated by KL that prevented cell death, and one of the putative targets could be PKB. A comparison of PKB- and Bcl-x_L-dependent cell survival was recently undertaken using interleukin-3-dependent progenitor cells [280]. Expression of constitutively active PKB allowed cells to survive for prolonged periods following growth factor withdrawal, and was comparable in magnitude to the protection provided by Bcl-x_L. Although both genes prevented cell death, PKB-protected cells could be distinguished from Bcl-x_L-protected cells on the basis of increased glucose transporter expression, need for glycolytic activity, stabilized mitochondrial potential, and larger cell size.

In our studies, a rather straightforward role of PKB was seen, as presented in paper I and II. In the hematopoietic progenitor cell lines FDC-P1 and FDCP-mix, and BM-derived Lin^- cells, c-kit signaling could not activate the antiapoptotic Bcl-2 family proteins investigated, which has also been seen in other cell systems [271,272]. Also, as seen at the mRNA level in RNAse protection assay, no proapoptotic Bcl-2 family proteins were down-regulated, although it still remains to be seen if cellular localisation, degradation, or conformational change of these occur. For example, it has recently been shown that PKB regulates cell survival and apoptosis by inhibiting Bax conformational change [221]. In any case, PKB is activated by c-kit signaling via activation of PI-3 kinase, and inhibition of PI-3 kinase leads to apoptosis.

In paper III, we also show that expression of kinase dead PKB leads to enhanced cell death compared to cells with overexpression of wtPKB or a constitutive active form of PKB (E40K). We speculated that we could substitute the combination of KL and Bcl-2 with PKB and Bcl-2. A similar approach was recently used in primordial germ cells, where KL-induced survival could be replaced with PKB [273]. Contrary, our results showed that although PKB protected cells from apoptosis, Bcl-2 was superior in antiapoptotic signaling, and the combination of KL and Bcl-2 was superior to the combination of PKB and Bcl-2. It still remains to be seen if the PKB constructs used in paper III has full activity in hematopoietic progenitor cells, as discussed in this paper. One of the construct used had a modification in its PH domain, with increased affinity

for PIs, but additional factors might be crucial. Most of all, there might be a need for further activation of PDK1 for full activity of PKB in some cell systems.

In paper II, we found that KL stimulation leads to phosphorylation of both PKB and FoxO3 in FDCP-mix cells as well as in Lin^- mouse progenitors. When we infected FDC-P1 cells with an inducible form of transcriptionally active FoxO3, we also showed that FoxO3 inhibited KL-mediated survival, and the same construct suppressed the colony-forming capacity of Lin^- cells. Thus, this suggests that the regulation of FoxO3 is a critical step in survival of hematopoietic progenitors via c-kit. However, other cytokines (e.g., IL-2, IL-3, IL-6, epo, and Tpo) can also induce phosphorylation of FoxO3 in committed progenitors [235,274-277].

We also demonstrated phosphorylation of FoxO1 in FDCP-mix cells, whereas FoxO4 was unphosphorylated. However, in BM-derived Lin^- cells, FoxO1 and FoxO4 appeared phosphorylated, even in cytokine-deprived cells. This could be due to activation by factors present in the serum, since several studies have shown that serum can lead to phosphorylation of downstream PKB targets [208,209,230,232]. Moreover, a recent report showed that when human erythroid progenitors were stimulated with epo or KL, this lead to phosphorylation of FoxO3 as well as FoxO1 and FoxO4 [278]. Thus, distinct cytokines can lead to phosphorylation of different forkhead family members, depending on what cell type is studied.

In paper IV, we initially speculated whether KL, with its antiapoptotic properties, could in the right physiological environment (hypoxia) support survival to an even higher extent than seen before, and also to be able to prevent differentiation. As shown recently, hypoxia can even increase LT-HSCs reconstitution in SCID mice [241]. In our study the more primitive progenitor cells were supported by the hypoxic conditions, whereas more differentiated cells were actually inhibited under low oxygen pressure. This fits a model, where the HSC niche in the BM is slightly hypoxic, and thus supports the long-term regeneration of the most primitive cells. Recruited and more differentiated cells leave the BM, and thus have optimal survival conditions in normoxic conditions. This would also be a means to eradicate differentiated cells in the BM, thus limit the use of the HSC niche to LT-HSCs only.

Activation of the PI-3 kinase/PKB pathway has been shown to increase expression of HIF-1α, by negatively regulate FoxO4 [260]. It would be interesting to investigate whether KL signaling and hypoxia synergize in the down-regulation of forkhead transcription factors in hematopoietic cells, and thus increase HIF-1 activity. Also,

recent data indicate that the gene for Pgp is hypoxia responsive, and it would be interesting to find out if the multidrug resistance transporters are mutually regulated by PKB and hypoxia [279].

FUTURE STUDIES

PKB-expressing hematopoietic cells seem to require high levels of extracellular nutrients to support cell survival, whereas cells expressing Bcl-2 family proteins do not [280]. This implies that metabolism is important in the outcome of the destiny of the cells. In our hands, PKB did not affect the mitochondrial potential nearly as much as Bcl-2, and it is tempting to speculate that PKB protects the cells via pathways not involving the mitochondria or cytochrome c release. Activation of PKB have been shown to increase HIF-1α protein synthesis and transcriptional activity [281], and it has been found that HIF-1α is involved in the transcriptional activation of genes encoding glycolytic enzymes [282]. It will be interesting to evaluate if HIF-1α is up-regulated in HSCs during hypoxia, and whether HIF-1α can be up-regulated at the physiological oxygen levels present in the BM after stimulation of additional signaling pathways.

Lactate and pyruvate have been found to stimulate the accumulation of HIF-1α [283]. Lactate is formed from pyruvate in cells under hypoxia, and under these conditions the rate of formation of NADH by glycolysis is greater than the rate of its oxidation by the respiratory chain. Continued glycolysis depends on the availability of NAD+ for the oxidation of glyceraldehyde 3-phosphate, so the accumulation of both NADH and pyruvate is reversed by lactate dehydrogenase, which oxidizes NADH to NAD+ as it reduces pyruvate to lactate. Thus, the conversion of glucose to lactate does not involve a net oxidation-reduction, and no reactive oxygen species that can induce apoptosis are formed. The plasma membrane of most cells is highly permeable to lactate and pyruvate and both substances can therefore diffuse out of the cell, but much more lactate than pyruvate is carried if there is a high NADH/NAD+ ratio in the cell [284].

Pyruvate dehydrogenase is the enzyme complex responsible for the entry of pyruvate to the oxidative phosphorylation chain, and it would be interesting to investigate the regulation of this complex in HSCs, and the oxidative phosphorylation activity. There has also been found a positive correlation between the induction of terminal differentiation and reduced lactate production elicited by retinoic acid [285]. Whether Pgp or BCRP are

responsible for protecting HSCs from differentiating hormones, like retinoic acid, and thus enhance self-renewal, remains to be seen. PKB signaling might be involved in this mechanism, since it was suggested in a recent paper that PKB signaling modulate the side population cell phenotype by regulating the expression of BCRP [286].

The importance of hexokinase involvement in the survival signals mediated by PKB would also be worthwhile studying, not only in its regulation of glycolysis, but also in its activity on the mitochondrial PTP. Activated PKB, like Bcl-2 and Bcl-x_L, prevents closure of the PTP component VDAC, and PKB does this via the promotion of hexokinase-VDAC interaction at the outer mitochondrial membrane [212]. Interestingly, lithium has been found to induce detachment of hexokinase from mitochondria [287], and after addition of lithium, apoptosis is induced in the hematopoietic progenitor cell line FDCP-mix (unpublished observation). These results can, though, be due to the effects of lithium on the PKB target GSK-3.

In *Figure 3* (adapted from [158]), the relations between c-kit, PKB, forkhead, Bcl-2, hypoxia and metabolism are summarized.

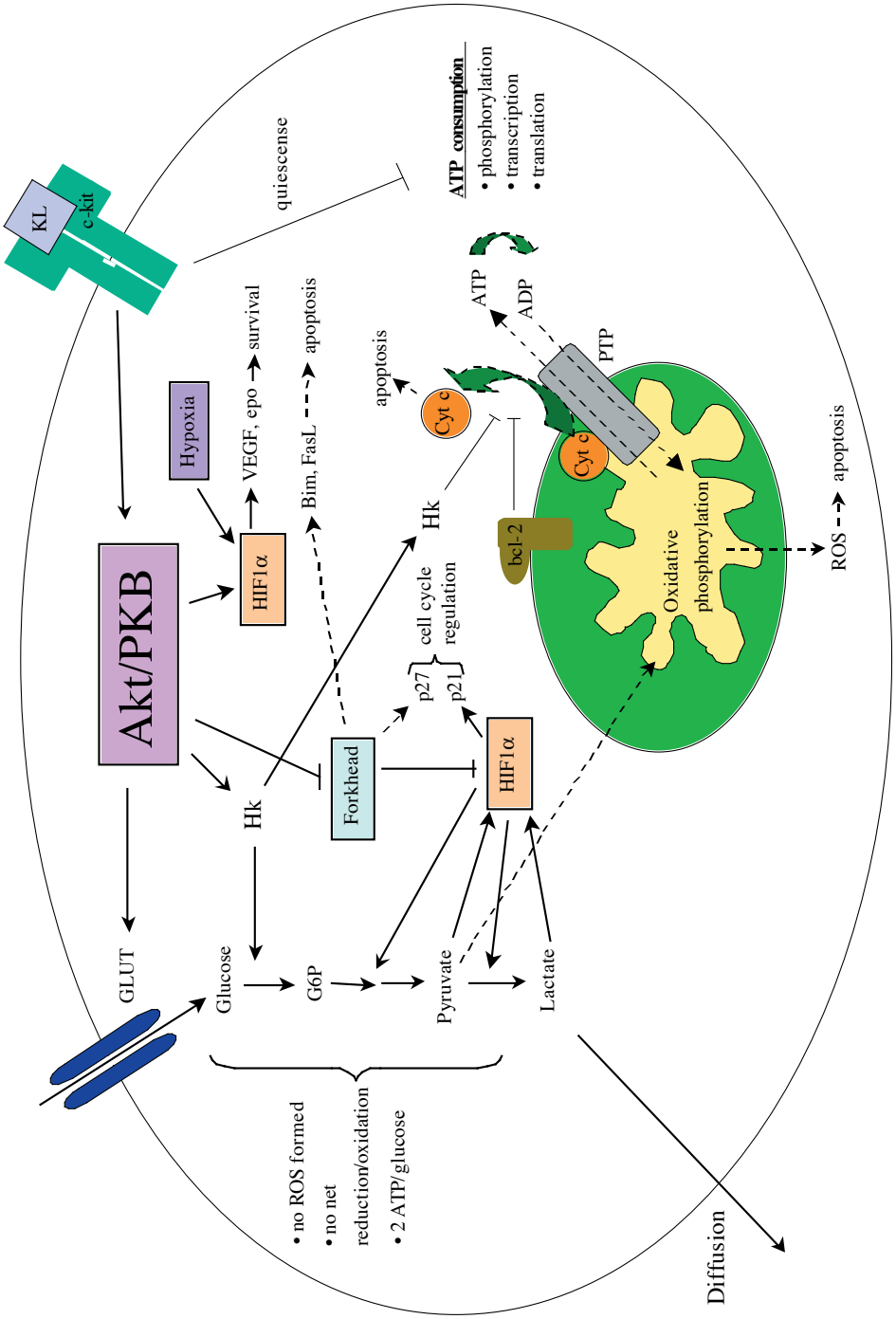

Figure 3: *Relations between c-kit, Akt/PKB, forkhead, hypoxia and metabolism.*

SAMMANFATTNING

Homeostasen, dvs. bibehållandet av ett konstant antal blodceller, pågår genom hela livet och har sitt urprung från en sällsynt cell som huvudsakligen finns i benmärgen, där den utgör mindre än 1 cell på 10000-100000. Denna cell, den hematopoetiska stamcellen, ger upphov till alla blodprogenitorer (snabbväxande celler som har bestämt vilken typ av blodceller som de kan utvecklas till) genom differentiering (förändring av cellens egenskaper genom utmognad), samtidigt som den kan fylla på poolen av stamceller på nytt. I en människa som väger 70 kg ersätts varje dag nästan 1 biljon (10^{12}) celler i det hematopoetiska systemet och ett viktigt mål med denna avhandling var att kartlägga hur olika faktorer (tex. cytokiner) påverkar stamcellernas celldelning, differentiering och överlevnad. Vi ville även finna de molekylära mekanismerna bakom blodstamcellers och blodprogenitorers överlevnad, vilka fortfarande är ofullständigt kända.

Vi har framför allt undersökt två hematopoetiskt aktiva cytokiner; stamcellsfaktorn (KL) resp. Flt3-liganden (FL), vilka utövar en signalöverförande förmåga via tyrosinkinasreceptorer (c-kit resp. Flt3-receptorn). Dessa receptorer uttrycks på flera typer av blodceller och har föreslagits vara viktiga vid blodstamcellernas överlevnad och bibehållande. Detta understryks av att c-kit uttrycks på de mest odifferentierade blodstamcellerna, vilka har visat sig kunna nybilda alla celler i blodsystemet vid benmärgs transplantationer. Med avseende på FL-medierad överlevnad finns ytterst få rapporter, men överlevnadsproteiner från Bcl-2-familjen har föreslagits vara involverade, medan överlevnad förmedlad via c-kit tycks vara beroende av vilken typ av hematopoetisk cell som har undersökts.

Vi har visat att KL förbättrar överlevnaden av hematopoetiska celler, men att KL inte aktiverar något protein från Bcl-2-familjen. Däremot uppreglerar FL Bcl-2-proteiner, vilket innebär att dessa för blodstamceller två centrala cytokiner tycks aktivera olika vägar för överlevnad. Med hjälp av blockerare för olika signalvägar identifierade vi ett viktigt protein för c-kit-förmedlad överlevnad av blodstamceller, nämligen PI-3 kinas. Nedströms från PI-3 kinas visade sig KL kunna aktivera protein kinaset Akt/PKB, vilkets funktion var nödvändigt för de hematopoetiska cellernas överlevnad. Akt/PKB har vidare visat sig inaktivera den genaktiverande forkhead faktorn FoxO3 och överuttryck av denna faktor visade sig kunna blockera KL-medierad överlevnad.

Vidare studerade vi kombinationer av överuttryck av Akt/PKB och Bcl-2 i blodprogenitorer för att se om överlevnaden kunde förbättras ytterligare. Bcl-2

visade sig vara bättre på att bibehålla cellerna än Akt/PKB och en kombination av överuttryck av Bcl-2 och tillsats av KL gav en bättre överlevnad än överuttryck av Bcl-2 tillsammans med Akt/PKB. Detta tyder på att andra signalvägar nedströms från c-kit än Akt/PKB är viktiga i dessa celler. Det återstår dock att visa hur de mer odifferentierade blodstamcellerna skulle påverkas.

I benmärgen hos möss har det visats att snabbt växande differentierade progenitorceller är lokaliserade nära blodkärlen, medan mer omogna blodstamcellerna är belägna i områden med lägre syretryck (hypoxi) än normalt (normoxi). Hypoxin har också visat sig vara en komponent av den hematopoietiska mikromiljön och en regulator av balansen mellan viloläge och tillväxt. Vad som gjorde detta speciellt intressant för oss var att hypoxi har visat sig kunna aktivera Akt/PKB i vissa celler.

Initialt visade vi att progenitorceller odlade i närvaro av KL har en ökad överlevnad under hypoxi jämfört med normoxi. Vi fortsatte därför med att undersöka celler tagna direkt från benmärg. Vi fann att hypoxi gynnar en bibehållning och en eventuell förökning av blodstamceller, medan progenitorcellerna minskade i antal. Detta fynd skulle kunna vara viktigt kliniskt med avseende på hur man odlar celler vid benmärgs transplantationer.

Ny kunskap kring mekanismerna vid hematopoetiska stam- och progenitorcellers tillväxt, val av differentiering och överlevnad kommer avsevärt att förbättra möjligheten att påverka stamceller till förlängd tillväxt *ex vivo* och på sikt leda till förfinade benmärg stransplantationer och till stamcellsbaserad genterapi. Eftersom blodcancer ofta uppstår på grund av genförändringar i de hematopoetiska stamcellerna kan våra resultat även leda till en bättre förståelse av de biologiska aspekterna på dessa förändringar.

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