CHAPTER 7

General discussion

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Maintaining a correct number of cells in the hematopoietic system, as well as having sufficient differentiated cells to carry out specialized tasks and to be able to respond in changes in the extracellular environment requires a highly organized network to coordinate these events. Terminally differentiated cells generally do not divide, but arise from precursors (stem cells), which can either proliferate, giving rise to more precursors, or differentiate, which in general is accompanied with a loss of their proliferative capacity; reviewed in [1,2]. Somewhere in the life of a stem cell the decision is made to proliferate or to undergo differentiation. This decision can be governed by a specific combination of extracellular factors, cytokines, which activate a network of intracellular signaling pathways. Mutations in components of this signaling network can disturb the balance between proliferation and differentiation, as well as abrogate the dependence of the cell for survival factors. This can result in leukemia, characterized by a block in differentiation of hematopoietic precursors, accompanied with inappropriate proliferation and expansion. Thus, an important feature of leukemic cells is that they exhibit a block in differentiation and have gained the capacity for unlimited proliferation, without being restrained by the normal mechanisms that promote apoptosis upon inappropriate division. Generally, in addition to exhibiting unrestrained proliferation, tumor cells also need to have adapted to overcome apoptosis. The differentiation block in leukemias is often a result of chromosomal translocations of genes encoding transcription factors (reviewed in [3,4]). The resulting protein products of these translocations can interfere with the normal growth factor activated signaling network and transcription factors that act in concert to promote differentiation.

Terminal differentiation is in general accompanied with an arrest in the cell cycle and evidence is accumulating that this arrest is mediated by CKIs acting together to induce a G_1 arrest and exit from the cell cycle (known as G_0, reviewed in [5]). In tumor cells, expression of CKIs is often lost or substantially decreased (reviewed in [6,7]). Elevation of the CKI $p27^{KIP1}$ is an adverse prognostic indicator in cancer in that low levels are correlated with a poor prognosis[8-10]. Although it is not clear whether this simply reflects the fact that relatively undifferentiated cells have a higher tumorigenic capacity and lower levels of CKIs, it is worth noticing that induction of differentiation in some leukemias in combination with chemotherapy increases the chance of ablating the leukemia[11]. In addition, lower levels of $p27^{KIP1}$ might also reflect a defect in the pathways regulating $p27^{KIP1}$ expression, which might also include other proteins, whose dysregulation is more directly related to oncogenesis.

To obtain more insight into the mechanisms by which cytokine-activated signaling pathways could promote proliferation and survival and potentially obtain insight into strategies of clinical interference in leukemias or inflammatory diseases, we studied IL-3, IL-5 and GM-CSF-induced signaling. Receptors for IL-3 and GM-CSF are present on a wide variety of hematopoietic cells, whereas expression of the IL-5 receptor is restricted to eosinophils and basophils in man, and in mice also on B cells[12,13]. As eosinophils are

believed to be involved in the pathogenesis of allergic diseases[14,15], potential eradication by interference with signaling pathways involved in IL-5-mediated survival might alleviate the disease symptoms. A useful therapeutic agent might result from the recent development of peptides that bind to and antagonize the IL-5 receptor[16]. However, the requirement for this strategy is that cells are still responsive to IL-5, and have not developed mechanisms that result in cytokine-independent growth or survival.

Upon analyzing which signaling pathways are essential for IL-3, IL-5 and GM-CSF-mediated proliferation utilizing specific pharmacological inhibitors, only inhibition of PI3K abrogated both cytokine-mediated survival and proliferation (Chapter 3, Fig. 1). We have demonstrated that PI3K target PKB can account for the anti-apoptotic effect of PI3K (Chapter 3, Chapter 5, Fig. 5). Previously identified targets of PKB, caspase-9 and Bad, whose activity is inhibited following PKB phosphorylation, have been described in PKB-mediated survival[17,18]. However, phosphorylation of these targets is not required for survival in all cellular systems[19,20]. Therefore we set out to investigate other targets of PKB. This resulted in the observation that PKB-mediated phosphorylation of Forkhead transcription factor FKHR-L1 appears to be required for IL-3, IL-5 and GM-CSF-mediated rescue from apoptosis (Chapter 3 Fig. 4). Phosphorylation of FKHR-L1 by PKB promotes association of FKHR-L1 with 14-3-3 proteins, resulting in cytoplasmic retention, thus inhibiting its transcriptional activity[21]. Upon investigation as to how FKHR-L1 activity promotes cell cycle arrest and induction of apoptosis in cells, two FKHR-L1 targets were identified, $p27^{KIP1}$ and pro-apoptotic Bcl-2 family member Bim (Chapter 3, Chapter 4). These findings are summarized in Fig. 1.

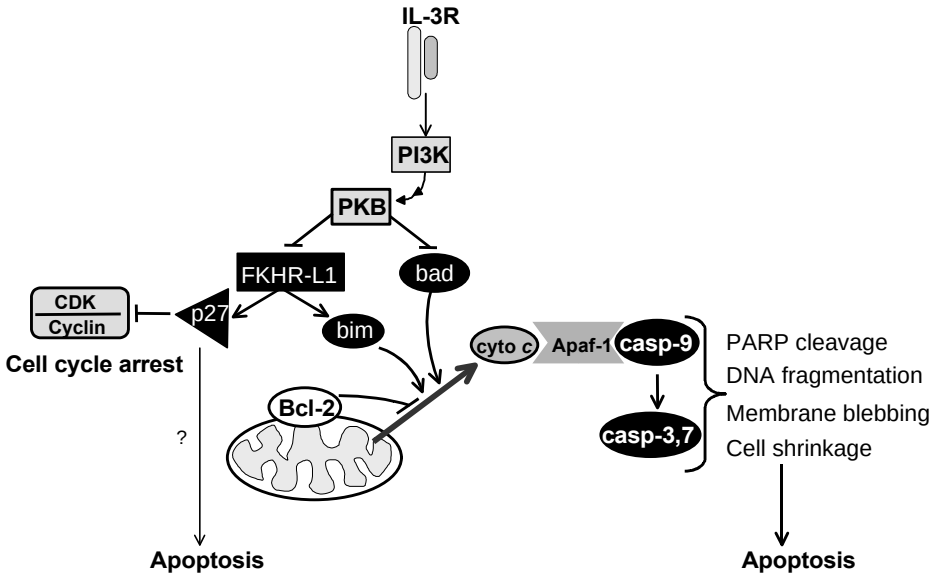

Figure 1 Model.

Cytokine-mediated rescue from apoptosis can be mediated by activation of PKB, which phosphorylates pro-apoptotic Bcl-2 family member Bad and Forkhead transcription factor FKHR-L1, thus inhibiting their activity. In the absence of cytokines, FKHR-L1 is active, promoting transcription of $p27^{KIP1}$ and pro-apoptotic Bcl-2 family member Bim. $P27^{KIP1}$ can promote either cell cycle arrest by inhibiting G_1 CDK-cyclin complexes or inhibit apoptosis through an unidentified mechanism. Bim can promote apoptosis by inhibiting the activity of anti-apoptotic Bcl-2 family members. FKHR-L1 activity results in subsequent activation of caspases, executing the apoptotic program through proteolytic cleavage of substrates, such as PARP.

$p27^{KIP1}$, a critical mediator of proliferation and apoptosis ?

$p27^{KIP1}$ was initially identified as an inhibitor of CDK/ cyclin complexes that are present in G_1 and constitute an important mediator of the "restriction point" (Chapter 1, Fig. 3) which can be overcome by growth factors[22,23]. At the end of G_1, levels of $p27^{KIP1}$ decrease, which is predominantly mediated by phosphorylation by cyclin E/CDK2, resulting in degradation by the ubiquitin system[24-26]. Transcriptional regulation of $p27^{KIP1}$ has, in contrast to our observations in Chapter 3, never been considered an important mechanism of regulation of $p27^{KIP1}$ levels. These differences might be

explained in that many previous studies have been carried out in fibroblasts, in which mRNA levels of $p27^{KIP1}$ do not or only mildly increase in response to growth factor deprivation[23,27]. With respect to this it is also interesting to note that overexpression of $p27^{KIP1}$ in fibroblasts results only in a G_1 arrest, whereas in hematopoietic cells overexpression can in addition promote induction of apoptosis[28,29]. A potential explanation of induction of the apoptotic program by $p27^{KIP1}$ in hematopoietic cells has been provided by Boussiotis and coworkers, who observed that $p27^{KIP1}$ associates with c-Jun co-activator JAB1, resulting in defective AP-1-mediated transcription in T cells[30]. Increases in $p27^{KIP1}$ levels may then result in a decrease in transcription of anti-apoptotic genes, resulting in apoptosis (Fig. 2A). To examine whether this might be an explanation for the induction of apoptosis that we observed upon overexpression of $p27^{KIP1}$, we analyzed the effect of overexpression of $p27^{KIP1}$ on the luciferase activity of an AP1-responsive luciferase construct. Indeed, luciferase activity of cells overexpressing $p27^{KIP1}$ was substantially decreased compared to control cells (Fig. 2B), whereas overexpression of $p27^{KIP1}$ did not alter luciferase activity of an unrelated cyclin D1 luciferase construct.

While this could indicate that displacement of JAB1 from AP-1 complexes is a potential mechanism by which $p27^{KIP1}$ could promote apoptosis in hematopoietic cells, this still awaits further analysis. As an alternative explanation for induction of apoptosis, we cannot exclude that overexpression of $p27^{KIP1}$ in cells which have already passed the restriction point might result in induction of the apoptotic program as a result of simultaneously receiving both proliferative and anti-proliferative signals, as usually levels of $p27^{KIP1}$ decrease towards the end of G_1[22,23]. Further evidence for a function of $p27^{KIP1}$ other than inducing G_1 arrest is provided in that $p27^{KIP1}$ expression is regulated in terminally differentiated eosinophils (Chapter 3 Fig. 2). Although expression of CKIs in terminally differentiated cells has been suggested to be required for the maintenance of their non-proliferative status[2], $p27^{KIP1}$ is upregulated upon IL-5 withdrawal, suggesting that in some cell types $p27^{KIP1}$ might indeed be involved in induction of the apoptotic program.

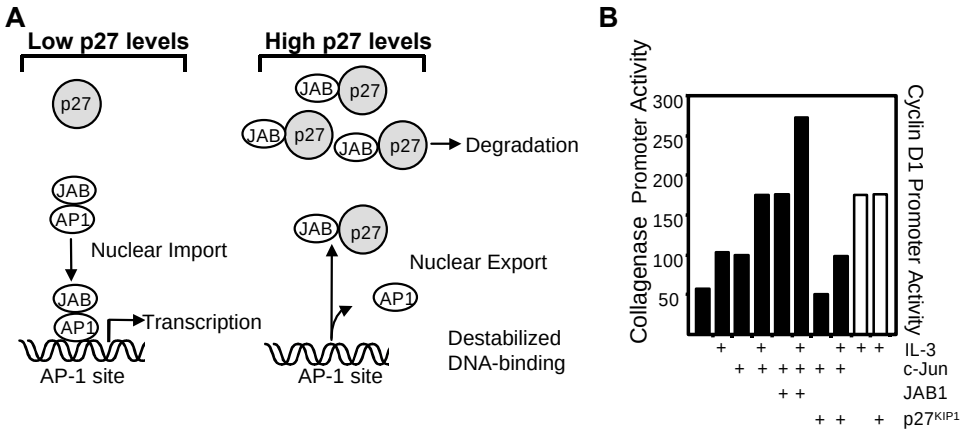

Figure 2 Expression of p27KIP1 decreases collagenase promoter activity.
(A) Model for $p27^{KIP1}$ in induction of apoptosis. When levels of $p27^{KIP1}$ are low, JAB1 translocates to the nucleus and serves as a cofactor for AP-1 complexes. High levels of $p27^{KIP1}$ promote translocation of JAB1 to the cytoplasm, resulting in a destabilization of AP-1 complexes and reduction of AP-1-mediated transcription. (B) Ba/F3 cells were electroporated with either 2 μg of collagenase-luciferase construct (black bars), or cyclin D1 luciferase constructs (white bars) together with 500 ng of renilla as an internal transfection control, together with 8 μg of c-Jun, JAB1 or $p27^{KIP1}$ as indicated. Cells were cultured in the absence or presence IL-3 for 24 hours and luciferase activity was analyzed as described in Chapter 3.

Function of pro-apoptotic Bcl-2 family members in development and potential mechanism of regulation.

A second FKHR-L1 target that we identified was the BH3-only protein Bim (Chapter 4). In addition to Bim there are other BH3-only proteins that interact with anti-apoptotic Bcl-2 family members through their BH3-region and thus inhibit their activity, resulting in induction of the apoptotic program[31]. Interestingly, expression and regulation of Bim was only observed in hematopoietic cells[28], but not in fibroblasts (G. Kops et al., personal communication). Moreover, activity of FKHR-L1 results in a G_1 arrest in fibroblasts, and induction of apoptosis in hematopoietic cells[27,28], suggesting that Bim is indeed a critical mediator of the apoptotic program in hematopoietic cells.

Regulation of activity of BH3-only proteins in mammals can occur posttranslationally, such as for Bad[18,32]. In addition, evidence is accumulating for transcriptional regulation of some of these proteins. DNA damage-induced activation of transcription factor p53 promotes transcription of BH3-only protein Noxa, concomitant with elevation of CKI $p21^{CIP1}$(33; reviewed in [34]). This is similar to our own observations of FKHR-L1-mediated upregulation of a CKI and a BH3-only protein. NGF withdrawal-induced apopto-

sis of neuronal cells, but also survival factor withdrawal of several hematopoietic cell lines and bone marrow-derived CD34(+) cells, induces elevation of the BH3-only protein Harakiri (Hrk, also known as DP5)[35,36]. However, the transcription factor(s) responsible for this upregulation still remains to be elucidated. We have set out to investigate whether FKHR-L1 might be involved in regulation of Hrk, but failed to detect Hrk expression in Ba/F3 cells (data not shown). It is still of course interesting to examine this in the context of other hematopoietic cells.

Could there be a function for BH3-only proteins in apoptosis during development, for example to eliminate unwanted cells during T and B cell selection, where autoreactive cells undergo apoptosis ? The observation that B and T cell development is perturbed in *Bim (-/-)* mice and is accompanied with autoimmune diseases suggests that this might indeed be the case[37]. Here, research on model organisms such as *C. elegans* can shed light on BH3 only proteins in development, as many components of the apoptotic machinery are conserved through evolution[38]. Moreover, the recently elucidated sequence of the human genome will be of help in identifying additional components in human[39]. In *C. elegans*, the BH3-only protein EGL-1 has been demonstrated to be essential for the majority apoptotic cell deaths during development, since loss of function of the *egl-1* gene abrogated apoptosis this process[40]. Additional research suggested that regulation of EGL-1 might be cell-type dependent[41]. In some cells, transcription of *egl-1* can be repressed by the zinc-finger protein CES-1, which in turn is repressed by CES-2, a member of the basic-leucine zipper family of transcription factors[42] (reviewed in [38], see Fig. 3).

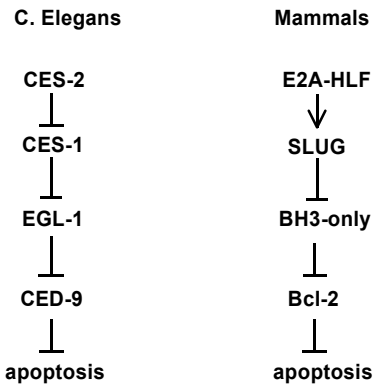

Figure 3 Evolutionary conservation of players of the apoptotic program.
In C. elegans, as well as in mammals, members of the Bcl-2 family play an important role in mediating apoptosis. Upstream regulators of the BH3-only protein EGL-1 in C. elegans have orthologs in mammals, although their function in regulating the expression of BH3-only proteins in mammals still needs to be established.

Upstream regulators of *egl-1* transcription have orthologs in mammals. Recently, a mammalian homolog of CES-1, Slug, was identified, which exhibits anti-apoptotic effects[42]. This suggests that transcriptional repression of pro-apoptotic Bcl-2 members could also be an important means of regulation in mammalian cells. Indeed, IL-3 signaling was recently found to repress protein levels of Bim, which might be due to transcriptional repression[43,44]. Interestingly, chromosomal translocation of a potential mammalian ortholog of CES-2, hepatic leukemia factor (HLF), resulting in the fusion gene E2A-HLF, has been associated with perturbed development of lymphoid cells and leukemia[45-47]. Furthermore, whereas Slug expression appears to be restricted to lymphoid progenitors, expression of E2A-HLF results in sustained expression of Slug, which might be linked to the leukemogenic effects of E2A-HLF[42]. However, the normal activity of HLF or other potential mammalian orthologs of CES-2, as well as regulation of BH3-only proteins by Slug still remains to be demonstrated.

FKHR-L1 and its potential involvement in disease.

In our studies, FKHR-L1 activity promoted G_1 arrest and apoptosis concomitant with an elevation of $p27^{KIP1}$ and Bim[28,43]. The PI3K- PDK-1-PKB- FKHR-L1 pathway is evolutionarily conserved and has orthologs in *C. elegans*. However, a role for the Forkhead ortholog Daf-16 in apoptosis has not yet been demonstrated, although its activity is associated with an increase in life span[48-50].

There are many examples of tumors, and also autoimmune diseases that result from increased activity of PI3K and its downstream effectors[51-54]. In addition to promoting cell division and survival through inhibiting members of the Forkhead family AFX, FKHR and FKHR-L1, rearrangements of FKHR and AFX, have also been demonstrated in certain cancers (reviewed in [55]). However, a potential role for FKHR-L1 in the clearing of inflammatory cells remains to be established. Inflammation in allergic diseases due to enhanced activity, but also enhanced numbers of inflammatory cells, such as neutrophils or eosinophils. Enhanced numbers of these cells might be a result of overproduction or increased mobilization, but also of defective regulation of apoptosis. To discriminate between those possibilities, we analyzed eosinophils of a hypereosinophilic patient having a marked elevation of eosinophil numbers in the peripheral blood (white blood cell count $23x10^9$ cells/l, 95% eosinophils). Cytokine-deprived cells from this patient failed to undergo apoptosis (Fig. 4A, kindly provided by M. Rosas), suggesting that the default apoptotic pathway is affected. Interestingly, whereas in the absence of cytokines activity of FKHR-L1 is induced through dephosphorylation, phosphorylation of FKHR-L1 in eosinophils was sustained even in the absence of cytokines and not altered upon addition of IL-5 (Fig. 4B), in contrast with eosinophils derived from a healthy donor (Fig. 4C). The observation that phosphorylation of ERK decreased in cytokine-starved eosinophils from the patient (Fig. 4A, right) suggests that sustained survival is not simply a result from autocrine survival factor secretion. These data provide evidence that aberrant regulation of FKHR-L1 may not only be related to oncogenesis,

but also to the pathogenesis of inflammatory diseases. We have shown that apoptosis induced by FKHR-L1 is a result from a decrease of mitochondrial transmembrane potential, resulting in release of cytochrome c and activation of caspases (Chapter 5). Although mitochondria are present in low numbers in eosinophils, it has recently been shown that also in these cells mitochondria play a central role in the induction of apoptosis[56]. Indeed, we have recently demonstrated that removal of IL-5 from eosinophils results in decrease of mitochondrial transmembrane potential (data not shown).

Although negative regulation of PI3K and its effectors is mediated by lipid phosphatases[57,58], inhibitory regulation of PKB targets by phosphatases is largely elusive. Recently, the PKB target Bad has been demonstrated to be dephosphorylated by the serine-threonine phosphatases PP1A and PP2A, raising the possibility that these phosphatases could also dephosphorylate FKHR-L1[59,60]. As dysregulation of the activity of these phosphatases has been associated with malignancies (reviewed in [61]), it would be of interest to investigate whether this is associated with constitutive phosphorylation of FKHR-L1.

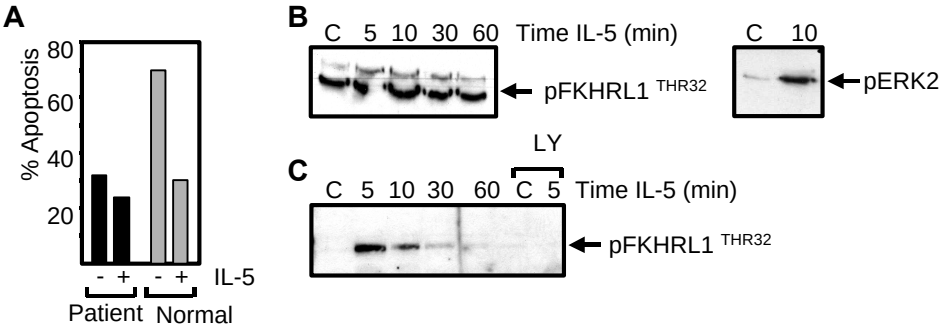

Figure 4 Constitutive phosphorylation of FKHR-L1 correlates with enhanced survival of eosinophils.

(A) Eosinophils from a hypereosinophilic patient (black bars) or a healthy donor (grey bars) were cultured in the absence of presence of IL-5 (10^{-10} nM) for 48 hours and the percentage of apoptotic cells was analyzed as in Chapter 3. (B) Left: cells from a hypereosinophilic patient were stimulated with IL-5 (10^{-10} nM) and the phosphorylation of FKHR-L1 was determined using FKHR-L2-Thr-32 phosphospecific antibody as described in Chapter 3. Right: Cells from the same patient were stimulated with IL-5 (10^{-10} nM) for 10 minutes and ERK2 phosphorylation was analyzed using an ERK2 phospho-specific antibody. (C) Eosinophils from a healthy donor were treated with IL-5 (10^{-10} nM) for indicated times either without or with pretreatment of LY294002 (10 μM) analyzed as in (B).

Induction of apoptosis in response to DNA damage has been attributed to p53 through upregulation of pro-apoptotic Bcl-2 members Bax and Noxa[33,62]. However, mechanisms of transcriptional upregulation of pro-apoptotic proteins in development or in the absence of growth factors is still largely elusive. In addition to FKHR-L1-induced apoptosis in hematopoietic cells, another putative transcription factor, Requiem, might also be involved. Requiem was identified in a screen to identify genes mediating programmed cell death triggered by IL-3-deprivation and encodes a zinc-finger protein, but its mechanism of action still remains to be elucidated[63]. The transcriptional upregulation of pro-apoptotic proteins after cytokine withdrawal might prove to be a common mechanism regulating survival of hematopoietic cells.

Strategies for the development of therapeutic agents.

Cancer cells exhibit unrestricted proliferation independent of environmental regulatory mechanisms having adapted to the apoptotic restraints that are normally be imposed on them. These cells are often more sensitive to irradiation and chemotherapeutic agents than their normal counterparts. Given the side effects of these treatments, it would be desirable to develop a therapy that is more specific and restricted to malignant cells. For many leukemias, this would require a better characterization of the different developmental stages in order to specifically target the therapy. Although this still requires additional research, knowledge of apoptosis is currently being used for clinical interference. For example, adaptive mechanisms in cancer cells often involve the Bcl-2 family, where the activity of anti-apoptotic members is enhanced over pro-apoptotic members (reviewed in [31,64]). Interestingly, treatment of melanoma cells that have elevated levels of Bcl-2 with Bcl-2 antisense oligonucleotides enhanced their susceptibility to chemotherapeutic agents[65]. Studies of the inhibitory effects of BH3-only proteins have resulted in the observation that only a small peptide derived from the BH3 domain, which specifically interacts with and inhibits the activity of anti-apoptotic Bcl-2 family members, is sufficient to trigger apoptosis of cells[66,67]. Recently, research towards developing therapeutic agents to inhibit anti-apoptotic Bcl-2 family members has taken an additional step with the characterization of cell-permeable small molecule inhibitors that compete with the binding of the BH3 domain of BH3-only proteins to anti-apoptotic Bcl-2 family members[68-70]. Addition of these small molecules to cells promotes apoptosis similar to the mechanism of action of BH3-only proteins. Although development of such molecules is promising and has advantage over peptides in that they are more stable, it still requires the development of strategies to be able to specifically target these inhibitors to malignant cells. Taken together, the knowledge generated by fundamental research into the regulation of apoptosis allows the development of more specific means for clinical interference in disease. Further research could contribute to increase the specificity of the therapeutic agent, and limit its effects to malignant cells.

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