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Regulation of Apoptosis in Hematopoietic Progenitor Cells: Involvement of Different Signaling Pathways

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Regulation of Apoptosis in Hematopoietic Progenitor Cells: Involvement of Different Signaling Pathways

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Abstract

Proliferation, differentiation, and survival of hematopoietic stem cells and multipotent progenitor cells are regulated by cytokines and cell-cell interactions. Kit ligand (KL) and Flt3 ligand (FL) have pleiotropic effects, promotes survival, but are nonredundant. Using myeloid progenitor cell lines and mouse bone marrow-derived Lin- progenitors, we demonstrate that inhibition of phophatidylinositol (PI) 3-kinase abolish survival mediated by KL, whereas survival via FL is only partially affected. KL and FL both activate Akt, leading to inhibitory phosphorylation of the transcription factor FoxO3. Overexpression of constitutively active FoxO3, FoxO3(A3):ER, induced apoptosis even in the presence of KL or FL, indicating that inactivation of FoxO3 is crucial for signaling via both c-Kit and Flt3. Induction of FoxO3(A3):ER also inhibited myeloid and erythroid colony formation of Lin- progenitors. In addition FL, but not KL, induced expression of Bcl-2 and Bcl-xL. By overexpressing Akt and Bcl-2 we demonstrate that Bcl-2 is the better mediator of survival than Akt. However, Akt was crucial for KL-mediated survival since overexpression of dominant negative Akt induced apoptosis. We also conclude that Akt and Bcl-2 have synergistic effects since their coexpression was a far better mediator of survival than either one acting alone. In AML, Flt3 is commonly mutated via internal tandem duplications, rendering it constitutively active. Introducing Flt3-ITD into an IL-3 dependent progenitor cell line rendered it factor-independent. Both Akt and FoxO3 were phosphorylated in the absence of FL and several Bcl-2 family members were upregulated. In fact, Flt3-ITD activated additional Bcl-2 family members not activated by normal Flt3. Finally we found that PI3-kinase and an unidentified Src kinase were important for survival via Flt3-ITD. Signaling via normal Flt3 and Flt3-ITD differs in some aspects and disruption of specific Flt3-ITD signals may be potential targets for treatment.

Key words: hematopoiesis, progenitor, mouse, cytokines, apoptosis, cell cycle, c-Kit, Flt3, Flt3-ITD, Akt, FoxO3, Bcl-2

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Signature _____ Date 2004-11-03

For my father

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LIST OF PAPERS

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

I Karlsson R, Engström M, Jönsson M, Karlberg P, Pronk C.J.H, Richter J, Jönsson JI. 2003. Phosphatidylinositol 3-kinase is essential for kit ligand mediated survival, whereas interleukin-3 and flt3 ligand induce expression of anti-apoptotic *Bcl-2* family genes. *J. Leukoc. Biol.* 74:923-931.

II Engström M, Karlsson R, Jönsson JI. 2003. Inactivation of the forkhead family transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by kit ligand. *Exp. Hematol.* 31:316-323.

III Jönsson M, Engström M, Jönsson JI. 2004. FLT3 ligand regulates apoptosis through AKT-dependent inactivation of transcription factor FoxO3. *Biochem. Biophys. Res. Commun.* 318:899-903.

IV Engström M, Karlsson R, Jönsson M, Jönsson JI. Cooperativity between Akt and Bcl-2 leads to prolonged anti-apoptotic signaling and cell survival but only to short-term cell cycle progression. Submitted.

V Engström M, Hummerdal P, Jönsson JI. Flt3-ITD induces factor-independent cell survival by activation of the PI3-kinase/Akt pathway and upregulation of anti-apoptotic Bcl-2 family members. Manuscript.

ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
AGM	aorta-gonad-mesonephros
AML	acute myeloid leukemia
BH	bcl-2 homology
BM	bone marrow
CD	cluster of differentiation
CFU	colony forming unit
CFU-GEMM	CFU-granulocyte/erythrocyte/megakaryocyte/macrophage
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSF	colony stimulating factor
DISC	death-initiating signaling complex
E	embryonic day
Epo	erythropoietin
ER	estrogen receptor
FACS	fluorescence activated cell sorter
FL	flt3 ligand
Flt3	fms-like tyrosine kinase 3
FLIP	flice-inhibitory protein
Fox	forkhead box
G-CSF	granulocyte-CSF
GM-CSF	granulocyte-macrophage-CSF
GMP	granulocyte/monocyte progenitor
GSK	glycogen synthase kinase
HPP-CFC	high proliferative potential colony forming cell
HSC	hematopoietic stem cell
IFN	interferon
IL	interleukin
ITD	internal tandem duplication

Jak	janus kinases
JM	juxta-membrane
KL	kit ligand
LTC-IC	long-term culture-initiating cell
LT-HSC	long-term repopulating HSC
MACS	magnetic activated cell sorter
MAP	mitogen-activated protein
MEP	megakaryocytic/erythroid progenitor
MKP	megakaryocyte-committed progenitor
MNC	mononuclear cell
MPP	multipotent progenitor
NK	natural killer
NOD/SCID	non-obese diabetic / severe combined immuno-deficient
PAS	para aortic splanchnopleura
PDK	3´-phosphoinositide-dependent kinase
PH	pleckstrin-homology
PI	phosphatidylinositol
PKB	protein kinase B
PTEN	phosphatase and tensin homologue
Rb	retinoblastoma
RTK	receptor tyrosine kinase
SGK	serum and glucocorticoid inducible kinase
SH	src homology
SHIP	src-homology-2 containing inositol 5´-phosphatase
Stat	signal transducers and activators of transcription
ST-HSC	short-term repopulating HSC
TNF	tumor necrosis factor
Tpo	thrombopoietin
wt	wild type

INTRODUCTION

The blood contains a large number of mature specialized blood cells with different functions. Red blood cells (erythrocytes) supply tissues with oxygen, platelets (thrombocytes) induce clotting and repair damaged blood vessels, and white blood cells (granulocytes and lymphocytes) protect us from foreign invaders such as bacteria and viral infections. Most of these cells are rather short-lived and we therefore need a constant supply of as much as 10^{12} new cells every single day to survive.[1] Hematopoiesis refers to the generation and development of mature specialized cells, and all mature blood cells are derived from a common cell type, the multipotent hematopoietic stem cell (HSC). These are located mainly in the bone marrow where they are tightly regulated by the availability of growth factors, cytokines, and interactions with other cells.[2] Cell division and maturation of HSCs are balanced by programmed cell death, apoptosis, leading to maintenance of a steady state of hematopoiesis. Several cytokines have been demonstrated to have anti-apoptotic abilities, among which especially two are of great importance for survival of HSCs, Kit ligand (KL) and Flt3 ligand (FL). In this thesis, the signaling pathways mediated via these two cytokines have been studied with special emphasis on anti-apoptotic pathways.

HSCs are being extensively studied and nowadays HSC transplantation is routinely used in the clinic. An increased knowledge of how stem cells are regulated, and development of methods to culture and expand them in vitro, will lead to improvements of bone marrow transplantations and stem cell-based gene therapy.

GENERAL BACKGROUND

Hematopoiesis

In an adult individual there is a continous production of mature blood cells all generated from highly proliferative multipotent hematopoietic stem cells (HSCs) located in the bone marrow (BM) cavities.[3,4] Blood cells are produced via stepwise maturation, through intermediate oligo-, bi-, and uni-potent progenitor cells.[4] Whether this occurs as a result of exogenous signals, such as cytokines (instructive model), or intrinsic signals permitting survival of already commited progenitors (permissive model), remains unclear,[5,6] but probably involves both models. Progenitor cells become increasingly restricted to a specific lineage and at the same time their proliferative potential decreases. Differentiation is considered to be a linear process. Once a cell has made a developmental choice it cannot revert and at each decision point during differentiation, genes associated with a specific lineage are upregulated whereas other genes are silenced.

HSCs can be separated into long-term (LT) HSCs, capable of long-term reconstitution of hematopoiesis for the entire life of a recipient, and short-term (ST) HSCs, only capable of reconstitution for a limited period of time. LT-HSCs are thought to be the predecessor of ST-HSCs which in turn generate multipotent progenitors (MPPs).[7] MPPs are capable of differentiation either along the myeloid lineage into a common myeloid progenitor (CMP) or along the lymphoid pathway to a common lymphoid progenitor (CLP).[8,9] CMPs differentiate further into either granulocyte/monocyte progenitors (GMPs), yielding granulocytes and monocytes, or into megakaryocytic/erythroid progenitors (MEPs), generating thrombocytes, and erythrocytes,[8] whereas CLPs differentiate via intermediate progenitors into B- or T-lymphocytes, or natural killer (NK) cells[9] (figure 1).

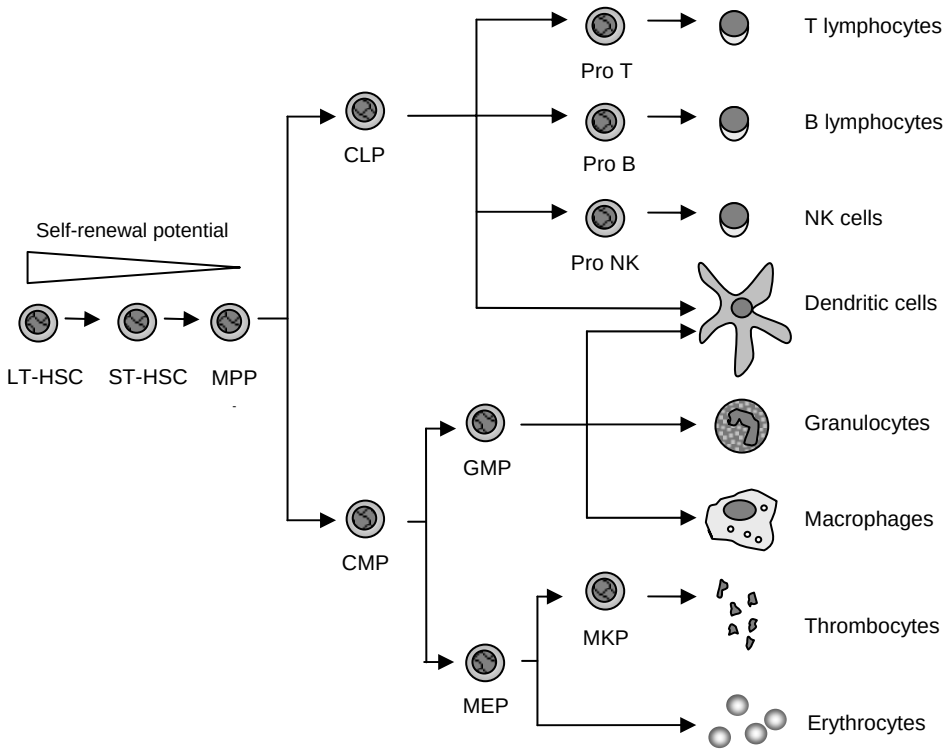

Figure 1. *The hematopoietic tree. (Modified from Reya et.al.[10])*

Development of the hematopoietic system

In the mouse embryo, the hematopoietic system is generated by cells migrating between varying hematopoietic tissues during development. Primitive hematopoiesis leads to production of large, nucleated erythroblasts, some megakaryocytes, and primitive macrophages,[11-13] whereas definitive hematopoiesis results in the production of all hematopoietic lineages. The major site of primitive hematopoiesis is the blood islands, which are derived on embryonic day 7 (E7) as ventral mesoderm migrates to the yolk sac. At E7.5 the first visible erythrocytes form in the center of the blood islands, surrounded by developing endothelial cells. Since these cells arise in close association, it has been suggested that they may descend from a common mesodermal progenitor, the hemangioblast.[14] At E8.5 the yolk sac circulation becomes directly linked to the embryo.[15]

HSCs with the potential of definitive hematopoiesis are also produced in the mature yolk sac,[16,17] which may seed intraembryonic tissues, such as liver and large arteries. Hematopoiesis continues in the yolk sac until approximately E13, when it starts to degrade. From E7.5 hematopoietic cells can also be found in the para aortic splanchnopleura (PAS)/aorta-gonad-mesonephros (AGM) region, in the trunkal and abdominal region within the mouse embryo.[15,18] PAS refers to this tissue at early stages of development whereas AGM defines this area when organogenesis has proceeded. The PAS/AGM region is the primary generator of definitive hematopoeitic stem and progenitor cells.[15,18] The AGM functions as a hematopoietic site until E11/E12 when it starts to degenerate. At midgestation, hematopoiesis shift to the fetal liver.[18,19] Erythrocytes and hematopoietic progenitors start colonizing the fetal liver at E9, and by E10.5 and E11 it is colonized by definitive hematopoeitic progenitors and stem cells.[18-20] On E16/E17 fetal liver HSCs migrate to the BM and around the time of birth the BM becomes the major site of hematopoiesis and remains so throughout life.[21]

Hematopoietic stem cells

HSCs are relatively few in number, but still they generate the enormous amount of cells produced every day in an adult individual. Stem cells contain the unique ability to produce identical daughter stem cells, a process called self-renewal. The hallmark of stem cells is their ability to balance self-renewal and differentiation and their ability to reconstitute lifelong hematopoiesis in transplanted hosts. HSCs reside in the BM at a frequency of approximately 1 stem cell per 10.000-100.000 bone marrow cells.[22] The BM provides a unique microenvironment for the stem cells, where the matrix juxtaposes stem and progenitor cells with cytokine producing stroma cells. Location seems to be of importance in controling hematopoiesis, with more immature cells located adjacent to the bone whereas the more differentiated cells reside closer to the sinuses of BM.[23] The HSC pool is also regulated by the availability of cytokines, either membrane bound or secreted as soluble factors.

Most HSCs in the BM have been considered to be maintained in a resting quiescent state, however BrdU incorporation assays have indicated that they divide slowly, completing approximately one cell division every second month.[24,25] Asymmetric cell division of HSCs generates one identical stem cell and one more mature cell. This is sufficient to sustain hematopoiesis and differentiation of mature cells necessary throughout life. Symmetric cell division generates two identical daughter cells. During development the number of stem cells increases due to symmetric cell division favoring stem cell expansion. In the case of an injury, HSCs may shift to symmetric division favoring differentiation to replace lost cells.[26] Every day an enormous number of cells are produced, exceeding the number of HSCs that are actually needed for steady state, and excessive cells may die by apoptosis.[27-29] Low numbers of HSCs, capable of re-engraftment, can be found in the peripheral blood of an animal.[30] The reason for this is not known but they could represent a source of rapidly recruitable stem cells in the case of hematopoietic stress.

Sources for hematopoietic stem and progenitor cells

When studying hematopoietic stem and progenitor cells in mice, BM derived from femur and tibiae is most commonly used as a source whereas in humans, BM derived from the pelvic bone is the main source of stem cells for BM transplantations. HSCs can be mobilized by systemic treatment with certain cytotoxic drugs or cytokines, either in combination or acting alone, which increases the frequency of HSCs in peripheral blood.[31-33] Prior to hematopoietic stem cell transplantation, systemic treatment of the donor with granulocyte-colony stimulating factor (G-CSF) is commonly used,[33] leading to HSC mobilization. Hematopoietic stem and progenitor cells are then isolated from peripheral blood and infused into the recipient. Another source of HSC is umbilical cord blood. About the time of birth, much of HSCs are located in the circulation due to homing to the BM via the blood. Isolation of stem cells from cord blood is primarily used for treatment of children. The risk for graft versus host disease, a condition where the donor T cells raises an immune response against host cells, is decreased because of the immaturity of the T cells in umbilical cord blood. Another advantage is the easy access. In addition HSCs

from umbilical cord blood is more effective than BM-derived HSCs in reconstituting hematopoiesis in NOD/SCID (non-obese diabetic/severe combined immuno-deficient) mice.[34]

Expansion of the hematopoietic stem cell pool

Ex vivo expansion of HSCs is desireable to optimize and develop HSC transplantation. In vitro studies have shown that certain cytokines are more effective than others in expanding LT-HSCs, either in combination or acting alone, among which are KL, FL, thrombopoietin (Tpo), interleukin-3 (IL-3), and IL-11.[35-40] Unfortunately, these responses often lead to induction of differentiation of HSCs. A few candidate mediators of self-renewal have been identified. The homeobox proteins HoxB4[41-43] and HoxA9[44] have been shown to have potential to expand HSC in vitro and in vivo. Notch and Wnt signaling pathways have also been shown to play important roles in HSC self-renewal,[45-47] and expression of telomerase and $p21^{Cip1/Waf1}$ seems to be crucial to avoid exhaustion of the HSC pool during serial transplantation assays.[48,49]

Transdifferentiation of hematopoietic stem cells

During the last couple of years researchers have investigated transdifferentiation, the potential for a stem cell from one given tissue to differentiate into cell types of other tissues. Cells from BM gave rise to a number of cell types such as neural cells, cardiac muscle, and hepatic cells.[50-53] This is a compelling area with the potential for treatment of various diseases with fairly easy accesible BM-derived stem cells, but many of these results can be explained by other mechanisms than transdifferentiation, such as cell fusion, where bone marrow derived cells adopt the phenotype of the recipient cells.[54] Recent evidence suggests that apparently pluripotent stem cells, which may contribute to nearly every tissue in the body can be isolated from adult BM.[55] The identification of such cells would be of great use, especially in terms of development of therapies for various diseases.

Isolation of hematopoietic stem and progenitor cells

The low frequency and difficulty to identify HSCs have made isolation of HSCs a challenging task. One common technique to enrich for hematopoietic stem and progenitor cells is density gradient centrifugation. Since hematopoeitic cells vary in size and density, it is possible to remove denser, more mature cells such as erythrocytes and granulocytes. This is usually followed by additional purification procedures such as positive and negative selection for cells expressing certain surface antigens.

No single molecular marker expressed exclusively on HSCs have yet been identified, however, several cell surface markers are up- or down-regulated as HSCs and progenitor cells differentiate. Combinations of antibodies to specifically expressed surface antigens, followed by cell sorting with either magnetic activated cell sorting (MACS) or fluorescens activated cell sorting (FACS), yield populations highly enriched for hematopoietic stem and progenitor cells. Terminally differentiated hematopoietic cells express lineage specific markers, such as Ter119 (erythrocytes), Mac-1 (macrophages), Gr-1 (granulocytes), CD4 and CD8 (T lymphocytes), and B220 (B lymphocytes) in mice. Depletion, or negative selection, of cells expressing such lineage markers yields a progenitor and stem cell enriched population of Lin^- cells. Sca-1 (stem cell antigen-1) is a surface marker commonly used for enrichment of HSCs from mice. Expression of this marker in combination with low expression of Thy-1 is highly enriched for long-term repopulating cells.[56] c-Kit expressing cells from mouse BM contain all multipotent progenitors and are capable of long-term reconstitution of all blood lineages.[57-59] Several progenitor cell populations have now been identified with the use of specific surface markers, including CLPs, CMPs, MEPs, and megakaryocyte-committed progenitors (MKPs).[8,9,60] CD34 is a surface marker expressed on early hematopoietic cells in humans, and is the most commonly used marker for both research purposes and clinical use of HSCs of human origin. Concommittant depletion of $CD34^+$ cells expressing the lineage marker CD38 is a widely used method to enrich for HSCs.[61,62] However, $CD34^-$ cells within the HSC population have also been identified and seem to be a predecessor of $CD34^+$ cells suggesting that some $CD34^-$ cells comprise an even more immature population of HSCs.[63]

Other methods for isolation of HSCs include culturing cells with cytotoxic drugs and isolation of cells capable of effluxing fluorescent dyes, such as Rhodamine-123 and Hoecsht 33342.[24]

Hematopoietic stem and progenitor cell assays

In vitro assays for evaluation of the proliferative and differentiation potential of multi-, bi-, and uni-potent progenitors have been developed and are commonly used. These assays include methods to identify colony forming units (CFU) in semi solid media, such as methylcellulose or soft agar. One advantage with such a system is that cells are immobilized and the emerging colonies originate from one single cell. CFU-GEMM and CFU-Mix are colonies comprised of granulocytes, erythrocytes, monocytes, and megakaryocytes. CFU-Blast forms small colonies of blast-like cells that can differentiate into several lineages and HPP-CFC (high proliferative potential colony forming cells) represent early, probably multipotent, cells with high proliferative potential forming colonies with thousands of cells. However, since most HSCs are quiescent, they are usually not detectable with this method. By cocultivation of HSCs and progenitor cells with feeder stroma cells for 5-8 weeks, mimicking the BM microenvironment, the more primitive cells can be recruited into cell cycle, generating colony forming progeny. These cells are called long-term culture initiating cells (LTC-IC).[64,65] Extended LTC-IC allows identification of even earlier HSCs by cocultivation for an extended period of time on the feeder stroma cell layer (8-14 weeks).[66] Now these assays have been modified to include cells with myeloid, erythoid, B-lymphoid, NK cell, and dendritic cell potential.[67]

The only conclusive assay to identify HSCs is via their ability to long-term multilineage repopulate the entire hematopoietic system in a BM ablated recipient following transplantation. In mice HSCs are assayed by competitive repopulation assays and long-term engraftment is assessed by secondary and tertiary transplantations.[68] For humans artificial models have been developed where human cells are transplanted into xenogenic hosts, such as NOD/SCID mice, after which human mature hematopoietic cells can be detected in the blood of these animals. This is the most commonly used method to study

human HSCs in vivo and cells capable of engraftment are called SCID repopulating cells.[36,69]

Apoptosis

Apoptosis is a genetic process of programmed cell death leading to depletion of dysfunctional cells from the body. An apoptotic cell is easily recognized by its distinct morphology with cell shrinkage, chromatin condensation, and DNA degradation leading to release of apoptotic bodies that is removed by surrounding phagocytic cells. This controlled way of removing damaged cells plays an important role in hematopoiesis, and in the regulation of HSCs.

Apoptosis can be triggered by two different mechanisms, the intrinsic and the extrinsic apoptotic pathways, and is executed by a family of cystein proteases called caspases.[70,71] The intrinsic (mitochondrial) pathway can be induced by cellular stress, such as DNA damage, growth factor deprivation and oxidative stress, leading to increased permeability of the mitochondrial outer membrane followed by release of cytochrome c and AIF (apoptosis inducing factor).[72,73] In the cytosol, cytochrome c forms a complex with Apaf-1 and procaspase-9, forming the mitochondrial apoptosome, which leads to activation of caspase-9,[74,75] and further activation of effector caspases resulting in cell death. The Bcl-2 family of proteins are important regulators of this pathway either inducing or inhibiting apoptosis. The extrinsic apoptotic pathway is triggered by ligand binding of members of the TNF superfamily to their cognate death receptor, such as the Fas receptor.[76] This leads to recruitment and activation of a procaspase, typically procaspase-8, to the death-initiating signaling complex (DISC). The caspase becomes activated via proteolytic cleavage and can then activate downstream effector caspases, executing the apoptotic program. This pathway can be inhibited by the flice-inhibitory protein (FLIP), which can prohibit formation of the DISC.[77]

The Bcl-2 family

The proto-oncogene Bcl-2 is the founding member of this family and was originally detected in a commonly occurring translocation in B cell lymphoma.[78] This family of proteins consist of both pro- and anti-apoptotic members, and the balance between these may determine whether a cell will live or die.[79] The mechanism by which Bcl-2 family members regulate apoptosis is not fully understood but may involve formation of channels in the mitochondrial membrane, allowing release of apoptosis-inducing factors into the cytosol, thereby Bcl-2 family members determines whether the apoptosome will assemble or not.

Bcl-2 family members contain one to four conserved Bcl-2 homology motifs (BH1-4). Anti-apoptotic members (Bcl-2 like factors), such as Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1, usually contain three to four of these, which seem to be crucial for their interaction with other proteins.[80,81] Anti-apoptotic Bcl-2 family members are either cytosolic or anchored in intracellular membranes, and their pro-survival effect seems to depend on their ability to bind, and sequester, pro-apoptotic Bcl-2 proteins. Pro-apoptotic Bcl-2 proteins can be divided into two distinct groups, the Bax-like factors and the BH3 only proteins. To the first group belong, among others, Bax, Bak, and Bok, which typically contain three BH domains, BH1-3, where the BH3 domain is responsible for binding to Bcl-2 like factors.[82,83] Upon apoptotic stimuli, Bax like factors insert into mitochondrial membranes, leading to increased permeability of the mitochondrial outer membrane,[84] possibly via formation of a channel or interaction with channel-forming proteins, releasing proteins and ions to the cytosol. The BH3 only proteins share only the BH3 domain with other Bcl-2 family members, and include, among others, Bim, Bid, Bik, Bad, and Hrk. BH3 only factors interact with Bcl-2 like proteins, leading to release of Bax-like factors, which can then induce apoptosis.[85] In addition to transcriptional regulation, BH3 only proteins can also be regulated post-transcriptionally. For instance, inhibitory phosphorylation of Bad may occur via either the phosphatidylinositol (PI) 3-kinase/Akt pathway[86,87] or the mitogen-activated protein (MAP) -kinase pathway.[88] This leads to sequestration of Bad in the cytosol by 14-3-3 proteins, thereby inhibiting apoptosis. The BH3 only protein Bid

integrates the death receptor pathway with the mitochondrial pathway. Some activated caspases, such as caspase-8, can cleave Bid to a truncated form, tBid, targeting it to the mitochondria where it associates with Bcl-2-like proteins, inducing apoptosis.[89,90]

Bcl-2 family members in the hematopoietic system

Transgenic expression of Bcl-2 in mice increases survival and repopulation of HSCs, protects cells from irradiation induced apoptosis and enhances resistance to chemotherapeutic drugs.[27,28,91,92] Mice deficient for Bcl-2 complete embryonic development, but usually die young. Hematopoiesis in these mice are initially normal followed by massive cell death of lymphoid cells.[93] HSCs from adult BM of Bcl-2 deficient mice can give rise to long-term reconstitution of myeloid lineages but not the lymphoid lineages,[94] suggesting that Bcl-2 is important for lymphoid development but not myeloid development and stem cells. Although HSCs seem to express low levels of Bcl-2, Bcl-x_L is more abundantly expressed and appears to be more important for their survival,[95-97] and deficiency of Bcl-x_L in mice is embryonic lethal, with massive cell death of immature hematopoietic cells.[98] Mcl-1 is expressed in HSCs from BM and mobilized peripheral blood,[99] and transgenic expression of Mcl-1 in mice leads to improved hematopoietic cell survival of both lymphoid and myeloid lineages.[100] Anti-apoptotic Bcl-2 family members are often up-regulated in response to cytokines, for instance KL have been shown to upregulate Bcl-2 expression in NK cells,[101] and Bcl-2 and Bcl-x_L in erythroid progenitors.[102] Among pro-apoptotic Bcl-2 family, especially Bim seems important for hematopoeisis, since mice deficient for this protein exhibit abnormal hematopoiesis and autoimmunity.[103] IL-3 induces MAP-kinase dependent phosphorylation of Bim in hematopoietic cells, leading to reduction of interaction between Bim and Bax, thus promoting survival.[104] Hrk, another pro-apoptotic Bcl-2 family member is also upregulated in hematopoietic progenitors after growth factor withdrawal[105] and Bad becomes phosphorylated in response to IL-3,[87] however the relevance of this in hematopoietic cells remains unclear.[106]

CYTOKINE RECEPTOR SIGNALING

Cytokines and their receptors

The fate of hematopoietic stem and progenitor cells is determined by the microenvironment in which they reside, and one important factor is the availability of cytokines. Cytokines are low molecular weight glycoproteins, that can be either secreted or membrane bound, generally acting locally in an autocine or paracrine fashion, and include the colony stimulating factors (CSFs), interleukins (ILs), interferons (IFNs), and tumor necrosis factors (TNFs). Most hematopoeitic cytokines are produced by BM stroma cells, and are constantly being generated at low levels, maintaining basal hematopoiesis. Upon hematopoietic stress, increased cytokine production can be induced by different stimuli, increasing blood cell formation. Cytokines can be divided into stimulatory and inhibitory factors, and they can be redundant, pleiotropic, synergistic, additive, or antagonistic in their effects. Cytokines comprise a network of factors with complex regulatory mechanisms. Some cytokines can directly affect target cells whereas others can induce the expression of certain cytokines.[107] The effects of cytokines have been debated, and it has been suggested that the function of cytokines is merely to keep cells alive and to induce proliferation, allowing proper differentiation of the cell via an intrinsic programme, whereas others mean that differentiation towards a specific lineage is induced by specific cytokines.[5,6] The truth probably lies somewhere in between where some cytokines seem to have the capacity to induce differentiation, whereas others promote proliferation and survival. Some factors promote survival of HSCs, but as single factors these cytokines seem to have little effect, merely promoting survival. However, in combination with other cytokines they can induce proliferation of early HSCs. KL, FL and Tpo are examples of such factors.[108-112]

The cytokine receptor family includes receptors for CSFs and ILs.[113] Most cytokine receptors form homo- or heterodimers, and sometimes heterotrimers, comprised of a α-, β- and/or a γ-subunit. The α-subunit is usually cytokine specific, leading to recruitment of the β-subunit or the γ-subunit, which stabilizes the complex. Their intracellular domain lacks intrinsic tyrosine kinase activity, but upon activation cytoplasmic kinases dock to the

receptor, transmitting signals to the cell interior. Receptor tyrosine kinases (RTKs) are a large family of receptors, which consist of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain containing intrinsic kinase activity.

Class III receptor tyrosine kinases

This family of receptors includes the c-Kit, Flt3, c-Fms, and PDGFα and β receptors.[114] The extracellular domain consists of five immunoglobulin like domains. The three N-terminal immunoglobulin domains mediate ligand binding whereas the fourth is involved in dimerization of the receptor. Class III RTKs contain one single transmembrane domain, and the intracellular domain has intrinsic tyrosine kinase activity, and inbetween these lies the juxta-membrane (JM) domain. The tyrosine kinase domain in the cytoplasmic region contains a kinase insert sequence dividing the cytoplasmic kinase domain into ATP-binding and phosphotransferase domains. Ligand binding induces homodimerization followed by autophosphorylation of the receptor and initiation of intracellular signaling cascades. All members of this family of receptors influence normal hematopoiesis.[114] In this study we have studied two class III RTKs, c-Kit and Flt3.

c-Kit and Kit ligand

c-Kit was first discovered as a cell surface marker on acute myeloid leukemia (AML) recognizable by a monoclonal antibody,[115] which later turned out to be the c-Kit receptor.[116] c-Kit maps to the white spotting (W) locus in mice,[117,118] mutations in which gave mice a characteristic phenotype including lack of hair pigmentation (white spots), anemia and mast cell deficiencies. A similar phenotype was observed with mutations at the Steel locus (Sl), which was shown to encode the gene for the ligand to c-Kit, Kit ligand (KL, also stem cell factor, steel factor and mast cell growth factor), cloned simultaneously by three different groups in 1990.[119-121] Lack of c-Kit or KL, results in death in utero or perinatally, with severe macrocytic anemia in mice.[122] This indicates an essential role for c-Kit in embryogenesis, and expression of c-Kit have been demonstrated on definitive HSCs from the AGM region in the developing fetus.[123]

c-Kit is expressed on both hematopoietic and non-hematopoietic cells. In hematopoietic cells the expression is highest in the stem and progenitor cell compartment, including LT-HSCs, CLPs, and CMPs,[8,9,124,125] and is required for survival, proliferation, and differentiation of early hematopoietic stem and progenitor cells. c-Kit is downregulated upon maturation of all hematopoietic lineages except mast cells which retain high levels of expression. Signaling via c-Kit involves several signal transduction pathways including PI3-kinase, the MAP-kinase cascade, members of the janus activated kinases/signal transducers and activators of transcription (Jak/Stat) pathway, and Src family members.[126] KL is generated in both membrane bound and soluble form, both of which are biologically active.[127] KL is constitutively produced by endothelial cells and fibroblasts, either presented on the cell surface or in soluble form,[128,129] and KL mRNA can also be detected in the stem and progenitor compartment of the hematopoietic system.[130] KL affects many cells within the hematopoietic system including stem and progenitor cells, precursors, and some mature cells. Acting alone KL mediates survival without proliferation,[108,109] but in combination with other cytokines KL may mediate self-renewal of HSCs and progenitor cells.[35-37,40] In vivo self-renewal of HSCs and progenitor cells is in part dependent on KL,[131] and ex vivo culturing of BM derived cells in the presence of KL and IL-11 supported quaternary BM transplantation, compared to only secondary BM transplantation of cells that had not been expanded.[132]

c-Kit has been associated with several forms of cancers, including gastrointestinal tumors, small cell carcinomas of the lung, and breast, cervical, and ovarian tumors. Mutations in the extracellular domain of c-Kit have been described in myeloproliferative disorders and AML.[133,134] Point mutations in the catalytic domain at position Asp816 are commonly observed in many cases of mastocytosis and certain subtypes of AML,[135-137] and c-Kit is expressed in as many as 60-90% of cases of AML cases.[138]

Flt3 and Flt3 ligand

Isolation of the Fms-like tyrosine kinase 3 (Flt3, also fetal liver kinase 2, Flk2) receptor was achieved simultaneously by two different groups.[139-141] Flt3 belongs to the class III

RTK family and is expressed on multipotent HSCs and progenitor cells[142] as well as on early lymphoid and myeloid progenitor cells,[143] but not on erythroid cells, megakaryocytes, or mast cells.[144-146] LT-HSCs do not express Flt3 and upregulation of the receptor within the stem cell compartment is correlated with loss of self-renewal capacity and commitment to the lymphoid pathway.[147,148] The receptor is an important regulator of hematopoietic stem and progenitor cells, since the repopulation potential of BM cells derived from Flt3 deficient mice is reduced compared to BM from wildtype (wt) mice. Otherwise Flt3 deficient mice appeared healthy, except for reduced numbers of pro B cells.[149] Activation of the Flt3 receptor via Flt3 ligand leads to recruitment and activation of several signaling molecules including PI3-kinase, Ras, PLCγ, Vav, Shc, Grb2, Cbl, and Stat5a.[150-152]

Flt3 ligand (FL) was cloned 1993,[153] and is expressed as a transmembrane protein, or as a soluble factor released by proteolytic cleavage of the membrane bound form, or alternative splicing of mRNA.[154,155] FL is expressed by a great number of tissues and cell types including fibroblasts and endothelial cells in the BM compartment,[156,157] and most immortalized hematopoietic cell lines express FL.[158,159] Disruption of FL in mice leads to deficient hematopoiesis affecting B cell progenitors, dendritic cells, and NK cells in vivo,[160] and administration of FL in vivo leads to increased numbers of dendritic cells.[161] FL can induce survival of hematopoietic progenitor cells without proliferation,[110,162] and can induce development of dendritic cells from myeloid and lymphoid progenitors in vivo.[163] In synergy with other cytokines FL may affect HSC self-renewal,[38,164] i.e., a combination of FL, KL, and IL-3 expand murine long-term reconstituting activity as well as human LTC-IC and extended LTC-IC.[165] In addition, an increased amount of SCID repopulating cells was seen with different combinations of FL with KL, IL-3, IL-6, and G-CSF[36,166].

Flt3 has gained a lot of interest during the last couple of years especially since it has been shown to be mutated in some cases of hematopoietic malignancies. As much as 30% of all AML cases carry an internal tandem duplication (ITD) in the JM domain of the receptor leading to FL independent receptor dimerization and activation,[167,168] and mutations in the

activation loop of Flt3 (Asp835) have been observed in several cases of AML.[169,170]Insertions of ITDs in the JM domain lead to instability, disrupting its autoinhibitory capacity on the receptor.[171-173] ITD mutations in Flt3 have transforming abilities on factor-dependent hematopoietic cells, and may result in activation of Stat3 and 5, Ras, MAPK, PI3-kinase/Akt, upregulation of anti-apoptotic Bcl-x_L and inhibitory phosphorylation of pro-apoptotic Bad.[174-177] Block of differentiation may be an alternative way by which Flt3-ITD excerts its transforming abilities, and block of myeloid differentiation by Flt3-ITD is reversed when inhibiting the activity of Flt3.[178]

c-Kit and Flt3 are closely related, as are their cytokines. Still they seem to have unique effects, since deficiencies in c-Kit results in death in utero whereas Flt3 deficient mice are healthy, except for a reduced number of pro B cells and decreased repopulation potential of HSCs.[122,149] KL promotes growth of mast cells, megakaryocytes, and erythrocytes, whereas FL does not.[144,145,179] On the other hand, FL seems to expand B cells, NK cells, and dendritic cells.[110,160,163] c-Kit is more highly expressed in the stem cell compartment than Flt3, and repopulating HSCs are highly enriched in the c-Kit^+ population of BM.[57-59] Expression of Flt3 on the other hand is associated with reduced repopulation potential.[147,148] If this is an effect of Flt3 signaling is not known but these differences may reflect activation of different signaling pathways. Alternatively, since c-Kit and Flt3 are expressed on different cell types, this may explain some of the differences.

Src kinases

Constitutive activation of Src kinases leads to cellular transformation, affecting several cellular processes including cell cycle progression and differentiation. Besides a kinase domain, Src kinases contain a Src homology (SH) 2 domain, a SH3 domain, and one domain that is unique for each Src family member. SH2 domains recognize sequences with phosphorylated tyrosine residues and associate with these, whereas SH3 domains bind specifically to proline-rich sequences.[180] In the C-terminal part of Src kinases, a conserved tyrosine residue is critical for regulation of the kinase activity, dephosphorylation of which

results in catalytic activity. This regulatory residue can be phosphorylated by kinases such as Csk (c-src kinase), thereby inactivating Src activity.[180] Src kinases are activated in response to signaling via several cytokine receptors, including RTKs.[181] Activation of receptors leads to phosphorylation of intracellular tyrosine residues, either within the receptor, or on associated tyrosine kinases. This leads to recruitment of Src family members via their SH2 domain, which can further recruit and activate a second level of signaling molecules, including Ras, PI3-kinase, and Stats, either directly or via adaptor molecules. The Src kinase family consists of eight members, five of which are mainly expressed in hematopoeitic cells, Blk, Hck, Lck, Fyn, and Lyn.[180]

Activation of Src kinases is implicated in proliferation. Src and Fyn associate with the PDGF receptor in vivo and induce progression through cell cycle. Microinjection with either catalytically inactive Src kinases, or specific antibodies for these, inhibited PDGF-dependent entry into S-phase.[182] The proliferative block induced by inhibition of Src kinases could be rescued by overexpression of c-Myc, suggesting that Src kinase activity is required for expression of c-Myc and concomittant progression through cell cycle.[183] Lyn has been found to be important for signaling via c-Kit, and influences KL-mediated proliferation and chemotaxis as well as c-Kit receptor trafficking.[184-187] Lyn associates with the JM region of c-Kit and addition of KL induces tyrosine phosphorylation and activation of Lyn. In addition, Lyn antisense as well as a Src-kinase inhibitor decreased KL induced proliferation remarkably.[185] After stimulation with KL, Lyn kinase activity peaks in the G_1/S transition of cell cycle, and Lyn associates with the cell cycle regulator cdk2 (cycline dependent kinase 2).[186] Moreover, proliferation of hematopoietic progenitors from Lyn deficient mice in response to KL was reduced compared to wt mice.[187]

Janus kinases

Janus kinases (Jaks) are non-receptor tyrosine kinses and their primary function seems to be to phosphorylate cytokine and growth factor receptors. This family consists of four members, Jak1, Jak2, Jak3 and Tyk2, which phosphorylates Signal transducers and activator of transcription (Stats) leading to their activation. Jak1, Jak2, and Tyk2 are

expressed ubiquitously whereas Jak3 is restricted to the myeloid and lymphoid lineages.[188] Most cytokine receptors signal via Jak kinases which are constitutively associated with receptors.[189] Cytokine binding leads to dimerization of the receptor, juxtaposing associated Jaks, enabling trans-phosphorylation and activation. This leads to Jak dependent phosphorylation of specific tyrosine residues within the receptor, creating SH2 docking sites for Stats and other signaling molecules.[189] Jak family members integrate components of diverse signal transduction pathways, and are required for optimal activation of Src-kinases, the Ras-MAP-kinase pathway, the PI3-kinase pathway, and Stat signaling, following cytokine receptor activation. Overactivation of Jaks has been implicated in tumorigenesis, and loss of Jak kinase function can result in diseases such as severe combined immunodeficiency.[190]

Jak2 and c-Kit are constitutively associated in human and murine cell lines, and in human progenitor cells, and KL induces activation of Jak2.[191] In addition, Jak2 antisense markedly decreased KL mediated proliferation.[191] Constitutively active Flt3 may involve signaling via Jak2.[192] However, another group studying normal Flt3 found that no Jak family members were phosphorylated in response to FL.[152] Jak2 might also be important for HSC self-renewal, since in combination with either KL or FL Jak2 was able to support self-renewal of multipotent progenitor cells.[193]

Signal transducers and activators of transcription

Signal transducers and activators of transcription (Stats) transmit signals from several cytokines. This group of transcription factors contains a DNA binding domain followed by a SH3-like domain, a SH2 domain, and a C-terminal transactivating domain. Upon receptor activation cytoplasmic Stats are recruited to SH2 docking sites in the receptor, where it becomes phosphorylated by Jaks or Src kinases on a conserved tyrosine residue. When phosphorylated Stats dissociate from the receptor and form homo- or hetero-dimers with other Stats, they translocate to the nucleus and activate transcription of target genes.[189] c-Kit associates with and induces tyrosine phophorylation of Stat1 in response to KL.[194] In addition, mutations of Asp816 in c-Kit leading to KL-independent activation of

the receptor, leads to constitutive activation of Stat1 and Stat3, and inhibition of Stat3 led to suppression of KL-independent survival and proliferation of human leukemia cells.[195] Stat3 has been found to be required for dendritic cell formation by FL.[196] Regulation of Stat family members seem to differ significantly between wt Flt3 receptor and Flt3 receptors with mutations rendering them constitutively active. In patients with AML, Flt3-ITD mutations are associated with activation of Stat5.[174,197] However, another study failed to demonstrate any association between Flt3-ITD expression and Stat5 phosphorylation.[198] Constitutively active Flt3 may involve signaling via Jak2, Stat3, Stat5a, and Stat5b,[174,192] whereas no Jak family members, and only Stat5a was activated by normal Flt3.[152] Interestingly, HSCs from Stat5 deficient mice exhibit defects in long-term repopulation activity.[199]

The Jak/Stat pathway regulates many different cellular processes such as proliferation, survival, and differentiation, and regulation of Bcl-2 family members seems to play an important role in anti-apoptosis via Stat family members. The anti-apoptotic Bcl-2 family member Mcl-1 is upregulated via IL-6 in human myeloma cells, and this was dependent of both Jak2 and Stat3 activity.[200] In neutrophils, stimulation with granulocyte-macrophage (GM)-CSF upregulates Mcl-1 expression through mechanisms involving both Stat3 and PI3-kinase.[201] Erythropoietin (Epo) inhibited apoptosis in hematopoietic progenitor cells, through activation of Stat5 and subsequent Stat5 dependent upregulation of Bcl-x_L.[202] Dominant negative Stat5 sensitized BaF3 cells to apoptosis via decreased Bcl-x_L expression, whereas Bcl-2 levels where unchanged,[203] and constitutively active Stat5 transformed the murine factor-dependent cell line BaF3 in a Pim1 (a serine/threonine kinase associated with proliferation) and Bcl-x_L dependent manner.[204]

Mitogen activated protein kinases

This family of kinases includes three major groups, the Erk family, the p38 MAP-kinase family, and the JNK family. The Erk family consists of Erk1 and Erk2 and primarily regulates cell growth and survival in response to cytokine signaling, whereas the stress-induced activation of JNKs and p38 MAP-kinases is primarily pro-apoptotic and growth

inhibitory.[205] Interestingly, p38 MAP-kinases can under certain circumstances promote survival and cell growth

Activation of this pathway occurs via a series of events culminating in the activation of MAP-kinases (MAPK), via dual phosphorylation of specific residues located in the activation loop, by MAPK-kinases (MAPKKs, or MEKs).[206] These are in turn activated by upstream kinases, the MAPKK-kinases (MAPKKKs). Several serine/threonine kinases act as MAPKKKs, including Raf for Erk family members and MEKK-kinases for JNK and p38 MAP-kinase family members. MAPKKKs are activated by small G proteins regulated by guanine exchange factor proteins, and include Ras for the Erk pathway and Rho family members for JNKs and p38 MAP-kinases.[206] Ras becomes activated when autophosphorylation of a receptor creates docking sites for SH2 containing adaptor proteins such as Shc and Grb2, leading to recruitment of Sos, a Ras guanine nucleotide exchange factor, to the plasma membrane, where it can convert Ras from an inactive GDP-bound form to an active GTP-bound form.[126] Activation of MAP-kinases ultimately leads to phosphorylation of specific substrates on serine and threonine residues, which regulates their activity positively or negatively.

Activation of the Raf/Mek/Erk pathway can abrogate cytokine dependence of some cell lines, possibly via induction of growth factors creating an autocrine loop.[207,208] In addition, the FDC-P1 cell line overexpressing constitutively active Raf1 was able to induce tumors in immuno-compromised mice.[208] Expression of Bcl-2, PI3-kinase, or Akt in combination with active Mek1 leads to abrogation of cytokine dependence of hematopoietic cell lines, suggesting that the Mek/Erk pathway may act in synergy with other pathways to induce leukemogenesis.[209,210] Ligand induced activation of c-Kit leads to interaction with several regulatory proteins for Ras/Raf/MAP kinase cascade, including Grb2 and Shc,[211] and c-Kit activation also activates Ras and Erk.[212-214] Interestingly, a recent report demonstrated that c-Kit-dependent activation of the Raf/Mek/Erk pathway, but not Ras, requires PI3-kinase activity in hematopoietic stem and progenitor cell lines, whereas more committed progenitor cells did not exhibit such PI3-kinase dependence.[215] Moreover, both normal Flt3 and Flt3-ITD induce activation of Erk1 and Erk2,[174,216] and in clinical samples from

patients with AML there was a correlation between mutated Flt3 and constitutive activation of Erk.[174]

Phosphatidylinositol 3-kinase

PI3-kinase is one of the major pathways activated upon cell surface receptor activation, especially those with tyrosine kinase activity. Activation of this pathway leads to the production of phosphatidylinositol-3 phosphorylated lipids, which are involved in the regulation of several cellular processes. The PI3-kinase pathway and downstream targets have been implicated in survival of hematopoietic cells, and several cytokine receptors are known to associate with PI3-kinase, including c-Kit.[217] Survival mediated by several cytokines, such as KL, IL-3, and IL-4, is dependent on a functional PI3-kinase pathway, as demonstrated by using the PI3-kinase specific inhibitors LY294002 and wortmannin.[218] PI3-kinases are a family of lipid kinases, and can be divided into class I, II, and III, based on sequence homology and substrate specificity.[219] Class I family members are heterodimers, composed of one catalytic (p110), and one regulatory subunit (p85), and is further subdivided into class IA and IB. The class IA family members, which are activated by growth factor receptors with tyrosine kinase activity, consist of three isoforms of the p110 subunit (α, β, and δ). p110 dimerizes with one of seven possible adaptor proteins (p85), generated by expression and alternative splicing, of three genes, p85α, p85β, and p55γ. Class IB members are activated by G-protein coupled receptors. This subgroup consists of p110γ, which differs from class IA in the N-terminal region and thereby does not interact with p85. Instead it associates with another regulatory subunit, p101.[219] Class II family members, consisting of PI3KC2α, β, and γ, contain a catalytic domain with great sequence homology to PI3 kinase class I. In addition, a C2 domain is located at the C terminus of the kinase. Human PI3KC2α is activated by insulin, and PI3KC2α and β associate with growth factor receptors such as the EGF receptor and the PDGF receptor.[220,221] Class III members share sequence homology with the p110 subunit of class I kinases, and seem to be involved in vesicle trafficking within cells. Only one mammalian class III PI3 kinase has yet been identified.[219]

Activation of growth factor receptors induces docking of PI3-kinase class IA to phosphorylated tyrosine residues, either within the receptor or on tyrosine kinases associated to the receptor, via one or two SH2 domains in the regulatory domain.[222] This induces a conformational change and activation of the catalytic domain. Located at the plasma membrane, PI3-kinase can phosphorylate the 3´-position in the inositol ring of phosphatidylinositol-4,5-bisphosphate (PI4,5P), generating PI3,4,5P, followed by generation of PI3,4P by 5´-phosphatases. PI3,4P and PI3,4,5P act as signaling intermediates regulating downstream signaling cascades and the levels of these are regulated by 3´-phosphatases such as PTEN (phosphatase and tensin homologue), and 5´-phosphatases such as SHIP (src-homology-2 containing inositol 5´-phosphatase), thus acting as negative regulators for PI3-kinase signaling.[222] PI3-kinase can also be activated by direct interaction with Ras.[223] PI3,4P and PI3,4,5P mediate specific binding to pleckstrin-homology (PH) domains, which are present in several key regulatory proteins, including 3´-phosphoinositide-dependent kinase 1 (PDK1) and Akt, both of which are central for the transforming abilities of aberrant PI3-kinase activity. Thus activation of PI3-kinase activates several signaling processes within the cell, affecting different cellular functions including inhibition of apoptosis. Akt is one downstream target of PI3-kinase that has been extensively studied, and its role in survival is well known. Other downstream targets for PI3-kinase are PKC, serum and glucocorticoid inducible kinase (SGK), and p70S6K.[224,225]

Akt / Protein kinase B

In 1991, three research groups simultaneously cloned the serine/threonine Akt (also protein kinase B, PKB).[226-228] The Akt family comprises Akt1, 2, and 3 (PKBα, β, and γ), which seem to be redundant in their functions. Akt1 is more ubiquitously expressed, compared to Akt2 and Akt3 that are restricted to certain tissues. The biological relevance of Akt kinases is supported by the fact that they are evolutionary conserved, with structural homologues in both *Drosophila melanogaster* and *Caenorhabditis elegans*.[229,230] Akt1 and Akt2 deficient mice are viable, which may be explained by redundancy among Akt family members, supported by the findings that Akt1/Akt2 deficient mice exhibit a more severe

phenotype resulting in neonathal lethality.[231] The importance of Akt is further supported by the notion that regulatory mechanisms for Akt activity is often dysregulated in malignancies, resulting in constitutive activation of Akt kinase activity. One example is that the tumor suppressor PTEN, which negatively regulates Akt activity, is frequently mutated in some human cancers,[232] and it has been shown that Akt expression may be required for the tumorigenic properties of PTEN mutations.[233] Although uncertain, Akt expression by itself does not seem to induce oncogenic transformation, but may act in combination with other pathways such as Mek1, to mediate transformation.[210]

Akt regulation

Akt contains an N-terminal PH domain, connected to the central catalytic kinase domain via a hinge region. The C-terminal domain contains a hydrophobic and a proline rich domain. Unlike PH domains in other proteins the PH domain in Akt binds to both PI3,4P and PI3,4,5P, which are generated by activated PI3-kinase, in the plasma membrane.[234] The relocation of cytoplasmic Akt and binding to PI3,4P and PI3,4,5P induce a conformational change, exposing two major phosphorylation sites, Thr308 and Ser473. Located by the plasma membrane Akt is placed in the vicinity of PDK1, which also contains a PH domain thus placing it by the inner surface of the plasma membrane. PDK1 is thought to be constitutively active and phosphorylates Akt at Thr308, stabilizing the activation loop in an open conformation. This allows access of ATP and substrate at the active site. Phosphorylation of the second phosphorylation site Ser473 is subsequently executed by a yet unidentified kinase, or via autophosphorylation, leading to full activation of Akt. The fully activated Akt is then translocated to both cytosol and nucleus where it can phosphorylate target proteins on serine and threonine residues within a Akt consensus phosphorylation sites.[234,235] Phosphorylation of two tyrosine residues near the activation loop of Akt also seems to be required for growth factor induced activation, and there are also indications that Src kinases may play an important role in Akt activation.[236] Other pathways not involving PI3-kinase, such as increases in cytoplasmic calcium levels, may also mediate activation of Akt.[237]

The involvement of Akt in survival signaling downstream of PI3-kinase has been demonstrated in several studies. Transfection of cerebellra neurons with dominant negative Akt led to decreased survival mediated via IGF-1, whereas wt Akt promoted survival even in the absence of IGF-1.[238] In response to IL-3, Akt kinase activity is rapidly increased, and overexpression of a dominant negative Akt interferes with IL-3 dependent proliferation, whereas wt Akt protects cells from apoptosis upon IL-3 withdrawal.[239] The PI3-kinase/Akt pathway can also protect cells from death receptor induced apoptosis, via TGF-β and Fas receptor activation.[240,241]

Akt targets

The first substrate identified for Akt was glycogen synthase kinase (GSK) 3, a protein involved in the regulation of glycogen storage and in signaling via the Wnt pathway. GSK3 activity was inhibited in a PI3-kinase dependent manner by phosphorylation of Akt on a N-terminal regulatory serine residue.[242] Later on it was demonstrated that active, unphosphorylated, GSK3 could trigger degradation of the cell cycle regulator $p21^{Cip1/Waf1}$, thus Akt activation leads to increased levels of $p21^{Cip1/Waf1}$ which is required for progression through cell cycle.[243] The pro-apoptotic Bcl-2 family member Bad is also a downstream target of Akt. Phosphorylation on Ser136 in Bad by Akt leads to dissociation from Bcl-x_L and sequestration of Bad by 14-3-3 proteins in the cytoplasm. This leads to release of anti-apoptotic Bcl-x_L, thus promoting survival.[86,87,244] In hematopoietic cells, however, cytokine induced activation of Akt does not seem to correlate with Bad phosphorylation.[106] Another pro-apoptotic Bcl-2 protein affected by Akt is Bax. Expression of constitutively active Akt in hematopoietic cells inhibits a conformational change of Bax and its translocation to the mitochondria, thereby maintaining mitochondrial potential and prohibiting cytochrome c release.[245] Other downstream targets for Akt phosphorylations are pro-caspase 9, phosphorylation of which leads to defective proteolytic cleavage to active caspase 9,[246] and IKK, which when phosphorylated promotes release of NFκB promoting survival via transcriptional activation of anti-apoptotic target genes, such as the anti-apoptotic Bcl-2 family member A1.[247,248]

Akt activity influences several cellular processes including metabolism, survival, and cell cycle progression. Especially two downstream targets for Akt are known to influence these processes, GSK3 and members of the Forkhead transcription factor family. In our studies we have turned our attention towards members of the FoxO subfamily of Forkhead transcription factors.

The Forkhead transcription factor family

The name originates from a two spiked-head structure in embryos of the *Drosophila melanogaster* Forkhead mutant.[249] The Forkhead box (Fox) family of transcription factors is highly conserved evolutionary and is expressed in species ranging from yeast to human. The unifying feature of this family is the Forkhead box DNA binding motif, which was first identified when comparing the DNA binding domains of the *Drosophila melanogaster* Forkhead protein and the mammalian hepatic nuclear factor-3, HNF-3.[250] This domain is highly conserved and confers a winged helix structure, a variant of the helix-turn-helix motif, whereas other parts, such as the transactivating domain, are divergent between different family members. Seventeen subclasses have been identified, of these the FoxO subfamily is the only one known so far to be regulated by the PI3-kinase/Akt pathway.[251]

Inactivation of the insulin receptor homologue, Daf-2, in *Caenorhabditis elegans*, induces dauer formation, a shift in metabolism to fat storage, and prolongs the life span of the animals. Mutations in another gene, Daf-16, suppress dauer formation, metabolic shift, and longevity, and Daf-16 is negatively regulated by Daf-2 via a mechanism dependent on the activity of AGE-1 (a PI3-kinase homologue), PDK-1 (homolog to mammalian PDK1), and Akt1/Akt2 (mammalian Akt).[252,253] This signaling pathway is conserved between worm and mammals, and the Daf-16 mammalian homologs identified are members of the FoxO subclass of Forkhead transcription factors. In mouse and human three FoxO family members FoxO1, FoxO3, and FoxO4 have been identified (also FKHR, FKHR-L1, and AFX) and in mouse a fourth family members, FoxO6, was recently identified.[254,255] Knockout mice for FoxO1, FoxO3, and FoxO4 have been generated and reveal functional diversity. FoxO1$^{-/-}$ animals were unable to complete embryonic development and did not

survive beyond E10.5, seemingly due to incomplete vascular development. FoxO3$^{-/-}$ and FoxO4$^{-/-}$ mice were viable, and whereas FoxO3$^{-/-}$ females exhibited an age-dependent reduced fertility, no obvious distinguishing phenotype was observed for FoxO4$^{-/-}$ mice.[256] FoxO proteins have been suggested to play a role in oncogenic transformation. A number of translocations involve Forkhead genes, including one in alveolar rhabdomyosarcoma resulting in fusion of the Pax3 or Pax7 DNA-binding domain with FoxO1 (FKHR) transactivating domain. The resulting chimeric protein retains the specificity conferred by Pax proteins driven by a strong transcriptional activator domain.[257] In acute lymphoid leukemia many of the translocations causing the disorder involves fusion of part of the mixed-lineage leukaemia, MLL, to the transactivating domain of another transcription factor, one of which is FoxO4.[258]

FoxO regulation

The main regulator for FoxO proteins are the PI3-kinase/Akt pathway. Mammalian FoxO1, 3, and 4 contains three highly conserved Akt phosphorylation sites. One N-terminal threonine residue, one central serine residue within the Forkhead domain, and one C-terminal serine, and impaired phosphorylation of these sites leads to increased transcriptional activity of FoxOs.[259-261] These residues have been demonstrated by several groups to be phosphorylated by the PI3-kinase/Akt pathway in response to a number of cytokines in hematopoietic cells, including Epo, Tpo, IL-2, and IL-3.[262-265] Akt inhibits FoxO transcriptional activity via control of subcellular localization. Akt enters the nucleus where it can phosphorylate FoxOs on the first two consensus Akt sites, followed by nuclear export. Export of FoxO3 depends on an intrinsic nuclear export signal sequence within the FoxO protein, 14-3-3 binding, and activity of the export receptor Crm1.[266,267] In addition, phosphorylation of the second Akt regulatory site may mask a nuclear localization signal, leading to cytoplasmic localiztion and decreased transcriptional activity.[267,268] Inhibition of the PI3-kinase/Akt pathway via treatment with specific inhibitors or overexpression of dominant negative PI3-kinase or Akt, retains FoxO in the nucleus, and activation of Akt leads to relocation of FoxOs to the cytoplasm.[269] Mutants

where the three consensus Akt phosphorylation sites have been replaced, fail to localize to the cytoplasm upon Akt activation.[269,270]

Akt-dependent phosphorylation of FoxOs not only leads to redistribution within the cell. Phosphorylation of the central serine residue within the Forkhead DNA binding domain also interrupt DNA-binding by a mechanism not involving relocation of the protein.[271] Also, phosphorylation of FoxOs by Akt targets the protein for proteasomal degradation, which has been demonstrated for FoxO1 and FoxO3 via overexpression of a constitutively active Akt, and via induction of the PI3-kinase/Akt pathway by IL-3.[272] FoxO family members are, however, not exclusively regulated by Akt. Other kinases may also phosphorylate FoxO family members. IKKβ interacts with and phosphorylates FoxOs independently of Akt activation, causing proteasome dependent degradation of the protein.[273] Serum and glucocorticoid regulated kinase (SGK), which is similar to Akt, phosphorylates the C-terminal consensus Akt phosphorylation site,[274] and two serine residues adjacent to this site can be phosphorylated by casein kinase 1, which is dependent on priming phosphorylation of the C-terminal Akt/SGK site.[275] An additional serine residue is phosphorylated by another kinase, DYRK1A, a site constantly phosphorylated, mutation of which leads to cytoplasmic retention and enhanced transcriptional activity of FoxO.[275,276] In addition, Ras/Ral signaling results in phosphorylation of two C-terminal threonine residues of FoxO4, which are essential for FoxO4 activity.[277]

FoxOs and cell cycle

Cell cycle progression relies on phosphorylation and inactivation of members of the retinoblastoma (Rb) family of nuclear pocket proteins. These bind to different members of the E2F family of transcription factors, leading to repression of genes that are required for cell cycle progression. The tumor suppressor pRb is hyperphosphorylated by cyclin/cdk complexes and thereby released from E2F leading to entry into S-phase. Inhibition of FoxO proteins by PI3-kinase/Akt is necessary for progression through the G_1/S checkpoint of the cell cycle. Unphosphorylated FoxOs activate transcription of the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$. This cell cycle inhibitor interacts with cyclinE/cdk2

complexes, which are essential for progression into S-phase, and inhibits their activity.[262,278,279] However, upregulation of $p27^{Kip1}$ is not the only cause of G_1 arrest, since FoxOs also repress transcription of cyclin D1 and D2. These cyclins are required for phosphorylation and inactivation of pRb, essential for progression through G_1.[280,281] Not only do FoxOs induce G_1 arrest but cells may also enter G_0 due to upregulation of the nuclear pocket protein p130, which is associated with quiescence.[282] However, FoxO phosphorylation must also be downregulated in G_2 to allow mitosis and re-entry into G_1. In its unphosphorylated state FoxOs upregulate transcription of the mitotic genes cyclin B and polo-like kinase, Plk, allowing progression through G_2/M into G_1.[283] Thus, in order for cell cycle to proceed, FoxO proteins must be phosphorylated in G_1 followed by dephosphorylation in G_2.

Another interesting finding is that activation of the PI3-kinase/Akt pathway potentiates the transforming abilities of Myc activation via inactivation of FoxO transcriptional activity. Constitutive activation of FoxO3 represses Myc target gene expression involved in proliferation and transformation, whereas dominant negative FoxO allows activation of Myc targets in the absence of PI3-kinase.[284] In addition, the constitutive activation of IKKβ seen in some tumors, inducing proliferation and tumorigenesis, can be reverted by FoxO3 transcriptional activity.[273]

FoxOs and apoptosis

Activation of FoxO proteins leads to cell cycle arrest in a number of cell systems, whereas in hematopoietic cells the major outcome of FoxO activation is apoptosis. Several pro-apoptotic target genes are activated by FoxO transcription factors, and the first one identified was the Fas ligand in Jurkat cells, capable of triggering death receptor induced apoptosis.[260] Another death receptor activator, TRAIL, a member of the TNF family, is also upregulated by FoxOs,[285] and more recently the caspase-8 inhibitor FLIP was shown to be downregulated by active FoxO3.[286] Interestingly, activation of Fas receptor mediated cell death leads to cleavage of FoxO3 by caspase-3 like proteases, releasing the N-terminal DNA-binding domain from the transactivating domain. In its unphsophorylated state the

N-terminal DNA-binding domain may then enter the nucleus inhibiting FoxO mediated transcription creating a negative feed-back loop.[287] Besides influencing the extrinsic apoptotic pathway FoxO3 has also been shown to activate the death receptor-independent pathway. The pro-apoptotic Bcl-2 family member Bim is upregulated in response to FoxO3 transcriptional activity,[263,288,289] and cells from $Bim^{-/-}$ mice exhibit reduced levels of apoptosis upon inhibition the PI3-kinase/Akt pathway.[288] FoxO4 can also trigger the intrinsic apoptotic pathway via upregulation of the transcriptional repressor Bcl-6, which inhibits transcription of anti-apoptotic Bcl-x_L. Thus transcriptional activation of FoxO leads to decreased levels of Bcl-x_L, further promoting apoptosis.[290] In addition, upon activation of neutrophils anti-apoptotic Mcl-1 was upregulated and found to associate with a complex containing phosphorylated FoxO proteins, which may sequester FoxO proteins further inhibiting apoptosis.[291]

FoxOs and stress

As mentioned earlier Akt protects cells from apoptosis, which has been shown to be dependent on the presence of glucose. The first step of glycolysis is sufficient for Akt mediated survival. Akt redistributes hexokinase to the mitochondria where it catalyses the conversion of glucose to glucose-6-phosphate by using intramitochondrial ATP, preventing hyperpolarization and release of cytochrome c.[292] In the absence of Akt activity, cells can be protected from oxidative stress via FoxO proteins. For instance, FoxO3 has been shown to upregulate the expression of two antioxidant enzymes, catalase and MnSOD (manganese superoxide dismutase), which protect the cell from damaging ROS (reactive oxygen species).[293,294] In addition, FoxOs upregulate Gadd45 in response to stress at the G_2/M checkpoint of the cell cycle, triggering DNA repair.[295,296] Activation of FoxOs can lead to quiescence or apoptosis. The decision to either die or enter G_0 may be detemined by the cells capability to handle cellular stress.

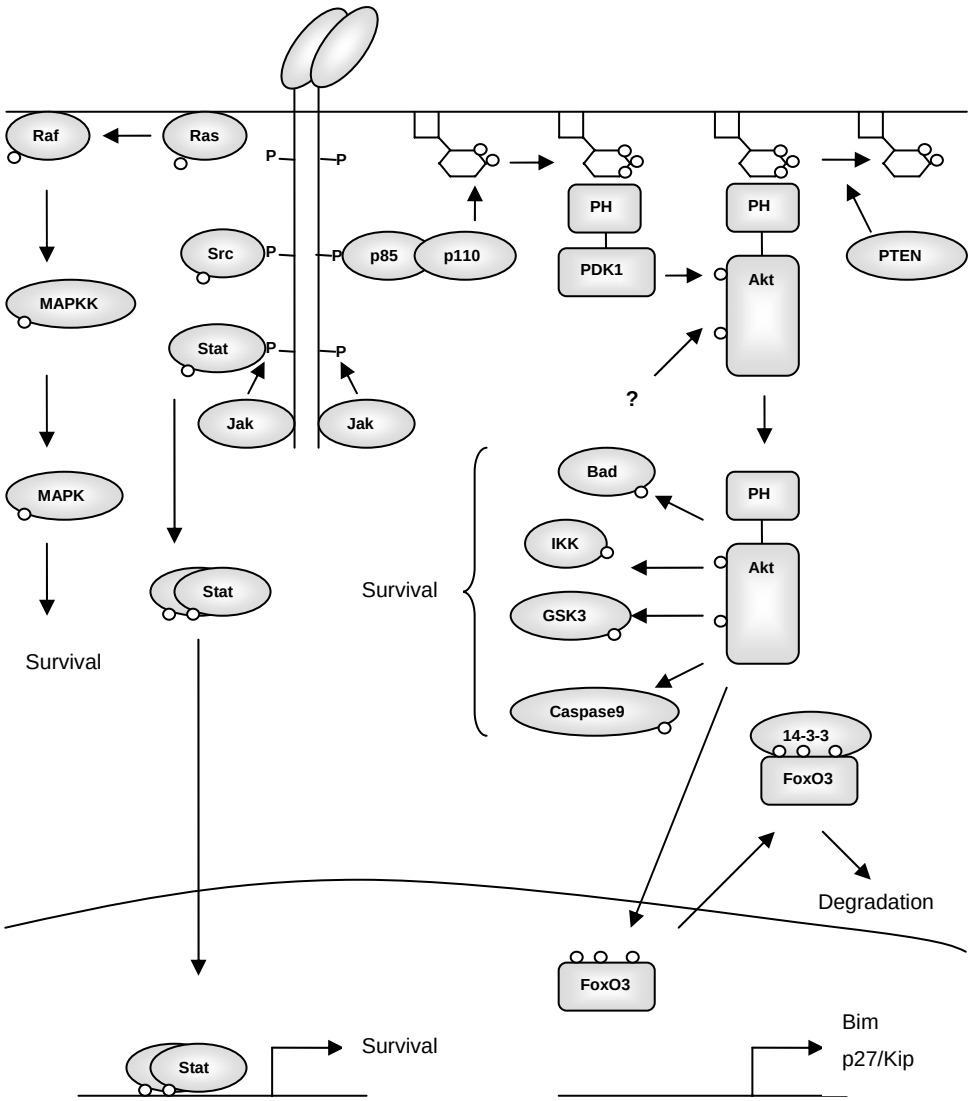

Figure 2. *Receptor activation activates several signal transduction pathways mediating survival. (○ phosphate)*

AIMS OF THE PRESENT INVESTIGATION

The general aim of this thesis was to gain an increased understanding of mechanisms regulating survival of hematopoietic stem cells and progenitor cells, with special emphasis on signaling through the c-Kit and Flt3 receptors.

The specific aims were to:

- Compare survival mechanisms activated by the cytokines KL and FL.
- Study the involvement of the PI3-kinase/Akt pathway and FoxO family members, especially FoxO3, in survival signaling via KL and FL.
- Investigate the role of Bcl-2 family members in the anti-apoptotic machinery activated by KL and FL in hematopoietic progenitor cells.
- Compare the survival effects mediated by Akt and Bcl-2, and to study possible synergistic interactions when Akt and Bcl-2 are coexpressed.
- Study similarities and differences in signaling via normal Flt3 and Flt3 with internal tandem duplication (ITD), and in particular to investigate which distinct signaling pathways that are activated and if Bcl-2 family members are involved.

RESULTS AND DISCUSSION

Survival mechanisms activated by KL and FL (paper I-III)

The first three papers are together a comparative study on signals mediating survival via the early hematopoietic cytokines KL and FL. Acting alone KL promotes survival of hematopoietic stem and progenitor cells,[108,109] and in synergy with other cytokines KL can promote self-renewal to some extent.[35,38,164] c-Kit, the receptor for KL is highly expressed on stem and progenitor cells,[124,125] and repopulating HSCs are highly enriched in the c-Kit$^+$ population of the BM.[57,58] In contrast, FL is not able to support any extensive survival of HSCs, and upregulation of Flt3, the receptor for FL is accompanied with a loss of self-renewal potential.[148] c-Kit and Flt3 belong to the same receptor tyrosine kinase family and activate several overlapping signaling cascades. We found that although signaling via these receptors are similar in many aspects, they do activate different survival pathways.

Throughout this work we have utilized immortalized cell lines as models for apoptotic regulation in hematopoietic cells as well as BM derived Lin$^-$ progenitor cells from mice. FDCP-mix is a non-leukemic, multipotent, and IL-3 dependent progenitor cell line derived from Src-infected mouse BM that exhibits many of the characteristics typical for hematopoietic stem cells.[297] By alternating the growth conditions between high IL-3 concentration and suboptimal IL-3 together with 100 ng KL/ml, we developed a KL-responsive variant called FDCP-mix 93.1, which shows increased survival but no proliferation in the presence of KL. In parallell, we have utilized the IL-3 dependent progenitor cell line FDC-P1.[298] To study FL signaling, we infected FDC-P1 cells with a retroviral vector containing the coding sequence for murine Flt3 to generate FDC-P1/Flt3 cells. We confirmed expression of Flt3 on the surface of these cells by staining with a PE-conjugated antibody for murine Flt3 followed by FACS analysis.

Some of our findings were also confirmed in freshly isolated bone marrow derived Lin$^-$ cells. These were prepared by crushing femur and tibiae from mice with a pestle and mortar, followed by isolation of mononuclear cells (MNCs) by density gradient centrifugation. Bone marrow cells were incubated with monoclonal antibodies for specific

lineage markers (B220, Mac-1, Gr-1, CD4 and CD8), followed by incubation with secondary antibodies conjugated to magnetic beads and depletion of lineage restricted cells (Figure 4).

Signal transduction pathways (paper I-III)

The PI3-kinase/Akt pathway is a known mediator of survival in many cell systems, and is often activated upon cytokine receptor signalling.[217,218] We demonstrate that three different cytokines, IL-3, FL and KL, are all dependent on functional PI3-kinase in order to mediate survival. When using a specific inhibitor for PI3-kinase, LY294002, we noticed a strong induction of apoptosis even in the presence of cytokine. Importantly, low concentration of LY294002 affected KL-mediated survival more severely than survival via IL-3 or FL. Higher concentration of the inhibitor (50 μM) completely blocked KL-mediated survival but only induced a partial block of cell survival mediated by IL-3 and FL. A plausible explanation for this would be that IL-3 and FL may activate additional survival signals not provided by KL. As a downstream survival mechanism for PI3-kinase we identified phosphorylation on Ser473 of Akt, which is associated with activation of its kinase activity. Akt has been identified as a survival factor in several systems,[238,239] and it was phosphorylated rapidly after one minute of KL or FL stimulation. Activation of Akt was dependent on a functional PI3-kinase, since preincubation of cells with LY294002 inhibited phosphorylation. In contrast, inhibition of the MAP-kinase pathway with the specific inhibitor PD98059 did not affect Akt phosphorylation at Ser473.

Another signaling pathway implicated in survival is the Erk pathway. All three cytokines induced phosphorylation of Erk1 and Erk2, which was abolished by pretreatment of the cells with PD98059 or another MAP-kinase inhibitor, U0126. However, inhibition of MAP-kinase activity did not have any effect on cell survival, since treatment with these inhibitors did not result in any substantial apoptosis of cells cultured with KL or FL, although a slight increase in apoptotic cells cultured in KL was seen.

Involvement of FoxO transcription factors (paper II and III)

Apparently the PI3-kinase/Akt pathway is crucial for survival via both KL and FL. Several possible Akt targets have been identified, including phosphorylation of Bad, a pro-apoptotic Bcl-2 family member, leading to inhibition of its apoptotic functions.[86,87] However, we were unable to detect Bad in the cell models used. Instead we turned our attention towards the FoxO transcription factor family, FoxO1, FoxO3 and FoxO4, which have been implicated in the control of apoptosis, cell cycle, and cellular stress. These are regulated by inhibitory phosphorylation by Akt, leading to cytoplasmic retention of the protein inhibiting its transcriptional activity, a mechanism shown to be involved in signaling via several cytokines.[262-265]

Both KL and FL mediated rapid and strong induction of FoxO3 phosphorylation (paper II and III). This phosphorylation was induced within one minute of cytokine stimulation and pretreatment of cells with LY294002 revealed that this was PI3-kinase dependent. Pretreatment with an inhibitor for MAP-kinase activity did not influence FoxO3 phosphorylation. KL stimulation led to a slight increase of FoxO1 phosphorylation, however, the background phosphorylation level were high, possibly due to the presence of serum in the media. FoxO4 was constitutively phosphorylated regardless of KL stimulation or inhibition of PI3-kinase. By immunofluorescent staining and subcellular fractionation, we also demonstrated that in cells stimulated with KL, FoxO3 was relocated from the nucleus to the cytoplasm. This process required PI3-kinase activity since LY294002 inhibited the relocation.

Although our results demonstrated inactivation of FoxO3 by KL and FL in a PI3-kinase dependent manner, a role for FoxO3 in KL- and FL-mediated survival had not been directly established. To prove this we utilized conditionally active FoxO3, FoxO3(A3):ER. In this construct the three regulatory phosphorylation sites Thr32, Ser253, and Ser319, have been replaced with alanine residues, and thus it cannot be inactivated via inhibitory phosphorylation by Akt. The FoxO3(A3) protein was fused to part of the estrogen receptor (ER) and is retained in the cytoplasm by heat shock proteins in the absence of ligands for

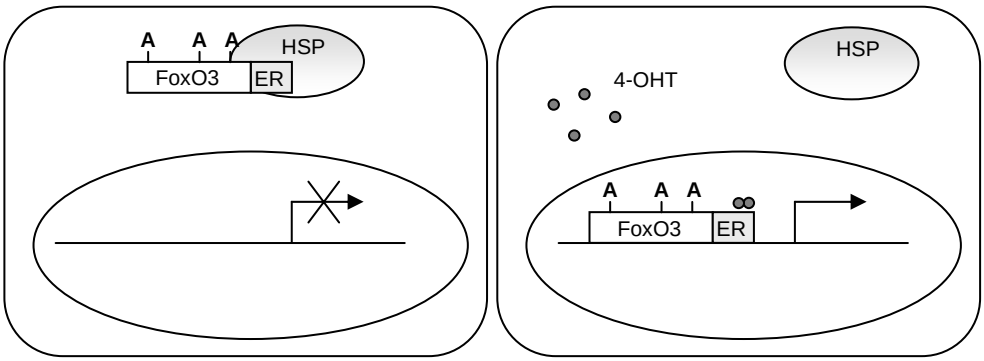

Figure 3. *Addition of 4-hydroxytamoxifen (OHT) leads to release of FoxO3(A3):ER from heat chock proteins (HSPs), relocation to the nucleus, and transcriptional activation of target genes. (ER: estrogen receptor)*

ER. When adding 4-hydroxytamoxifen (4-OHT) to cells expressing this construct, however, the chimeric protein is released and enters the nucleus where it activates transcription of target genes (Figure 3).[262] By retroviral gene transfer we overexpressed FoxO3(A3):ER in FDC-P1 and FDC-P1/Flt3 cells. Upon addition of 4-OHT survival via both KL (paper II) and FL (paper III) was clearly abrogated, thus proving the importance of inactivation of FoxO3 in survival mediated by these cytokines. In paper III we also demonstrated that in FL-stimulated cells, cell cycle arrest preceeded induction of apoptosis upon activation of FoxO3(A3):ER. In accordance with this we also demonstrated that induction of FoxO3 activity was accompanied by upregulation of both pro-apoptotic Bim, and the cell cycle inhibitor $p27^{Kip1}$.

The study of FoxO3 in KL-mediated survival was also repeated in Lin^- progenitor cells from mouse BM. Both Akt and FoxO3 were phosphorylated in response to KL, whereas no effects on either FoxO1 or FoxO4 was seen upon addition of KL. We also retrovirally transduced Lin^- cells with FoxO3(A3):ER by cocultivation on a retroviral producer cell line, followed by a colony assay to study the effect of constitutive activation of FoxO3 on early progenitor cells (Figure 4). Colony formation was clearly inhibited after induction with 4-OHT, both for cells cultured with KL alone, promoting myeloid colony formation, and for cells with KL in combination with Epo, inducing erythroid colony formation

(paper II). This indicates that FoxO3 inactivation is of importance for early hematopoietic cells. However, it is still uncertain whether this was dependent on increased apoptosis or cell cycle inhibition.

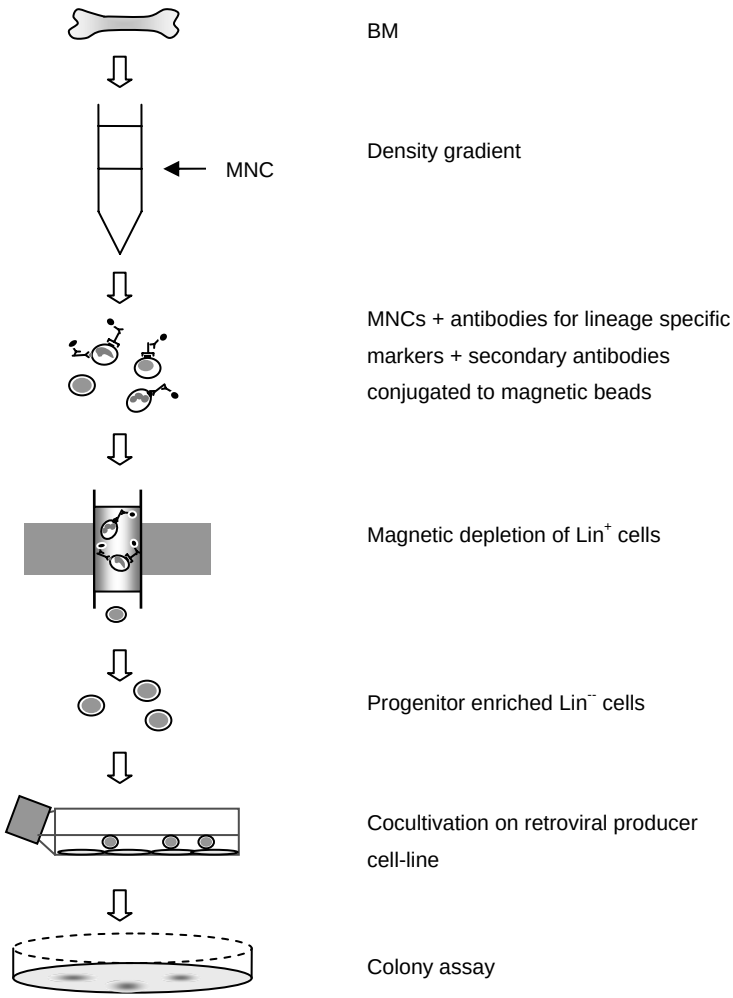

Figure 4. *Lin- progenitor cells from mouse bone-marrow were isolated, followed by retroviral gene transfer of FoxO3(A3):ER by cocultivation on a producer cell line, and in vitro-colony assay in methylcellulose with different cytokine combinations with or without 4-OHT was performed. (BM: bone marrow, Lin: lineage, MNC: mononuclear cells)*

Involvement of Bcl-2 family members (paper I).

The Bcl-2 family of proteins consists of both pro- and anti-apoptotic family members, and the balance between these determines the life and death of a cell.[79] The anti-apoptotic family members Bcl-2, Bcl-x_L, and Mcl-1 are important mediators of survival in the hematopoietic system,[27,92,96,100] and although many studies have indicated the failure of KL to induce expression of Bcl-2 and Bcl-x_L, recent studies have shown that KL can upregulate Bcl-2 in human NK cells and multiple Bcl-2 family members in erythroid progenitor cells.[101,102] Therefore we wanted to investigate any involvement of Bcl-2 family members in KL- and FL-mediated regulation of apoptosis in progenitor cells.

In FDC-P1/Flt3 cells, we could demonstrate that IL-3 and FL upregulated anti-apoptotic Bcl-2 and Bcl-x_L mRNA and protein, whereas KL did not. In addition, IL-3 was able to sustain protein expression of Mcl-1. FL upregulated Bcl-2 mRNA with slower kinetics than IL-3, and the induction of Bcl-x_L was not as high. These experiments were performed in FDCP-mix and FDC-P1 cells, but importantly, we could confirm our results in Lin^- progenitor cells in which IL-3 but not KL upregulated Bcl-x_L and sustained Bcl-2 expression. Treatment of cells with actinomycin D, a transcriptional inhibitor, resulted in loss of Bcl-2 proteins, and addition of the protein synthesis inhibitor cycloheximide, also reduced the levels of Bcl-2 family proteins. This suggested that Bcl-2 family members were regulated at the transcriptional level. The upregulation of Bcl-2 family proteins was not dependent on the PI3-kinase pathway, suggesting that this is an additional mechanism by which FL and IL-3 activate survival, which could explain the increased sensitivity for KL-mediated survival for inhibition of PI3-kinase activity.

Akt and Bcl-2 act in synergy (paper IV)

Based on the results presented above, it becomes apparent that different pathways activate distinct anti-apoptotic mechanisms. This could most likely be explained in two ways. Either Akt or Bcl-2 activation has the same consequence for the cell, or Akt and Bcl-2 activate distinct survival mechanisms, which may act in synergy when simultaneously utilized. Therefore we wanted to compare the survival effects mediated via Akt and Bcl-2.

Previously it has been reported that Akt and Bcl-x_L mediate distinct actions on survival, where Akt but not Bcl-x_L was dependent on the presence of extracellular nutrients.[299] In our case, the question then arises which of Bcl-2 and Akt mediate survival most efficiently?

Akt and survival

To address this question we infected FDC-P1 cells with a constitutively active form of Akt, myristoylated Akt (mAkt) in which Akt has been fused to the Src myristoylation signal targeting the protein to the cell membrane. This leads to constitutive activation of the protein due to an increase in its basal level of phosphorylation.[300] After cytokine deprivation of retrovirally infected FDC-P1 cells, mAkt led to phosphorylation of Akt even in the absence of cytokine, whereas this was not the case in control cells (uninfected cells or cells with wt Akt). Cells expressing mAkt survived better than control cells in the absence of cytokine, although the effect was limited. However, activation of Akt is crucial for survival since overexpression of a dominant negative Akt (K179M), in which the ATP-binding site has been mutated rendering it kinase dead,[301] clearly increased apoptosis even in the presence of KL.

Bcl-2 and survival

We also transfected FDC-P1 cells with human Bcl-2 and generated several clones, of which two individual clones expressing intermediate (clone 1) and high (clone 2) levels of Bcl-2 was utilized throughout the study. The survival effect was significant and reflected the relative expression of Bcl-2. Even after 6 days of cytokine deprivation the majority of the cells were still viable, and in combination with KL the survival was increased even more. This means that compared to Akt, Bcl-2 is far more effective in keeping the progenitor cells alive. One additional important finding was that cells expressing Bcl-2 arrested rapidly in the G_0/G_1 phase of cell cycle. This is in accordance with previous studies where Bcl-2 and Bcl-x_L was shown to reduce proliferation and delay S-phase entry.[302] To evaluate the effects of Bcl-2 in primary progenitor cells from mouse BM, we

used a transgenic mouse model where Bcl-2 is under control of the vav promoter. All hematopoietic cells from these mice express Bcl-2 and cells exhibit increased survival upon cytokine withdrawal.[92] In accordance, we found that cytokine deprivation of Lin^- cells from Bcl-2 transgenic mice exhibited increased survival compared to Lin^- cells from wt mice.

Akt and Bcl-2

Next we transduced the Bcl-2 clones with mAkt to establish whether their combination led to increased survival. Bcl-2 clones coexpressing mAkt exhibited a significant increase in survival compared to Bcl-2, supporting the notion that they may act in synergy. Importantly, combining Akt and Bcl-2 not only increased surivival, but also sustained proliferation for a limited time period. Thus, Akt itself may not mediate a proliferative signal, but in the presence of other signals, it may cooperatively induce proliferation. A recent study suggested that Akt and Bcl-x_L may cooperate to promote leukemogenesis,[303] and in another study Akt cooperated with the Mek pathway to promote proliferation.[210] We also confirmed our findings with Lin^- progenitor cells from vav-Bcl-2 transgenic mice coexpressing mAkt. These cells exhibited a substantial increase in survival compared to uninfected cells.

Bcl-2 and FoxO3

Since FoxO3 mediates apoptosis by upregulating the expression of Bim, we speculated that the pro-apoptotic functions of Bim would be counteracted by Bcl-2. We overexpressed FoxO3(A3):ER in the high-expressing Bcl-2 clone of FDC-P1, and induced FoxO3 activity by the addition of 4-OHT. This led to substantially increased apoptosis in control cells, whereas Bcl-2 expressing cells were protected from apoptosis induced by FoxO3(A3):ER, even with increasing amounts of 4-OHT accompanied by an increase in Bim protein. Thus overexpression of Bcl-2 protected cells from FoxO3-induced cell death, possibly by silencing the pro-apoptotic functions of Bim. In addition, induction of FoxO3 led to cell cycle arrest correlating with an increase in $p27^{Kip1}$ levels.

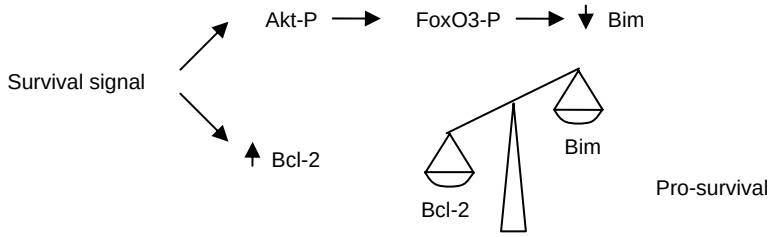

Figure 5. *Akt mediates inhibitory phosphorylation of FoxO3, leading to decreased transcription of the FoxO3 target gene Bim. Increased amounts of anti-apoptotic Bcl-2 in combination with activation of Akt leads to enhanced survival, where the balance between pro- and anti-apoptotic Bcl-2 family members are tilted towards pro-survival.*

Taken together Akt and Bcl-2 seem to cooperate in survival which could be explained as Akt inhibitory phosphorylation of FoxO3 leading to downregulation of Bim, and further tilting the balance between pro- and anti-apoptotic Bcl-2 family members towards pro-survival (Figure 5). In addition, Akt led to prolonged proliferation in the presence of a second more potent survival signal, which might be due to downregulation of $p27^{Kip1}$ via inhibitory phosphorylation of FoxO3.

Comparison of Flt3 and Flt3-ITD (Paper V)

Recent studies have indicated that signaling via normal Flt3 and Flt3-ITD differ. For instance, Flt3-ITD can activate Jak2, Stat3, Stat5a, and Stat5b, whereas wt receptor does not activate any Jaks and only Stat5a.[152,192]

We therefore decided to compare survival mechanisms activated by normal Flt3 and Flt3-ITD with regard to our previous results on the importance of the Bcl-2 family and the Akt/FoxO pathway. We transduced FDC-P1 cells with human Flt3-ITD,[304] generating FDC-P1/Flt3-ITD. After growth factor withdrawal FDC-P1/Flt3 died rapidly, whereas FDC-P1/Flt3-ITD cells survived and became factor-independent. This is in

accordance with other studies where IL-3 dependent cells became factor-independent after introduction of Flt3-ITD.[174-176] Signaling through normal Flt3 or Flt3-ITD was dependent on both PI3-kinase and Src kinase activity, since treatment of cells with inhibitors towards these pathways abrogated survival. Inhibition of MAP-kinases on the other hand had limited effects. We also noticed that both PI3-kinase and Src kinases were crucial for cell cycle progression since inhibition of these arrested cells in G_0/G_1. Akt and FoxO3 was constitutively phosphorylated in Flt3-ITD cells regardless of cytokine stimulation, and upon introduction of conditionally active FoxO3, survival was clearly abrogated. Thus, inhibitory phosphorylation of FoxO3 is crucial for Flt3-ITD mediated survival, and is in accordance with a previous study demonstrating that FoxO3 is a downstream target for Flt3-ITD.[305] Cytokine-deprived cells expressing a normal Flt3 receptor, downregulated expression of Bcl-2, Bcl-x_L, and Mcl-1. In contrast, Flt3-ITD cells showed increased levels of these three proteins even after weeks of cytokine deprivation, suggesting that one mechanism rendering these cells insensitive to induction of apoptosis is via upregulation of Bcl-2 family members. In paper I we demonstrated that signaling via Flt3 receptor led to upregulation of Bcl-2 and to some extent Bcl-x_L, but not Mcl-1. Thus, it appears as if signaling via Flt3-ITD activates additional signaling pathways not activated by wt Flt3, leading to a stronger induction of Bcl-x_L and upregulation of Mcl-1. Interestingly, expression of Bcl-2, Bcl-x_L, and especially Mcl-1, was dependent on Src kinase activity. Src kinases are known to be involved in the activation of Stat family members, and Stats regulate expression of Bcl-2 family members. For instance, Stat5 has been demonstrated to upregulate Bcl-2, Bcl-x_L, and Mcl-1, whereas Jak2 and Stat3 can upregulate Mcl-1.[200,202,306] Thus, this may be the molecular explanation to how Flt3-ITD sustains survival.

CONCLUSIONS

- KL and FL mediate survival via partially distinct mechanisms. Inhibition of PI3-kinase activity was more severe for KL-mediated survival than for FL. This is explained by FL-induced upregulation of anti-apoptotic Bcl-2 family members, which KL cannot execute.

- KL mediates survival via activation of Akt and inhibitory phosphorylation of the FoxO transcription factor FoxO3. Constitutive activation of FoxO3 disrupts survival via KL and inhibits colony formation of primary bone marrow-derived hematopoietic progenitor cells.

- Survival via FL is dependent on functional PI3-kinase/Akt pathway and inhibitory phosphorylation of FoxO3. Constitutive activation of FoxO3 leads to initial cell cycle arrest followed by apoptosis, accompanied by upregulation of Bim and $p27^{Kip1}$.

- Cooperation between Akt and Bcl-2 inhibits apoptosis more effectively than either one acting alone, and Bcl-2 protects cells from FoxO3 induced apoptosis. Bcl-2 leads to arrest in G_0/G_1, whereas coexpression of Bcl-2 and Akt led to prolonged cell cycle progression.

- Flt3-ITD mediated survival is dependent on inhibitory phosphorylation of FoxO3. In addition, Flt3-ITD upregulates expression of multiple Bcl-2 family members, an ability which the wt Flt3 receptor lacks. We also conclude that a Src kinase activity is crucial for survival via both normal and mutated Flt3 receptor.

GENERAL DISCUSSION

In this thesis, we have focused on the survival mechanisms induced by the two receptors c-Kit and Flt3. The importance of these receptors for HSCs is illustrated by the fact that repopulating HSCs are highly enriched in the c-Kit$^+$ population of the BM,[57,58] and that the repopulation potential of BM from Flt3 deficient mice are reduced compared to BM from normal mice.[149] Both KL and FL stimulation leads to suppression of apoptosis in HSCs and progenitor cells.[109,110] However, due to high expression of c-Kit on HSCs, this receptor appears to be more crucial. Although the signal pathways activated by c-Kit have been studied in great detail, the exact mechanisms by which c-Kit supports survival and maintenance of HSCs are still not well characterized. Alternative pathways have been suggested, e.g., the slug gene,[307] but still additional analysis of the signaling pathways activated in response to KL and FL are necessary and could further enhance our knowledge and understanding of HSC and progenitor cell regulation.

An attractive view to explain why one cytokine sustains survival of HSCs, whereas two cytokines would lead to cell cycle initiation was recently proposed by Weissman and colleagues. In this "two-signal survival" model the combination of two specific signals, Bcl-2 and KL, in serum-free conditions was demonstrated to mediate survival of LT-HSCs.[308] Neither one was able to mediate survival alone, but in combination they sustained survival and proliferation of LT-HSCs. Among many cytokines tested, only KL was capable of achieving cooperativity with Bcl-2. Thus, it was concluded that KL must activate unique survival mechanisms not activated by other cytokines. Although we have not been able to identify such pathways in the cell models utilized, important observations have been done. We found that both c-Kit and Flt3 activate the PI3-kinase/Akt pathway, leading to inhibitory phosphorylation of the FoxO transcription factor FoxO3. In addition FL led to upregulation of anti-apoptotic Bcl-2 family members. Thus, the increased sensitivity observed for cells cultured in the presence of KL towards inhibition of PI3-kinase activity may be a consequence of inability to handle FoxO3 induced Bim expression via anti-apoptotic Bcl-2 family members. Expression of anti-apoptotic Bcl-2 family members may initially protect cells from Bim-induced apoptosis, as demonstrated

in paper IV. The cells will eventually die possibly via activation of additional death inducing pathways. Taken together, classical signaling pathways such as the PI3-kinase or the MAP-kinase pathways did not account for the unique ability of c-Kit to sustain HSC survival and maintenance, and further studies of other possible molecular mechanisms are clearly required. One useful approach would be to identify different target genes for KL compared to other cytokines by microarray or suppressive subtractive hybridization. Studies on the effects of KL and FL in isolated stem cells would also further enhance our knowledge, since stem cell signaling may differ from signaling within more mature progenitor cells. [215]

In our study, we have not addressed the involvement and importance of Stats for the transmission of signals by c-Kit. Although activation of Stats has been demonstrated for both KL- and FL-mediated signaling, they seem to activate different Stat family members. c-Kit associates with and activates Stat1,[194] whereas Flt3 associates with Stat5.[152] Another interesting finding is that c-Kit can activate Jak2, and treatment of cells with Jak2 antisense reveals that activation of this kinase is important for KL signaling.[191] In contrast, Flt3 signaling does not seem to involve activation of any Jak family members.[152] Interestingly, Jak2 has been implicated in self-renewal of HSCs,[193] and thus the unique effects of KL on HSCs may involve activation of Jak2. Another finding supporting this theory is that Flt3-ITD activates Jak2,[192] and forced expression of Flt3-ITD leads to self-renewal of early hematopoeitic cells.[309]

In paper IV we found that activation of Akt alone did not mediate a strong enough anti-apoptotic signal, whereas Bcl-2 was a potent mediator of survival. A combination of the two had synergistic effects and could maintain survival of cells in the absence of growth factor for weeks. This is in agreement with other reports demonstrating that Akt and Bcl-x_L inhibit apoptosis by distinct mechanisms. Akt mediated survival required the presence of glucose and maintained the mitochondrial potential, whereas Bcl-x_L lead to decreased cell size and reduced mitochondrial potential due to a low glycolytic rate.[299] Although we have not thoroughly addressed this issue in paper IV, additional experiments have demonstrated that cells overexpressing Bcl-2 are significantly smaller than cells

overexpressing Akt (unpublished observations). An important finding in this study was the ability of Bcl-2 to prevent apoptosis induced by FoxO3. Thus, in the absence of Akt signaling anti-apoptotic Bcl-2 family members can still protect cells from apoptosis induced by increased transcriptional activity of FoxO3 leading to elevated Bim protein expression. This suggests that one mechanism by which Akt cooperates with Bcl-2 is by inhibitory phosphorylation of FoxO3, leading to downregulation of Bim. In such a scenario, the balance between pro- and anti-apoptotic Bcl-2 family members would be shifted towards pro-survival even more because of decreased levels of Bim (Figure 5).

A previous report has demonstrated that overexpression of Bcl-2 and Bcl-x_L delay entry into S-phase,[302] and we found that cells expressing Bcl-2 alone arrested cells in G_0/G_1. Coexpression of Akt and Bcl-2 on the other hand prolonged cell cycle progression after cytokine withdrawal. Thus Akt seems to be able to contribute with a proliferative signal in the presence of a strong survival factor. This is supported by the observation that Akt in combination with Bcl-x_L can cooperate to promote leukemic transformation.[303] Alternatively, Akt may mediate proliferation in the presence of a second proliferative signal, possibly via downregulation of $p27^{Kip1}$ because of inhibitory phosphorylation of FoxO3.

Akt/PI3-kinase, FoxO proteins, Bcl-2, c-Kit, and Flt3 have all been implicated in tumorigenesis. In the case of hematopoietic malignancies, a two-hit model for the development of AML has been suggested. This involves an activating mutation conferring proliferation and/or survival, and a second mutation blocking hematopoietic differentiation.[114] The first activating mutation often involves aberrant activation or overexpression of RTKs, such as c-Kit and Flt3. However, in the case of Flt3-ITD, aberrant activation of the receptor may actually lead to both a proliferative advantage and a block of differentiation.[174,176,178] This would imply that such mutations will have a greater penetrance in the development of AML due to its multiple effects. Inhibitors for RTKs are under development,[310,311] and identification of signaling pathways that differs between normal and mutated receptors is of potential interest for the design for specific therapies.

In paper V, we have identified differences between normal and mutated Flt3, where Flt3-ITD activates several anti-apoptotic Bcl-2 family members not activated by normal Flt3. Others have done similar observations between normal Flt3 and Flt3-ITD, involving for instance Jak and Stat family members.[152,192] Identification of potential phosphorylation sites within normal and mutated receptors would be valuable and may explain the differences in signaling that have been observed. One possibility is that phosphorylations within the receptor that are normally silenced by autoinhibition are aberrantly activated in mutated receptors. Identification of such sites is of interest and might be useful for the development of specific therapies in the future. Furthermore, application of different methods to analyze which proteins associate to and become activated by normal versus mutated Flt3 receptor may identify important differences.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Varje dag bildas ca en biljon nya blodceller i en vuxen person. Eftersom blodceller är relativt kortlivade sker denna nyproduktion ständigt. Alla dessa celler härstammar från en sorts cell, multipotenta blodstamceller. Genom en gradvis mognadsprocess, genereras röda blodkroppar (erytrocyter), vita blodkroppar (granulocyter, lymfocyter och monocyter) och blodplättar (trombocyter). Dessa tar hand om syreförsörjning av kroppens olika organ, infektioner i kroppen och koagulering av blod. Blodets stamceller kan tillgodose detta enorma behov av celler under en individs hela liv, och för att detta ska kunna ske krävs en kontrollerad reglering av deras funktioner. Defekter i denna reglering kan leda till att olika former av leukemier kan utvecklas.

Blodstamceller befinner sig i benmärgen. I denna unika miljö påverkas de av intilliggande celler via speciella ämnen, tillväxtfaktorer, som utsöndras av celler i deras närhet. På utsidan av celler, inklusive stamceller, finns ett antal olika receptorer. Tillväxtfaktorer i den omgivande miljön kan binda till dessa receptorer och då detta sker aktiveras ett antal processer inuti cellen. Detta kan i slutändan leda till att en cell bestämmer sig för att dela sig, mogna eller bara helt enkelt överleva. Celler kan inte dela sig ett obegränsat antal gånger. Därför är det viktigt för en stamcell att kunna befinna sig i ett vilande stadium, utan varken celldelning eller utmognad för att inte uttömma hela stamcellspoolen. Detta vilande stadium upprätthålls då cellen instrueras att förhindra programmerad celldöd. Programmerad celldöd, eller apoptos som det även kallas, är ett kontrollerat sätt för kroppen att göra sig av med defekta celler. För att upprätthålla jämvikten i blodbildningen balanseras ständigt celldelning, utmognad och vila av apoptos. Syftet med detta arbete har varit att studera olika överlevnadsmekanismer i blodets stamceller.

I denna avhandling har jag studerat två specifika tillväxtfaktorer, Kit liganden (KL) och Flt3 liganden (FL) och hur de kan instruera celler att överleva. Dessa två tillväxtfaktorer är speciellt viktiga för blodets stamceller, och receptorerna som de binder till är uttryckta på många olika sorters celler, inklusive blodstamceller. Då tillväxtfaktorer binder till en receptor aktiveras denna. Detta i sin tur påverkar olika signaleringsvägar inuti cellen.

I mina första tre delarbeten konstaterade jag att både KL och FL aktiverar PI3-kinas/Akt-vägen. Aktivering av denna väg leder i sin tur till att transkriptionsfaktorn, FoxO3, inaktiveras. Transkriptionsfaktorer kan binda in till DNA och reglera uttrycket av specifika gener. FoxO3 har visats aktivera ett flertal gener som leder till celldöd. Detta innebär att inaktivering av FoxO3 leder till förbättrad överlevnad. Då jag på konstgjord väg aktiverade FoxO3 visade det sig att detta resulterade i celldöd trots närvaro av KL eller FL. Detta innebär att inaktivering av FoxO3 är ytterst viktigt för att dessa båda skall kunna leda till överlevnad. Jag fann även att FL till skillnad från KL aktiverar ytterligare överlevnads-faktorer som kallas Bcl-2 proteiner. I delarbete IV kunde jag konstatera att Bcl-2 var en bättre överlevnadsfaktor än Akt, och att en kombination av de båda var ännu bättre.

Receptorer och de signaleringsvägar de aktiverar är ofta förändrade vid olika tumörsjukdomar. Man har konstaterat att Flt3 receptorn är förändrad i upp till 30% av alla patienter med akut myeloid leukemi, AML. Denna förändring innebär att receptorn är aktiverad oavsett om dess ligand binder till den eller inte. I det sista delarbetet undersökte vi signaleringen via en förändrad Flt3 och kunde konstaterade att ett flertal överlevnadsvägar var aktiverade oavsett om FL hade bundit till receptorn eller ej. Detta innebär att oavsett vilka signaler de får från sin omgivande miljö, så kommer celler som uttrycker en sådan receptor att överleva och dela sig.

Hur stamceller regleras studeras flitigt. Genom ökad förståelse av hur stamceller reagerar på olika stimuli kan man i slutändan utveckla effektivare metoder för t ex benmärgs-transplantationer. I vissa fall genomför man så kallade stamcellstransplantationer, då man isolerar stamceller från donatorns blod och för in i mottagaren. Det hade varit önskvärt att kunna veta hur man ska få dessa celler att överleva en längre tid och dela sig både utanför och i kroppen. Genom fortsatta studier av hur stamceller regleras kan man kanske i slutändan åstadkomma detta, vilket skulle innebära ett stort framsteg för stamcells-forskningen och för eventuella framtida terapier.

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