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# The role of STATs in myeloid differentiation and leukemia

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**Myeloid differentiation is a highly regulated process governed by various cytokines, such as EPO, TPO, G-CSF, IL-3, IL-5 and GM-CSF. These cytokines act in part through activation of the STAT transcription factor family. In particular, various isoforms of STAT3 and STAT5 are activated during myeloid differentiation in a cell-type and maturation-state dependent fashion. *In vitro* studies have shown that STAT proteins are essential for cytokine-regulated processes such as cellular proliferation, differentiation as well as survival. Similarly, various STAT knock-outs have highlighted the role of STATs in myeloid differentiation *in vivo*. STATs also appear to play an important role in various myeloid malignancies, which are characterized by arrested maturation and cytokine-independent proliferation of myeloid progenitors. Constitutive activation of STAT3 and/or STAT5 resulting in enhanced transcription of anti-apoptotic-cell-cycle progression genes is likely to contribute to the pathogenesis of various myeloid leukemia's.** *Oncogene* (2000) **19,** 2511 – 2522.

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## Introduction

The production of mature blood cells, hematopoiesis, occurs predominantly in the adult bone marrow. Differentiation of a limited population of self-renewing pluripotent stem cells results in the generation of distinct lineage-committed myeloid or lymphoid progenitor cells (Ogawa, 1993). These cells can then be further induced to differentiate, in the case of myeloid progenitors, into granulocytes, monocytes, megakaryocytes and erythrocytes. A large family of hematopoietic cytokines are able to strictly control the proliferation, differentiation, survival and effector functions of these myeloid progenitors. IL-3, for example, stimulates the growth of multipotential progenitor cells that will eventually differentiate into granulocytes, macrophages, megakaryocytes and erythrocytes. This ability to stimulate proliferation works primarily during early hematopoiesis without specifying the final differentiation fate of the progenitor. Other cytokines act at a later stage to direct the differentiation process towards specific myeloid cells. For example, IL-5 is critical for the terminal differentiation of myeloid progenitors to eosinophils, while erythropoietin (EPO) stimulates differentiation of erythroid lineages. In a similar fashion, granulocyte colony stimulating factor (G-CSF) plays a critical role in granulopoiesis resulting in the production of neutrophils. The critical role of these cytokines in hematopoiesis is highlighted by gene knockout studies. G-CSF-deficient mice, for example, develop chronic neutropenia and have a greatly impaired response to infection (Lieschke *et al*., 1994). These late-acting cytokines initially stimulate cellular proliferation followed by differentiation to a non-proliferating mature cell. It is the combinations of cytokines acting on a myeloid progenitor that will initiate a specific developmental program determining the final differentiation fate of the target cell.

Hematopoietic cytokines act on specific cell surface receptors resulting in the initiation of intracellular signal transduction events (reviewed in Smithgall, 1998). The first step in this process usually commences with receptor dimerization upon ligand binding. Cytokine receptors lack an intrinsic kinase-domain and instead bind to members of the Janus Kinase (JAK) family of tyrosine kinases (Liu *et al*., 1998). Receptor dimerization results in JAK activation through cross-phosphorylation followed by tyrosine phosphorylation of specific residues present on the intracellular domains of cytokine receptor sub-units. This results in the recruitment of SH2-domain containing signaling molecules which include members of the STAT family of transcription factors. The mechanisms of activation and recruitment of STATs is the subject of many of the reviews in this issue and will not be discussed here in detail.

The role of STATs in influencing the proliferation, differentiation and survival status of hematopoietic progenitors has been the subject of extensive research over the last few years. This work has initially focused on identifying the STAT isoforms activated by specific cytokines, their role in the regulation of target genes and has recently been followed up by analysis of STAT null-mutant mice to define the critical players in hematopoietic differentiation and immune function. The first part of this review will focus on the current understanding of the role of STATs in myeloid differentiation. The second section discusses how deregulated STAT activation is involved in the disruption of the normal process of myeloid differentiation resulting in uncontrolled proliferation, a block in differentiation and the development of myeloid leukemia.

## STATs and myeloid differentiation

### In vitro *studies*

The availability of various myeloid cell lines has allowed analysis of the potential role of STATs in proliferation, differentiation and survival *in vitro*. The

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ability to activate or inhibit transcription through overexpression of mutant STATs has provided evidence for a role for this family of transcription factors in hematopoiesis. Myeloid leukemia cells are blocked at an early stage of differentiation and proliferate aberrantly. In culture, these cells can be induced to differentiate terminally by a variety of agents and cytokines providing a good model system for investigating differentiation. An essential role for STAT3 in IL-6 induced macrophage differentiation of myeloid M1 cells was first suggested by the inability of IL-6R mutants lacking the STAT3 binding motif to generate growth arrest and differentiation (Figure 1) (Yamanaka *et al*., 1996). A critical role for STAT3 was further investigated through the use of a dominant-negative STAT3 mutant (STAT3DN). In M1 cells, overexpression of STAT3DN abolished the differentiation of these cells in response to IL-6 and LIF (Nakajima *et al*., 1996; Minami *et al*., 1996). As mentioned above, IL-6 exposure normally leads to morphological changes and induction of specific gene expression characteristic of terminally differentiated macrophages. In M1 STAT3DN-transformants these changes did not occur suggesting a block in macrophage differentiation. This differentiation is accompanied by a gradual inhibition of cell growth which again was not observed in the STAT3DN cell lines. Two genes known to be important for this IL-6 induced growth arrest and induction of differentiation are c-*myc* and c-*myb* (Hoffman-Liebermann and Liebermann, 1991). Indeed STAT3DN inhibited the normal IL-6 induced repression of c-*myc* and c-*myb* suggesting that these two genes may be *bona fide* targets of STATs regulating hematopoietic proliferation versus differentiation. Interestingly, the presence of dominant-negative STAT3 converted the action of IL-6 from a differentiation to proliferative agent suggesting that IL-6 generates antagonistic signals in which STAT3 is critical in determining whether cells differentiate or proliferate in response to cytokine challenge. In another study using a mouse myeloid cell line, LGM-1, exogenously expressing the G-CSF receptor (G-CSFR), STAT3 was implicated in neutrophil differentiation (Shimozaki *et al*., 1997). Addition of G-CSF to the G-CSFR cells resulted in a block in proliferation, down-regulation of c-*myc* and differentiation into cells with lobulated nuclei. Introduction of a dominant-negative STAT3 prevented both the growth arrest and morphological changes observed by G-CSF treatment.

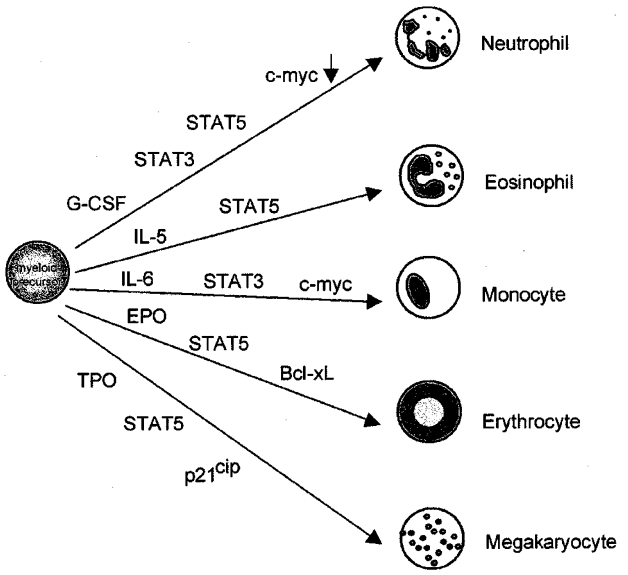


**Figure 1** Schematic representation of the role of STAT3 and STAT5 in '*in vitro*' myeloid differentiation. Cytokines important for the different lineages, the STATs activated by these cytokines and potentially important target genes are indicated

Erythropoietin (EPO) plays an important role in the proliferation and differentiation of erythroid progenitors. EPO has been found to induce the phosphorylation and activation of STAT5 but not STAT1$\alpha$ or STAT3 in the UT-7 subline UT-7/GM (Kirito *et al*., 1997). In these cells EPO treatment also stimulates erythroid differentiation. Interestingly, treatment of UT-7/GM with GM-CSF activates both STAT1$\alpha$ and STAT3 and inhibits EPO-induced erythropoiesis. Overexpression of STAT1$\alpha$ or STAT3 also suppressed EPO-induced differentiation, while dominant-negative forms of these two STATs prevented the inhibitory effect of GM-CSF on erythropoiesis (Kirito *et al*., 1998). This inhibitory effect of STAT1$\alpha$ on differentiation appears to be related to the regulation of cell cycle progression, a process thought to be critical for development of erythroid progenitors (Carroll *et al*., 1995). It has also been demonstrated that a dominant-negative STAT3 induced morphological differentiation of embryonic stem cells (Boeuf *et al*., 1997). These data are in contrast to the STAT3 induced differentiation of M1 cells to macrophages discussed above (Nakajima *et al*., 1996). Thus the ability of a specific STAT to induce or inhibit the process of myelopoiesis appears to be cell type specific. In a similar study utilizing the erythroleukemic cell line TF-1, STAT5 has been implicated in erythropoietin-induced proliferation (Chretien *et al*., 1996). TF-1 cells express a truncated EPO-receptor (EpoR) that is defective in STAT5 activation and induces erythroid differentiation rather than proliferation. Transduction of a wild-type receptor into TF-1 cells restores the ability of EPO to activate STAT5 and results in proliferation rather than differentiation. These data suggest that EPO-induced differentiation correlates with impaired EPO-induced STAT5 activation. In contrast, utilizing EpoR mutants, Iwatsuki *et al*. (1997) found that the activation of STAT5 leads to erythroid differentiation in the murine ELM-I-1 erythroleukemia cell line. Furthermore, overexpression of a dominant-negative STAT5 suppressed EPO-dependent erythroid differentiation. These discrepancies may be due to differences in cell lines and highlight the problems of an *in vitro*, cell line-based analysis of STAT function, where cells may be lacking other critical components of the differentiation process. Indeed, the expression of mutant EpoRs into fetal liver cells derived from EpoR (−/−) mice has revealed that the receptor that best reconstitutes erythropoiesis does not correlate with the ability to activate STAT5 but rather the phosphatidylinositol lipid kinase, PI3K (Klingmuller *et al*., 1997).

The role of STAT5 during the differentiation of myelomonocytic cells has been investigated using both the U937 promonocytic cell line and promyelocytic HL-60 cells (Woldman *et al*., 1997). STAT5 was found to be activated by several inducers of monocytic and granulocytic differentiation. Myelomonocytic growth

factor (cMGF) induced proliferation and differentiation of chicken myeloid progenitor cells was also analysed. Addition of cMGF resulted in the rapid activation of STAT5 and the onset of myelomonocytic differentiation. These data hint at a role for STAT5 in the functional changes accompanying the maturation of myeloid progenitor cells to a terminally differentiated stage. A role for STAT5 in regulating proliferation is further supported by studies in which a dominant-negative STAT5 partially inhibited growth of an IL-3 dependent cell line (Mui *et al*., 1996). Expression of a dominant-negative STAT5A in the murine myeloid cytokine-dependent line, 32D, inhibited both IL-3 dependent proliferation and G-CSF dependent differentiation without the induction of apoptosis (Ilaria *et al*., 1999). Importantly, expression of this interfering mutant in primary murine bone marrow inhibited G-CSF dependent granulocyte colony formation *in vitro*.

Thrombopoietin (TPO) is the major regulator of megakaryocyte differentiation and platelet production acting through the TPO-receptor, c-mpl. A 10aa intracellular deletion of c-mpl was found to completely abrogate STAT activation while permitting proliferation and survival of transfected cell lines (Dorsch *et al*., 1997). The ability of TPO to induce megakaryocytic differentiation of UT-7 cells expressing wild-type (c-mpl) or mutant (c-mpl$\Delta$10) receptors was recently analysed (Dorsch *et al*., 1999). Whereas TPO-treated cells expressing c-mpl show a striking increase in size, adherence and expression of the megakaryocytic marker CD41a, c-mpl$\Delta$10 UT-7 cells remained morphologically unchanged. In the CMK megakaryoblastic leukemia cell line, TPO treatment results in megakaryocytic differentiation and induction of the cell cycle inhibitor $p21^{CIP}$. Ectopic expression of $p21^{CIP}$ is in itself sufficient to induce differentiation (Matsumura *et al*., 1997) and STAT5 was found to be able to interact with oligonucleotide probes corresponding to STAT-binding sites within the $p21^{CIP}$ promoter. This suggests that a possible mechanism by which TPO can induce megakaryocytic differentiation is through STAT5-dependent induction of $p21^{CIP}$ resulting in cell cycle inhibition and induction of a specific developmental program.

The contradictory results often observed between cell lines utilizing ectopic overexpression of wild-type or dominant-negative STAT proteins may be explained by considering a role for the 'level' of activation governing a specific response. In this model, high-level activation may in fact be inhibitory by the induction of 'aspecific' genes not normally associated with activation of endogenous STAT proteins (Mui, 1999). This is demonstrated by the low level of STAT5 activation and induction of differentiation observed when TF1 cells are treated with EPO due to the expression of a mutant receptor (Chretien *et al*., 1996). However, as mentioned above, introduction of a wild-type receptor results in high-level STAT5 activation and stimulates proliferation rather than differentiation. This may also explain the ability of IL-3 to inhibit EPO-dependent differentiation in transfected BaF3 cells since in this system IL-3 is a potent STAT5 activator, while EPO is a weak STAT5 activator (Krosl *et al*., 1996). The concept of activation levels as being important for the regulation of a differentiation outcome has been demonstrated for other transcription factor families. It is also likely that the interplay between STATs and their regulators e.g. CIS, PIAS will govern the strength of a cytokine-induced transcriptional response defining the decision to proliferate or differentiate.

### *Specificity in STAT function*

With four JAK kinases and six STAT proteins there seems to be a limited specificity in this signaling pathway. However, specificity can be enhanced by several different mechanisms. Firstly, upon cytokine stimulation the activated STAT transcription factors are present both as homo-dimers and hetero-dimers (Leonard and O'Shea, 1998). Secondly, different STAT binding sequences have different affinities for the different homo- and hetero-dimers (Lamb *et al*., 1995; Seidel *et al*., 1995). Thirdly, besides tyrosine phosphorylation, serine phosphorylation of the STAT proteins seems to be essential for STATs to trans-activate target genes (Wen *et al*., 1995; Yamashita *et al*., 1998). Although the precise kinases involved in serine phosphorylation of all the different STATs have yet to be defined it is very likely that fine-tuning of the response can take place via controlled activation of these kinases.

Interestingly, the cellular context of different cytokine receptors seems to be involved in determining the usage of the different STAT proteins. A clear example for this level of control is found in neutrophils. The IFN$\gamma$R activates STAT3 in this cell type (Caldenhoven *et al*., 1999b), whereas this receptor only activates STAT1 in human eosinophils (van der Bruggen *et al*., 1995). In addition, GM-CSF can activate STAT1 in eosinophils (van der Bruggen *et al*., 1995), whereas the GM-CSFR does not engage STAT1 in normal human neutrophils (Al-Shami *et al*., 1998). This remarkable level of control of the pathway is not caused by the mere availability of the different STAT proteins, because in both neutrophils and eosinophils sufficient amounts of the STAT1 and STAT3 proteins are present. Currently no data are available to explain these phenomena, but these findings clearly point to the importance of the relevant cellular context.

### *Differential splicing, protein processing and functional diversity*

As described above, STAT3 activation by G-CSF is implicated in neutrophilic differentiation (Shimozaki *et al*., 1997; Chakraborty and Tweardy, 1998a,b; Ward *et al*., 1999a,b). A more detailed examination of STAT3 activation by G-CSF showed that multiple isoforms of STAT3 are activated by G-CSF. Three distinct isoforms of STAT3 have been described, that all arise from a single gene (Figure 2). STAT3$\beta$, an alternatively spliced form of STAT3$\alpha$ (full length STAT3), lacks the c-terminal 55 amino acids (Schaefer *et al*., 1995; Caldenhoven *et al*., 1996). In addition, a 72 kDa STAT3$\gamma$ protein was described that is also truncated at its c-terminus and is derived from STAT3$\alpha$ by limited proteolysis (Chakraborty and Tweardy, 1998a). In immature human myeloid cells capable of differentiation into neutrophils in response to G-CSF, STAT3$\beta$ is the predominant isoform activated by G-CSF (Chakraborty *et al*., 1996). Interestingly, in leukemic

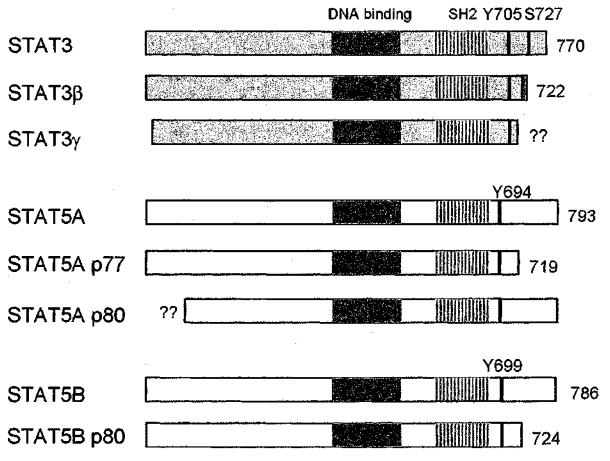


**Figure 2** Schematic representation of STAT isoforms implicated in myeloid differentiation. Y, tyrosine residue phosphorylated by JAKs. S727, serine residue implicated in modulation of STAT3 transcriptional activation potential. ??, precise nature of isoform unknown

cells which are refractory to G-CSF induced differentiation, both STAT3$\alpha$ and STAT3$\beta$ are activated. Overexpression of STAT3a even renders cells partially resistant to G-CSF induced differentiation (Chakraborty *et al*., 1999). By contrast, in terminally differentiated neutrophils, STAT3$\gamma$ is activated by G-CSF (Chakraborty and Tweardy, 1998a). Finally, there is a marked change in STAT3 isoform expression during the myeloid differentiation of normal human CD34+ cells (Biethahn *et al*., 1999). STAT3$\beta$ is functionally distinct from STAT3$\alpha$ and behaves as a dominant negative repressor in some, but not all cell types (Schaefer *et al*., 1997; Sasse *et al*., 1997; Caldenhoven *et al*., 1996). Together with the finding that STAT3$\alpha$ activation is closely linked with the regulation of proliferation, these findings suggest that composition of the STAT3 dimer is relevant for the decision between proliferation and differentiation. STAT3$\beta$ containing dimers might well regulate differentiation-related genes, while STAT3$\alpha$ containing dimers are likely to induce genes that are part of the G-CSF-induced proliferation program (Chakraborty and Tweardy, 1998b).

For STAT5, the situation is even more complex. STAT5A and STAT5B are encoded by two distinct genes, that clearly have both distinct as well as overlapping functions (Udy *et al*., 1997; Liu *et al*., 1997; Teglund *et al*., 1998). Splicing variants of STAT5A (STAT5A1 and A2) and STAT5B (STAT5B$\delta$40C) have been cloned from rat mammary gland and liver, but the functional significance of these isoforms has not been addressed yet (Ripperger *et al*., 1995; Kazansky *et al*., 1995). The first suggestion that different STAT5 isoforms contribute to IL-3 mediated signaling in myeloid cells was made by Azam *et al*. (1995). In mature murine myeloid cell lines, IL-3 activates STAT5A and STAT5B (Azam *et al*., 1995). By contrast, in more immature murine myeloid cell lines, IL-3 activates c-terminally truncated forms of STAT5A (p77) and STAT5B (p80) (Figure 2). Similar activation of p77 and p80 was also observed with IL-2 and EPO in murine cell lines (Wang *et al*., 1996). Moreover, p77 and p80 expression was also observed in immature mouse macrophages, while full-length STAT5 was more predominant in mature macrophages (Lokuta *et al*., 1998). However, although peritoneal macrophages express far more full length STAT5 than p77/p80, GM-CSF only activates p77/p80 in these cells (Welte *et al*., 1997). These c-terminally truncated p77 and p80 appear to have distinct properties compared to full length STAT5. In transient transfection experiments, they fail to support prolactin-induced activation of the $\beta$-casein promoter, but rather act as dominant negative regulators of transcription (Moriggl *et al*., 1996). Similarly, overexpression of synthetic p77 or p80 in 32D or BaF3 cells blocks the IL-3 induced expression of STAT5 dependent genes, such as CIS and OSM (Wang *et al*., 1996; Mui *et al*., 1996). The same phenomenon is observed when IL-3 induced gene expression is compared between immature and mature murine hematopoietic cells (Azam *et al*., 1997). In murine cells, p77 and p80 do not result from alternative splicing, but are generated by protein processing. STAT5 is processed by a nuclear serine endopeptidase which is expressed in early murine myeloid, but not lymphoid progenitor cell lines (Azam *et al*., 1997; Meyer *et al*., 1998; Lee *et al*., 1999). This protease cleaves STAT5A and 5B in a perfectly conserved c-terminal motif ATY*MDQA. When this motif was mutated, STAT5 becomes resistant to proteolytic cleavage (Lee *et al*., 1999). In a preliminary characterization, cleavage-resistant STAT5B partially induced differentiation of murine FDC-P1, while wild-type STAT5 supported proliferation and self-renewal of these cells. These observations clearly demonstrate that different STAT5 isoforms are likely to perform different functions in immature and mature murine cells.

In freshly isolated human monocytes, a p80 STAT isoform was activated by GM-CSF (Rosen *et al*., 1996). However, since this p80 reacts with antibodies directed to the c-terminus of STAT5, this p80 protein is likely to differ from the p80 observed in early murine myeloid cells. A c-terminally truncated p80 STAT5 was found to be activated by IL-5 and GM-CSF in freshly activated human eosinophils and neutrophils (Caldenhoven *et al*., 1999a). Activation of full length STAT5 by GM-CSF in human neutrophils was also reported, but due to the use of c-terminal antibodies p80 activation might have been missed (Al-Shami *et al*., 1998). Surprisingly, expression as well as GM-CSF/IL-5 induced activation of this p80 STAT5 protein is enhanced during differentiation of human primary CD34+ stem cells into eosinophils and neutrophils (Caldenhoven *et al*., 1998). p80 containing STAT5 complexes are able to bind to elements which STAT5A or 5B dimers are unable to bind, such as the IRE from the ICAM-1 promoter (Caldenhoven *et al*., 1998; 1999a). This should greatly enhance the spectrum of genes that can be regulated by GM-CSF in these cells. Although further experiments are needed to explain the different regulation of p80 during human versus mouse myeloid differentiation, these data strongly suggest that differential usage of STAT5 isoforms generated by protein processing or RNA splicing is likely to contribute to the regulation of STAT5-dependent gene expression in myeloid cells.

*Hematopoeisis in STAT null-mutant mice*

Recently the physiological role of each individual STAT protein has been examined through the analysis of STAT 'knockout' mice harboring a null allele for the particular gene. Interestingly, the generation of several STAT (−/−) mice has revealed a surprising specificity in the role of members of this transcription factor family in hematopoietic development and function. While STAT1 has been shown to be activated by a variety of cytokines and growth factors, the generation of STAT1 (−/−) mice demonstrated selective defects in their response to interferons (Durbin *et al*., 1996; Meraz *et al*., 1996). Mice were highly susceptible to opportunistic infections and extraordinarily sensitive to viral pathogens. They exhibited normal lymphoid and myeloid development suggesting that STAT1 does not play a critical role in hematopoiesis but rather is necessary for induction of genes required for normal host defence.

STAT4 has been reported to be fairly widely expressed including during early myeloid differentiation (Yamamoto *et al*., 1994). However, it appears to be exclusively activated by IL-12 and indeed in STAT4 (−/−) mice the major IL-12 mediated functions are perturbed (Kaplan *et al*., 1996a; Thierfelder *et al*., 1996). These include IFN$\gamma$ production, cellular proliferation, enhancement of natural killer cell cytotoxicity and Th1 cell differentiation. The mice were viable, fertile and had no defects in hematopoiesis suggesting that the developmentally regulated myeloid expression fulfils a redundant, non-essential function. In a similar fashion, STAT6 (−/−) mice have defects in IL-4 mediated functions including immunoglobulin class switching, T-cell proliferation and Th2 differentiation demonstrating the essential role of STAT6 in IL-4 signaling (Shimoda *et al*., 1996; Takeda *et al*., 1996; Kaplan *et al*., 1996b). Again, the lack of effects of STAT6 deletion on the development of granulocytes, monocytes and erythrocytes argues against a role for this transcription factor in regulating myeloid differentiation.

STAT3 has been demonstrated to be activated by a variety of cytokine receptors including G-CSF and members of the IL-3/IL-5/GM-CSF family (reviewed in Chakraborty and Tweardy, 1998b; de Groot *et al*., 1998). STAT3-deficient mice were found to die early in embryogenesis prior to gastrulation (Takeda *et al*., 1997). Since STAT3 mRNA is expressed in the extraembryonic visceral endoderm, the principle site of nutrient exchange, it is thought that lethality may be due to nutritional insufficiency. An elegant investigation of STAT3 function was performed by Takeda *et al*. (1999) who generated mice with a cell type-specific disruption of STAT3 in macrophages and neutrophils utilizing the Cre-*lox*P recombination system. These mice have allowed an analysis of the role of a specific STAT in the effector functions of specific subsets of myeloid cells. The mutant mice were highly susceptible to endotoxin shock with increased production of inflammatory cytokines such as TNF$\alpha$, IL-1 and IFN$\gamma$. Production of these cytokines from STAT3-deficient macrophages is dramatically augmented in response to lipopolysaccharide. Furthermore, the suppressive effects of IL-10 on macrophages and neutrophil cytokine production was completely abolished. These results indicate that STAT3 plays a critical role in controlling the anti-inflammatory response of certain myeloid cells although it doesn't provide answers as to the role of this STAT in myeloid differentiation.

While STAT5 was initially identified in mammary gland tissue as a prolactin-induced transcription factor, it has subsequently been found to be activated by a plethora of cytokines, growth factors and hormones. STAT5 is encoded by two highly related genes whose proteins, STAT5a and STAT5b, are 96% homologous (Liu *et al*., 1995). There are subtle differences in tissue expression of these two isoforms and they also exhibit distinct DNA-binding specificities. STAT5a knockout mice developed normally but female mice failed to lactate after parturition due to a lack of mammary gland terminal differentiation (Liu *et al*., 1997). These mice have been further analysed to study the effects of STAT5a deletion on the activation of myeloid cells by GM-CSF (Feldman *et al*., 1997). STAT5a null mice had normal numbers of nucleated cells from bone marrow (BM) suggesting no specific defects in myeloid development. BM culture-derived macrophages were found to be deficient in the GM-CSF induced expression of the *CIS* and the bcl-2 like *A1* gene which are thought to be important for regulating cellular growth, survival and differentiation. Analysis of the proliferative responses of BM-derived macrophages revealed no differences between wild-type and null-mutant mice at low concentrations of GM-CSF ($<0.3$ ng/ml). However, at higher GM-CSF concentrations ($>0.5$ ng/ml), there was a significant decrease in growth in STAT5a (−/−) mice. A more recent study has analysed the effect of deleting STAT5a and STAT5b either individually or simultaneously (Teglund *et al*., 1998). The STAT5a/b (−/−) mice were much smaller than their wild-type littermates probably due to significant defects in growth hormone function. In addition, the STAT5a/b double mutant female mice were found to be infertile, unlike the single allele deletions. This correlated with a lack of $p27^{KIP1}$ expression in the ovaries of these animals, a gene previously shown to be required for ovarian function. There were no significant differences in the number of red cells, hemoglobin levels or numbers of platelets between the various mice. The lack of any effect on erythroid development is in contrast to studies that have implicated STAT5 in erythropoiesis (Damen *et al*., 1995; Iwatsuki *et al*., 1997). By contrast, Socolovsky *et al*. (1999) have shown that STAT5a/b (−/−) mice suffer from fetal anemia and apoptosis of red cell progenitors. This is likely to be caused by a defect in Epo-induced expression of the anti-apoptotic Bcl-xL gene. The normal steady-state adult hematocrit observed in adult mutant mice might be caused by compensatory mechanisms, such as the extramedullary expansion of erythroid tissue (Morrigl *et al*., 1999) or upregulation of STAT1 or STAT3 (Teglund *et al*., 1998).

In STAT5a/b (−/−) mice but not STAT5a or STAT5b knockouts, there was a decrease in the white cell count that was associated with a decrease in lymphocytes. Conversely there was an increased percentage of neutrophils. Bone marrow hematopoietic progenitor colony assays revealed no difference in erythroid colony forming units in concurrence with the normal number of erythrocytes observed in the

peripheral blood. However, there was a reduction in the number of colonies induced in response to IL-3, IL-5, GM-CSF or G-CSF and the colonies were smaller. While this implicates STAT5 in mediating IL-3/IL-5/GM-CSF mediated regulation of proliferation it does not seem to play a major role in differentiation as such.

The data obtained from knockout mice has demonstrated surprisingly specific roles for the various STAT transcription factors *in vivo* despite redundancy in their activation profiles by cytokines *in vitro*. These data challenge many of the *in vitro* studies discussed above which have implicated STATs in myeloid differentiation. It will require further work analysing combinations of STAT null-mutants to determine whether there may be functional redundancy in the knockout mice masking an important role for STATs in myeloid hematopoiesis.

### *STAT target genes in myeloid differentiation and function*

Several STAT target genes have been identified that play a role in regulating proliferation and these have been discussed briefly in previous sections of this review. As previously discussed, these genes are likely to play a critical role in deciding the balance between proliferation and differentiation thereby regulating myeloid cell fate decisions. This is discussed here further. One of the first STAT target genes to be identified was the cytokine-inducible SH2-containing protein (CIS), subsequently found to be part of a large family of regulatory molecules (Yoshimura, 1998). CIS was found to be induced by a wide variety of cytokines including GM-CSF, IL-3 and EPO, and there are STAT-binding sites present in the *CIS* promoter (Matsumoto *et al*., 1997; Verdier *et al*., 1998). In bone marrow derived macrophages from STAT5a $(-/-)$ mice, GM-CSF induced expression of *CIS* was greatly reduced demonstrating a critical role for STAT5a in its transcriptional regulation (Feldman *et al*., 1997). CIS associates with the phosphorylated tyrosine residues of cytokine receptor signaling sub-units and overexpression acts to reduce the growth rate of cells. By regulating expression of CIS, or CIS-related proteins, cells may be able to control the level and duration of STAT activation which may play a critical role in the decision to proliferate or differentiate. Of course regulation of differentiation by STATs is unlikely to be limited to the regulation of just one or two genes. In an analysis of IL-3 induced genes the introduction of a dominant-negative STAT5 reduced the expression of c-*fos*, *CIS*, *OSM* and *pim-1* (Mui *et al*., 1996). This is accompanied by a partial inhibition of IL-3 dependent growth. Thus it is likely that the regulation of multiple targets rather than a single 'master gene' that defines a cells choice to proliferate or differentiate.

Further players regulating apoptosis have also been demonstrated to be regulated in a STAT-dependent manner. In the STAT5a deficient macrophages, described above, GM-CSF induced expression of the bcl-2 like gene, *A1*, is greatly reduced (Feldman *et al*., 1997). Overexpression of *A1* in the IL-3 dependent 32Dc13 cell line leads to a reduction of cell death upon cytokine withdrawal (Lin *et al*., 1996). Interestingly, ectopic expression of A1 permits the accumulation of differentiated myeloid cells both before and after IL-3 withdrawal. Thus regulation of A1 expression may not only protect cells against apoptosis but also play a role in deciding the differentiation state. In mouse neutrophils, A1 is constitutively expressed and essential for neutrophil survival (Orlofsky *et al*., 1999; Hamasaki *et al*., 1998).

STAT5a/b $(-/-)$ mice suffer from fetal anemia and apoptosis of red cell progenitors (Socolovsky *et al*., 1999). A principle function of EPO is to rescue committed erythroid progenitors from apoptosis. The null mutant embryos are severely anemic with low numbers of erythroid progenitors which show higher levels of apoptosis and are less responsive to EPO. This is thought to be due to the inability of EPO to activate specific STAT5 target genes. One of these was identified as the anti-apoptotic Bcl-xL gene through direct binding to its promoter.

At least in lymphocytes, the cell cycle inhibitor $p27^{KIP1}$ appears to be a target of STAT activation (Kaplan *et al*., 1998). In STAT6 $(-/-)$ mice the normal proliferative response of lymphocytes to IL-4 is compromised. This appears to be due to dysregulated expression of $p27^{KIP1}$ caused by an inability of STAT6 to down-regulate $p27^{KIP1}$ expression. As previously noted, the induction of myeloid differentiation is preceded by a reduction in proliferation. STAT3 is critical in the IL-6 induced growth arrest and differentiation of M1 leukemic cells (Nakajima *et al*., 1996). Among the genes regulated by IL-6, the repression of c-*myc* and c-*myb* expression are likely to play a crucial role in the process of growth arrest and differentiation (Hoffman-Liebermann and Liebermann, 1991; Selvakumaran *et al*., 1992). IL-6 was also found to induce the cell cycle inhibitor $p19^{INK4D}$ in a STAT3 dependent fashion (Narimatsu *et al*., 1997). Growth arrest and megakaryocytic differentiation of CMK cells by TPO has also been reported to be accompanied by the induction of $p21^{WAF/CIP}$ most likely through STAT5 (Matsumura *et al*., 1997). Furthermore, ectopic overexpression of $p21^{WAF/CIP}$ was sufficient to induce megakaryocytic differentiation of CMK cells dependent on its ability to induce cell cycle arrest. $p21^{WAF/CIP}$ has also been found to be an immediate early response gene induced during the differentiation of HL-60 cells along the granulocyte or monocyte-macrophage pathway (Jiang *et al*., 1994).

Fukada *et al*. (1998) have analysed the role STAT3 plays in $G_1$ to S phase cell cycle progression induced by the gp130 cytokine receptor sub-unit. The gp130-mediated growth signaling was due to the upregulation of the CDK-activators cyclins D2, D3 and A and the concomitant down-regulation of $p21^{WAF/CIP}$ and $p27^{KIP1}$. Furthermore, STAT3 was found to rapidly activate the c-*myc* gene by directly binding to its promoter (Kiuchi *et al*., 1999). This regulation is specific for STAT3 and, for example, the c-*myc* promoter is not regulated by STAT5. A recent study has employed a constitutively active STAT5a mutant in the IL-3 dependent BaF3 cell line to analyse the regulation of proliferation and apoptosis (Nosaka *et al*., 1999). This mutant STAT5a was constitutively tyrosine phosphorylated and induced expression of *Bcl*-xL, *pim*-1, and c-*myc* in the absence of IL-3. Surprisingly, IL-3 treatment of cytokine-deprived BaF3 cells expressing active STAT5a resulted in apoptosis or differentiation followed by death. This was preceded by high expression of the growth

inhibitory genes such as *CIS*, *JAB* and $p21^{WAF/CIP}$. Constitutive expression of JAB proved sufficient to induce apoptosis while $p21^{WAF/CIP}$ could induce differentiation. Furthermore, constitutive expression of *pim*-1 resulted in IL-3 independent growth of BaF3 cells and *pim*-1 expression was found to be elevated in factor-independent leukemic cells, such as K562, harboring constitutively active STAT5. These findings suggest that STAT5a can regulate cell fate by varying the intensity and expression of components of the cell cycle machinery as well as anti-apoptotic genes.

Besides genes that play a role in myeloid differentiation, several genes that encode for important modulators of mature myeloid cells contain STAT binding sides in their promoter regions. The high affinity Fc$\gamma$RI (CD64) is activated by G-CSF in human neutrophils by STAT1 and STAT3 binding to the IFN-inducible GRR in the Fc$\gamma$RI promoter (Bovolenta *et al*., 1996). Similarly, Intercellular adhesion molecule 1 (ICAM-1), an important regulator of leukocyte adhesion, is induced by IFN$\gamma$ and IL-6 in a STAT-dependent fashion (Naik *et al*., 1997; Caldenhoven *et al*., 1994). Therefore, STATs are also likely to be of importance for terminally differentiated myeloid cells.

## The role of STATs in dysregulated myelogenesis

As described above, STAT proteins are essential for cytokine-regulated processes such as cellular proliferation, differentiation as well as survival. However, more recently it has become evident that aberrant activation of STAT proteins is often associated with cellular transformation by various oncoproteins. More importantly, activation of STATs 1, 3 and 5 was reported in several human malignancies, including lymphomas, leukemia's and breast carcinoma (reviewed in Garcia and Jove, 1998). In this section we will describe the role of activated STATs in myeloid malignancies.

### In vitro *transformation of myeloid cells by v-Src*

Myeloid malignancies are often characterized by arrested maturation and cytokine-independent proliferation of myeloid progenitors. This process can be mimicked *in vitro* with the myeloblastic 32Dcl3 cell-line. Expression of v-Src in these cells completely abrogates IL-3 dependence of these cells and blocks their ability to terminally differentiate into granulocytes in response to G-CSF (Kruger and Anderson, 1991). 32Dcl3 cells expressing v-Src contain constitutively active STAT1, STAT3 and STAT5, of which only STAT3 could be co-immunoprecipitated with v-Src (Chaturvedi *et al*., 1997). Activation of these STATs by v-Src is critically dependent on the SH2 and SH3 domains of v-Src, and strictly correlates with its ability to cause cytokine-independent growth (Chaturvedi *et al*., 1997). Although direct evidence that STAT activation by v-Src contributes to the abrogation of IL-3 dependence is currently lacking, IL-3 induced STAT3 activation was reported to contribute to the proliferation of these cells (Chaturvedi *et al*., 1998). Interestingly, c-src rather than JAK2 might be the kinase that activates STAT3 in normal IL-3 stimulated 32Dcl3 cells (Chaturvedi *et al*., 1998). More direct evidence that STAT3 activation can contribute to cellular transformation comes from experiments in fibroblast cell lines. In these cells, v-Src expression causes the constitutive activation of STAT3 (Yu *et al*., 1995; Cao *et al*., 1996). Interestingly, experiments using dominant negative STAT3 mutants strongly suggest that STAT3 activation is required for transformation by v-Src (Bromberg *et al*., 1998; Turkson *et al*., 1998, 1999). Recently, it was shown that interfering with STAT3 signaling *in vivo* induces potent anti-tumor activity in mice. Gene therapy by electroinjection of DN-STAT3 into pre-existing B16 melanoma tumors caused inhibition of tumor growth as well as tumor regression (Niu *et al*., 1999). Moreover, a synthetic constitutively active (CA) STAT3 mutant can mediate cellular transformation in fibroblasts (Bromberg *et al*., 1999). More recently, STAT5 activation by v-Src was also implicated in the transformation of fibroblasts (De Groot *et al*., 1999). These results strongly suggest that constitutive activation of STATs in myeloid leukemia model systems are likely to be involved in cellular transformation.

### *Activation of STATs in chronic- and acute myelogenous Leukemia (CML and AML)*

CML can be divided in a stable phase characterized by excessive proliferation and accumulation of myeloid cells in all differentiation states, such as myelocytes, metamyelocytes and neutrophils, followed by a blastic phase that resembles acute leukemia. CML is caused by the BCR/ABL chimeric oncogene, a constitutively active tyrosine kinase which is generated from the Philadelphia chromosome translocation (t(9;22)(q34;q11)) (Rowley, 1973). There are two distinct forms of Bcr/Abl, the consequence of different breakpoints on chromosome 22. p210 is the hallmark of CML, while p190 is found mostly in acute lymphoblastic leukemia (ALL). Although it is clear that the tyrosine kinase activity of BCR/ABL is essential for transformation (Daley *et al*., 1987; Daley and Baltimore, 1988), the actual mechanism by which BCR/ABL transforms cells remains largely unknown. Interestingly, BCR/ABL constitutively activates several signaling pathways (reviewed in Sattler and Salgia, 1997) that are also used by several cytokines, including those of the IL-5/IL-3/GM-CSF cytokine family (reviewed in De Groot *et al*., 1998). These pathways include the RAS-Erk2, the PI3Kinase and the JAK/STAT pathways. In addition, BCR/ABL activates the phosphatases SHP1 and SHP2, which also play a pivotal role in IL-5/IL-3/GM-CSF signaling.

The first notion that activation of STATs might be of importance to CML came from experiments performed by Carlesso *et al*. (1996), who demonstrated constitutive activation of STAT1 and STAT5 in human CML cell lines as well as in cytokine dependent cell lines transformed by Bcr/Abl. Interestingly, the JAK kinases were not constitutively active in any of these cells, suggesting that STAT1 and STAT5 can be directly activated by Bcr/Abl (Carlesso *et al*., 1996). Since then, multiple reports have confirmed the activation of STAT5 (and to a lesser extent STAT1 and 3) in both primary blood cells and cell lines from CML patients as well as cell lines transformed *in vitro* by Bcr/Abl (Ilaria and Van Etten, 1996; Frank and Varticovski, 1996; Weber-Nordt *et al*., 1996; Shuai *et*

*al*., 1996; Chai *et al*., 1997; Nieborowska-Skorska *et al*., 1999). Although some of these reports also observe constitutive JAK activation in Bcr/Abl transformed cells, the failure of dominant-negative JAK mutants to block Bcr/Abl induced STAT5 activation strengthens the notion that Bcr/Abl directly activates STATs (Ilaria and Van Etten, 1996). Interestingly, although Bcr/Abl expression in myeloid cells frequently results in the induction of IL-3/GM-CSF autocrine loops, blocking antibodies to these cytokines failed to inhibit constitutive STAT5 activation in several CML cell lines (Chai *et al*., 1997). Both p210 and p190 were able to activate STATs, although some smaller difference in efficacy were reported between the two (Ilaria and Van Etten, 1996; Frank and Varticovski, 1996). Initial Bcr/Abl mutagenesis studies demonstrated that STAT5 activation is strictly dependent on Bcr/Abl kinase activity, and strongly correlates with its ability to confer cytokine independent growth in hematopoietic cells (Shuai *et al*., 1996).

Since STAT5 was shown to be necessary for cellular proliferation induced by the IL-5/IL-3/GM-CSF cytokine family (Mui *et al*., 1996) and overexpression of constitutively active (CA) STAT5 stimulates cell proliferation (Onishi *et al*., 1998; Nosaka *et al*., 1999), it seems conceivable that it might also contribute to cellular transformation by BCR/Abl. Recently, it was indeed shown that dominant negative STAT5 decreases STAT5-dependent transcription as well as growth in soft agar in K562 CML cells (De Groot *et al*., 1999). Using a different approach, a similar conclusion was drawn by Nieborowska-Skorska *et al*. (1999) who showed that STAT5 induction by Bcr/Abl is strictly dependent on its SH2 and SH3 domains. An SH2/SH3 mutant Bcr/Abl molecule functions as tyrosine kinase and activates Ras, but fails to induce IL-3 independence in 32D cells or cause leukemia in SCID mice (Nieborowska-Skorska *et al*., 1999). Importantly, these features could be rescued by a dominant active mutant of STAT5. Moreover, dominant negative mutants of STAT5 also inhibited IL-3 independence and leukemogenesis by wild-type Bcr-Abl. Taken together, these results strongly suggest that STAT5 regulated pathways contribute to Bcr/Abl induced leukemogenesis.

The role of STATs in AML is less clear than in CML. AML is characterized by a maturation arrest resulting in the accumulation of undifferentiated myeloid cells in bone marrow and peripheral blood (Pinto *et al*., 1998; Lowenberg and Touw, 1993). Growth and differentiation of AML cells can still be influenced by various cytokines, which in some patients are produced by the leukemic cells themselves. In contrast to CML, multiple different chromosomal translocations can be found. The first evidence for a potential role of activated STATs in AML was found by Gouilleux-Gruart *et al*. (1996) who found constitutive DNA binding of STAT3 (5/5) and STAT5 (2/5) in leukemic cells in the peripheral blood of five AML patients. These data were extended shortly after by others showing activation of STAT1, STAT3 and STAT5 in primary cells from patients with AML (Aronica *et al*., 1996; Weber-Nordt *et al*., 1996; Hayakawa *et al*., 1998; Xia *et al*., 1998). In some AML patients, STAT activity could be further regulated by exogenous cytokines, while others were completely cytokine-insensitive (Xia *et al*., 1998). In some AML cells, constitutive STAT3 phosphorylation is dependent on the autocrine/paracrine action of IL-6 produced by these cells (Schuringa *et al*., 1999). Activation of MAP kinase however, which is also frequently found in AML cells, did not correlate with STAT activity (Hayakawa *et al*., 1998). Further experiments are necessary to examine whether STAT activation plays a broad role in the leukemic properties of AML cells.

The first human tumor bearing a structurally abnormal STAT gene was recently reported (Arnould *et al*., 1999). In a patient with acute promyelocytic leukemia (APL), they found that the retinoic receptor alpha (RARA) gene, which is fused to the promyelocytic leukemia (PML) gene in most APLs, was fused with STAT5B. Interestingly, the RARA-STAT5B fusion protein was found in the nucleus of APL cells, where it might well act as a constitutive transcriptional activator. In AML cells containing constitutively active STAT3, a point mutation was identified in STAT3, resulting in a H-Y substitution in the n-terminus (Weber-Nordt *et al*., 1999). Whether this mutation is causally involved in STAT3 activation and AML is at present unclear.

### *STAT target genes and oncogenesis*

The results described above suggest the existence of STAT target genes that contribute to the transformed properties of CML and possibly also AML) cells. Since in CML cells, Bcr-Abl often causes resistance to apoptosis as well as cytokine-independent cellular proliferation, it seems conceivable that STAT target genes might be found among these genes involved in regulating cellular survival or cell cycle regulation (Figure 3).

Cell cycle progression is regulated by a complex molecular machinery of positive and negative regulators (Sherr and Roberts, 1999). Of these, cyclinD1, a

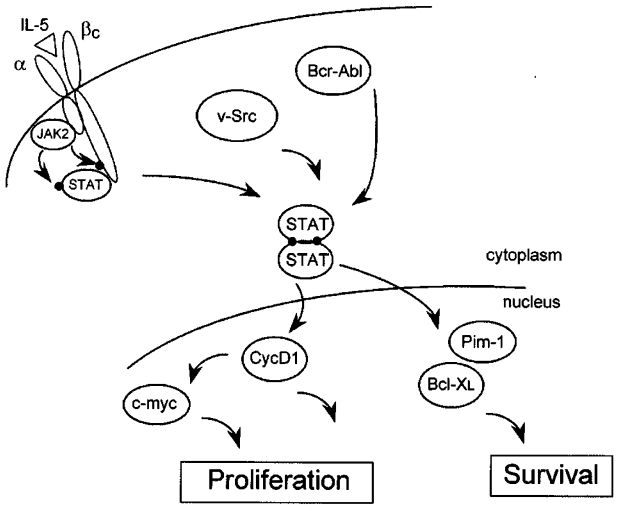


**Figure 3** STATs and oncogenic transformation: a model. Schematic representation of the role of activated STATs in cellular transformation by Bcr-Abl and v-Src. Upon activation in the cytoplasm by Bcr-Abl, v-Src of JAK kinases, STATs migrate into the nucleus and induce transcription of anti-apoptotic (pim-1 and bcl-xl) and proliferative (cyclinD1 and c-myc) genes. Signaling by the IL-5 receptor is included for comparison

positive regulator of G1 phase progression, is thought to play a major role in Bcr/Abl transformed cells. Cyclin D1 expression in Bcr/Abl transformed cells is dependent on the tyrosine kinase activity and the SH2 domain of Bcr/Abl (Afar *et al*., 1995; Yamada *et al*., 1996). Importantly, a transformation-defective SH2 mutant of Bcr/Abl could be rescued by overexpression of cyclinD1, but not cyclinE (Afar *et al*., 1995). Similarly, cyclinD1 was shown to be essential for G1 progression in Bcr/Abl transformed cells (Garcia and Jove, 1998; Williamson *et al*., 1997). More recently, it has become clear that cyclinD1 is a direct target gene for STATs. In BaF3 cells, IL-3 induced cyclinD1 expression was shown to be inhibited by DN-STAT5, while CA-STAT5 induced IL-3 independent cyclinD1 promoter activity (Matsumura *et al*., 1999). Similarly, CA-STAT3 also induces cyclinD1 expression (Bromberg *et al*., 1999). Indeed, the cyclin D1 promoter was shown to contain binding sites for STAT3 and STAT5 (Matsumura *et al*., 1999; Bromberg *et al*., 1999). Moreover, we have recently shown that in Bcr/Abl transformed cells, cyclinD1 promoter activity is up-regulated by a STAT5-dependent mechanism (de Groot *et al*., submitted).

Increasing evidence suggests that Bcr/Abl induced cyclinD1 expression is linked to up-regulation of c-myc expression. The cyclinD1-cdk4 kinase complex was shown to phosphorylate pRb in Bcr/Abl transformed cells, resulting in the release of active E2F1 (Williamson *et al*., 1997). E2F1 can enhance c-myc expression by direct binding to the c-myc promoter (Stewart *et al*., 1995). Since the transformation deficient Bcr/Abl SH2 mutant that can be rescued by cyclinD1 expression can also be complemented by c-myc (Afar *et al*., 1994) and DN-c-myc blocks transformation by Bcr/Abl (Afar *et al*., 1994; Sawyers *et al*., 1992), these results suggest the importance of the linear pathway Bcr/Abl – STAT5 – cyclinD1 – c-myc. However, recent evidence also suggests that STATs might directly regulate c-*myc* expression (Kiuchi *et al*., 1999).

CML cells generally exhibit enhanced resistance to apoptosis induced by chemotherapeutic drugs. Similarly, expression of Bcr/Abl in IL-3 dependent cells renders these cells resistant to apoptosis induced by cytokine-deprivation (Daley and Baltimore, 1988). Proteins of the Bcl-2 family are important regulators of the decision between survival or death in response to an apoptotic stimulus (Reed, 1998). Recent studies have shown that Bcr/Abl expression causes an upregulation of the level of Bcl-xL, an anti-apoptotic Bcl-2 family member, while the expression of bcl-2 was down-regulated (Amarante-Mendes *et al*., 1998a,b; Belhoussine *et al*., 1999). Similar observations were made in the CML cell line K562 (Amarante-Mendes *et al*., 1998b). Interestingly, enhanced Bcl-xL expression was recently reported to be crucial for the malignant progression of multiple myeloma by preventing apoptosis (Catlett-Falcone *et al*., 1999). This upregulation of Bcl-xL was shown to be mediated directly by constitutively activated STAT3 (Catlett-Falcone *et al*., 1999). Similarly, CA-STAT3 can induce Bcl-xL expression (Bromberg *et al*., 1999). A number of reports show that Bcl-xL expression can also be regulated directly by STAT5. IL-3 induced Bcl-xL expression can be blocked by DN-STAT5, resulting in enhanced apoptosis in BaF3 cells (Dumon *et al*., 1999). DA-STAT5 can induce Bcl-xL expression in the absence of cytokine stimulation (Nosaka *et al*., 1999). Finally, in STAT5A/5B knock-out mice, Epo-induced Bcl-xL expression is completely lost (Socolovsky *et al*., 1999). Our recent results suggest that this process might also contribute to apoptosis resistance in CML, since we have found that Bcr/Abl induced Bcl-xL promoter activity is dependent on activated STAT5 (de Groot *et al*., submitted). Although these results strongly suggest that up-regulation of Bcl-xL is important in CML, other anti-apoptotic proteins might also be dysregulated in CML. In this aspect it is noteworthy to mention that expression of the anti-apoptotic serine kinase pim-1 is also induced by DA-STAT5 in a cytokine-independent manner (Nosaka *et al*., 1999).

## Future prospects

As described above, STATs are thought to play an important role in the regulation of normal myeloid differentiation. In addition, accumulating evidence suggests that activated STATs might also play a pivotal role in the pathogenesis of human leukemia's, such as AML and CML. The identification of critical target genes regulating hematopoietic proliferation and differentiation will uncover the mechanisms by which the same cells become transformed. The recent availability of dominant active STAT molecules will be of great help in determining the genes which are involved in these diverse biological processes. Similarly, conditional STAT knockouts will provide valuable information on the precise function of distinct STATs in different cell types. Another challenging field will be the development of molecules with STAT-inhibiting properties, which might prove of great help in understanding and even controlling diseases such as AML and CML.

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