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The Synergy Between Stem Cell Factor (SCF) and Granulocyte Colony-stimulating Factor (G-CSF): Molecular Basis and Clinical Relevance

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Stem cell factor (SCF), an essential growth factor in normal hematopoiesis, exerts potent effects when combined with cytokines. In particular, its synergy with granulocyte colony-stimulating factor (G-CSF) results in important biologic responses. These include enhancement of *ex vivo* long-term expansion of human primitive hematopoietic cells and increased mobilization of peripheral blood progenitor cells (PBPC) for transplantation. Despite the clinical importance of the interaction between SCF and G-CSF, the absence of a model system in which it could be studied at the cellular level had impaired the ability to understand the basis of their co-operation. To overcome this impediment, a system was recently generated which recapitulates the biologic synergy between SCF and G-CSF. MO7e-G cells have allowed the identification of key events in the synergistic actions of these cytokines on proliferation and gene expression. Among the biochemical and molecular events mediated by these cytokines are the down-regulation of $p27^{kip1}$ and the independent phosphorylation of STAT3 on tyrosine and serine residues. Recent work has provided increasing evidence for the clinical importance of the combination of SCF and G-CSF. The elucidation of the intracellular events triggered by their receptors is now shedding light on key mediators of their synergistic effects. The identification of these pathways is of considerable importance for understanding fundamental aspects of hematopoiesis, and as potential targets for therapeutic intervention.

Keywords: Stem cell factor; Granulocyte colony stimulating factor; STAT3 Phosphorylation; p27kip1; Signal transduction

INTRODUCTION

Hematopoietic cell development and function involves a complex array of cytokines and growth factors. These molecules modulate the survival and proliferation of progenitors, drive differentiation commitment, and regulate end-stage cell functions. Stem cell factor (SCF) is one critical growth factor in hematopoiesis [1–3]. Reflecting its indispensable role in blood cell formation, the absence of SCF or its receptor c-kit results in perinatal lethality in the mouse [4,5]. In combination with cytokines, SCF results in a synergistic enhancement of the proliferation, differentiation, and survival of various hematopoietic lineages [6–21]. Its synergy with granulocyte colony-stimulating factor (G-CSF) is of particular significance in normal hematopoiesis. In addition, this interaction has important implications for *ex vivo* long-term expansion of human primitive hematopoietic cells and for mobilization of peripheral blood progenitor cells (PBPC) for transplantation. Despite the biologic and clinical importance of the synergistic effects of SCF and G-CSF, no cellular system had existed in which to study this interaction. Consequently, the integration of their receptor-triggered signaling pathways had remained poorly understood. The generation of the first cell line which recapitulates the biologic synergy between SCF and G-CSF, has allowed the identification of a number of intracellular pathways that mediate their biologic actions [22]. Recent work has provided increasing evidence for the clinical importance of the combination of SCF and

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TABLE I Biological and clinical synergy between SCF and G-CSF

System	Biological effect	References
Preclinical studies in vitro and ex vivo		
Bone marrow	Increased growth of:	
	Myeloid and erythroid lineages from normal BM	[12]
	Megakaryocyte lineage from normal BM	[17]
	Progenitor cells from BM failure syndromes	[9,10]
	Enhanced long term culture of BM-derived progenitor cells	[23]
Cord blood	Enhanced *ex vivo* expansion of cord blood progenitors	[24–28]
Preclinical studies in vivo in animals		
Rodents	Increase in neutrophils, lymphocytes, and reticulocytes	[6–8]
	Enhanced mobilization of PBPC	[29,32–34,45]
Dogs	Accelerated hematopoietic recovery after lethal irradiation	[35,36]
	Increased mobilization of PBPC	[31]
Primates	Increased mobilization of PBPC	[30,37]
Clinical trials in humans		
Enhanced mobilization of PBPC in patients with:		
Multiple myeloma		[43]
Breast cancer		[40–42]
Lymphoma		[38,39]

Abbreviations: BM, bone marrow; PBPC, peripheral blood progenitor cells.

G-CSF. Dissection of the signaling events downstream of their receptors is now revealing the mechanism of this synergy, and may provide the basis for therapeutic intervention.

THE SYNERGY BETWEEN SCF AND G-CSF HAS IMPORTANT BIOLOGIC AND CLINICAL IMPLICATIONS

SCF and G-CSF are crucial factors for long-term culture of human primitive hematopoietic cells [23], and play an important role in many protocols for the *ex vivo* expansion of human cord blood progenitors for hematopoietic transplantation [24–28] (Table I). *In vivo*, the combination of SCF and G-CSF increases the mobilization of PBPC over that seen with G-CSF alone [29–37]. Over the last few years, several clinical trials have proven this effect to be beneficial for cohorts of patients at risk for poor mobilization after highly cytotoxic pre-treatment for lymphoma [38,39], breast cancer [40–42], and multiple myeloma [43]. The combined use of SCF and G-CSF in the mobilization protocols used in these patients led to an increased median yield of CD34 + cells per leukopheresis, and to the subsequent reduction in the number of leukophereses required and in the proportion of patients failing to reach the target yield of CD34 + cells. In addition, a recent report has shown a trend toward a lower level of tumor cell contamination in the leukopheresis products of breast cancer patients mobilized with SCF and G-CSF compared to those mobilized with G-CSF alone [44]. In mice, the combination of SCF and G-CSF enhances the anti-leukemic effect mediated by the mobilized progenitors *in vivo* [45]. However, this potentially interesting finding has not been confirmed in humans.

Relatively little is known about the mechanism of mobilization of normal and malignant cells. Several groups showed that CD34 + cells mobilized by a variety of cytokines and/or regimens have a reduced expression of some adhesion molecules and a consequent modification of their capacity to adhere to bone marrow (BM) stroma [46–53]. Most of these studies have focused on phenotypic and functional modifications after G-CSF treatment. Moreover, the large number of adhesion molecules expressed during progenitor cell differentiation make these results difficult to interpret. A recent report showed a significant down-regulation of CD49d (VLA-4), CD11a (α subunit of LFA-1), and CD62L (L-selectin) after mobilization of PBPC with G-CSF, which correlated with reduced adherence of CD34 + cells to normal BM stroma [54]. In addition, the authors analyzed the effect of various cytokines on the phenotype and cell adherence properties of normal peripheral blood (PB) CD34 + cells *in vitro*. Surprisingly, this study found that SCF induces a significant increase of CD34 + cell adherence to preformed stroma and a significant enhancement of the expression of CD49d on these cells. However, in response to G-CSF, the expression of CD49d *in vitro* was also higher than that in the control, although this difference was not significant. It is noteworthy that these *in vitro* experiments were performed with normal PB CD34 + cells, while CD34 + cells derived from BM might have correlated better with the *in vivo* findings. No data regarding the combined effect of G-CSF and SCF were reported in this study.

Another recent report provided the first *in vivo* evidence that β1-integrins are involved in mobilization by showing that anti-VLA4 treatment induces mobilization of

progenitor cells [55]. Interestingly, mobilization induced by antibodies against CD49d/VLA-4 requires co-operative signaling events downstream of c-kit, the SCF receptor. Furthermore, these authors propose that SCF/c-kit signaling is a major common pathway involved in mobilization, integrating signaling through integrins and initiating cell migration. Indeed, mobilization of progenitor cells from BM into PB involves not only changes in the adhesive interaction between these progenitors and the BM stroma, but also their transmigration through the subendothelial basal lamina and the endothelial cell layer into the PB. This latter step requires the production of matrix-degrading enzymes such as type IV collagenases, also known as gelatinases or matrix metalloproteinases (MMPs). In this regard, one group recently reported that PB CD34 + cells have a high level of expression of MMP-2 and MMP-9, regardless of whether they were mobilized or not. In contrast, steady state BM CD34 + cells do not express MMP-2 and MMP-9, but can be induced to secrete them in response to several growth factor and cytokines. Moreover, this cytokine-induced secretion of gelatinases in BM CD34 + cells correlated closely with their capacity to transmigrate through reconstituted basal membrane (Matrigel) [56]. Interestingly, this study also showed that among the several cytokines and growth factors analyzed, only SCF and G-CSF induced significantly higher expression of MMP-2 and MMP-9 in BM CD34 + cells than in those from PB. Again, the synergistic effects that SCF and G-CSF might exert at this level were not examined. Further dissection of these molecular pathways remains a current and future challenge. However, this work is beginning to uncover the systems that govern the trafficking of progenitor cells from the BM to PB. By shedding light on the mechanisms involved in this process, it will be possible to understand the molecular basis for the synergistic effects of SCF and G-CSF in mobilizing PBPC.

MO7E-G CELLS RECAPITULATE THE PROLIFERATIVE SYNERGY BETWEEN SCF AND G-CSF

The absence of a model system in which to explore the synergy between SCF and G-CSF at the cellular level had impaired the ability to understand this interaction. To overcome this impediment, retroviral transduction was employed to introduce the human G-CSF receptor into the SCF-responsive human hematologic cell line, MO7e, which expresses c-kit endogenously [22]. The resultant MO7e-G cells could then be assessed for their biologic response to these factors. SCF exerts synergistic actions on the proliferation, survival, and differentiation of hematopoietic cells. These processes are critical for the adequate expansion and development of progenitor cells as well as for more complex biologic functions such as mobilization. Initial studies on MO7e-G cells focused on the synergistic induction of proliferation by SCF and G-CSF [7,8,15,17]. It was found that the transduced G-CSF receptor is functionally active, in that it not only supports G-CSF-dependent proliferation of these MO7e-G cells, but also recapitulates the proliferative synergy between SCF and G-CSF, thus validating the new MO7e-G cell line as a model system for its study.

Further analysis revealed that the synergistic proliferation induced by SCF and G-CSF co-treatment of MO7e-G cells was associated with a direct effect of these cytokines on cell cycle distribution, specifically a marked shortening of the duration of G0/G1 [22]. This shortening was mediated at least in part by a greater than 90% decrease of expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$, which is known to set a stoichiometric inhibitory threshold of cdk activity that prevents cdk-induced phosphorylation of the retinoblastoma protein (Rb) and cell cycle progression [57–61].

The observed shortening of G0/G1 in response to the combination of SCF and G-CSF correlated not only with down-regulation of $p27^{kip1}$ expression, but also with a maximized phosphorylation of Rb and a synergistic enhancement of the induction of *c-fos*, an immediate-early proto-oncogene involved in cell cycle progression [62]. In concordance with these data, two recent papers have reported down-regulation of $p27^{kip1}$ to be a key regulatory step in SCF-induced proliferation of mast cells [63] and erythroid progenitor cells [64]. $p27^{kip1}$ has been also implicated in the proliferative synergy between SCF and GM-CSF in wild type MO7e cells [65].

STAT3 IS A PIVOTAL ELEMENT IN THE COMPLEMENTARY SIGNALING PATHWAYS DOWNSTREAM OF THE SCF AND G-CSF RECEPTORS

Immediate-early genes, such as *c-fos*, are induced by mitogenic cytokines through activation of transcription factors downstream from their receptors. The promoter for *c-fos* contains DNA sequences or "elements" which confer responsiveness to a number of transcription factors. Among these, attention was focused on STATs, which are known to be key mediators of cytokine signaling in hematopoietic cells [66–70], and have been shown to control cellular proliferation by regulating $p27^{kip1}$ expression [71]. STATs are a family of latent transcription factors which reside in the cytoplasm of cells under basal conditions. When a cytokine interacts with its receptor, it activates an associated tyrosine kinase of the Janus kinase (Jak) family which can phosphorylate a STAT molecule on a single tyrosine residue toward the carboxy-terminus (Fig. 1). Once tyrosine phosphorylated, STATs form dimers and translocate from the cytoplasm to the nucleus.

There they bind to specific DNA elements in the regulatory promoter regions of target genes thereby activating their transcription. Individual cytokines can activate different subsets of STATs depending on their receptor structure. While G-CSF is known to activate

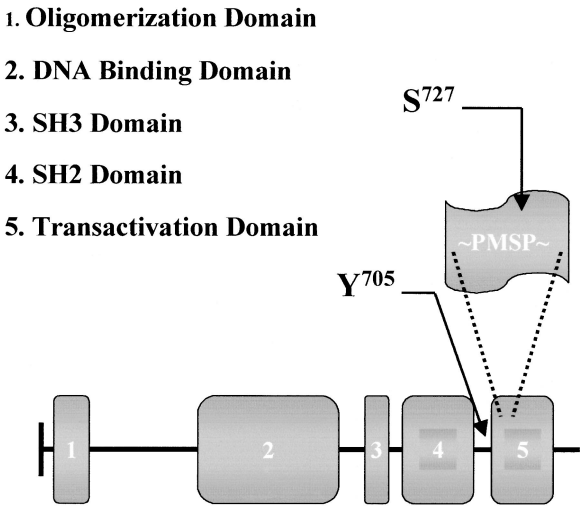

FIGURE 1 The structure of STAT3. The functional domains of STAT3 are indicated. These include: (1) the conserved oligomerization domain in the amino terminus which is necessary for the formation of STAT tetramers; (2) the central DNA-binding domain; (3) the Src homology 3-like region (SH3); (4) the highly conserved SRC homology 2 region (SH2) which allows STAT3 to dimerize and to bind to specific phosphotyrosine residues on receptors and kinases; and (5) the carboxy-terminal transactivation domain. Of critical importance are the tyrosine 705 site, whose phosphorylation is necessary for dimerization and activation, and the regulatory serine727 residue in the transactivation domain which is located in a proline-directed serine/threonin kinase consensus site.

STAT1 and STAT3 [72], STAT3 has never been found to be activated by SCF in any cell type, including wild type MO7e [73], and the ability of SCF to activate STAT1 remains controversial [73–76]. Accordingly, no evidence was found that the synergy between SCF and G-CSF in MO7e-G cells occurred at the level of STAT1 or STAT3 tyrosine phosphorylation, nuclear translocation, or DNA binding [22]. However, it had previously been reported that SCF could induce phosphorylation of STAT3 on serine727 [73], which increases the magnitude of the gene transcription mediated by the tyrosine-phosphorylated STATs and is necessary for maximal transcriptional activity [77]. Consistent with the importance of the phosphorylation of STAT3 on this serine residue, it was shown that SCF induces serine727 phosphorylation of STAT3 in MO7e-G cells. Furthermore, the combination of SCF and G-CSF maximized the induction of serine727 phosphorylation. In fact, all of the STAT3 detectable by western blot is completely phosphorylated on this residue after treatment with SCF and G-CSF, whereas neither factor alone induces complete serine727 phosphorylation of STAT3. Of note, SCF had no effect on the phosphorylation of STAT1 on serine727 [22].

Initially, there was conflicting evidence as to whether STAT3-dependent gene activation played a role in the induction of proliferation in response to G-CSF. Using dominant negative forms of STAT3 and truncation mutants of the G-CSF receptor, it had been shown that G-CSF clearly plays a role in differentiation and survival [78–81]. G-CSF-dependent induction of proliferation was, according to some reports, primarily associated with activation of $p21^{ras}$/MAPK-ERK or PI3K [80–82]. However, a number of studies in the last several years has provided clear evidence of a role for STAT3 in mediating proliferation of hematologic cells [83–85]. More recently, definitive evidence of the importance of STAT3 in the transduction of proliferative signals downstream of the G-CSF receptor has been provided. McLemore and co-workers generated transgenic mice with a targeted mutation of their G-CSF receptor. This abolishes G-CSF-dependent STAT3 activation, but preserves activation of other signaling cascades [86]. In these mice, which are severely neutropenic, G-CSF-induced proliferation and differentiation of hematopoietic progenitors is severely impaired. The authors concluded that STAT3 activation in response to G-CSF is critical for the transduction of normal proliferative signals and contributes to signals for differentiation. In addition, recent data give further support to the notion that phosphorylation of serine727 is important for STAT3-dependent biological responses. Constitutive STAT3 serine727 phosphorylation has been reported in B-cell-derived tumors [87] and in Src-mediated transformation [88,89], and its specific inhibition resulted in reduced transformation [88,89]. In the MO7e-G system, SCF allows the uncoupling of tyrosine and serine phosphorylation of STAT3. This suggests that single phosphorylation on serine727 is a means of priming STAT3 for an increased response, once a second stimulus, in this case driven by G-CSF, induces its tyrosine phosphorylation. Although most reports indicate that serine727 phosphorylation of STAT3 leads to maximal transcriptional activity [77], one group has reported a negative effect on transcription following ERK-mediated serine phosphorylation of STAT3 [90]. It is worth noting that these authors also reported serine727-dependent and independent down regulation of tyrosine phosphorylation of STAT3, which indicates that the negative effect on STAT3-transcription may not be secondary to phosphorylation of serine727 *per se*. Conversely, in MO7e-G cells, maximal serine727 phosphorylation of STAT3 resulting from combined treatment with SCF and G-CSF did not alter the level of STAT3 tyrosine phosphorylation or DNA binding [22]. Taken together, these findings support an important role for STAT3 and, in particular, its phosphorylation on serine727 in cellular growth control. Clearly, the biological implications of phosphorylation of STATs on serine residues may vary with the target promoter and/or the cellular context, and thus may differ in specific systems.

BIOCHEMICAL AND BIOLOGICAL SYNERGY BETWEEN SCF AND G-CSF IS MEDIATED BY P13 KINASE AND MAP KINASE

Serine727 of STAT3 is located within a proline-directed serine/threonine kinase consensus site [91,92]. Such a

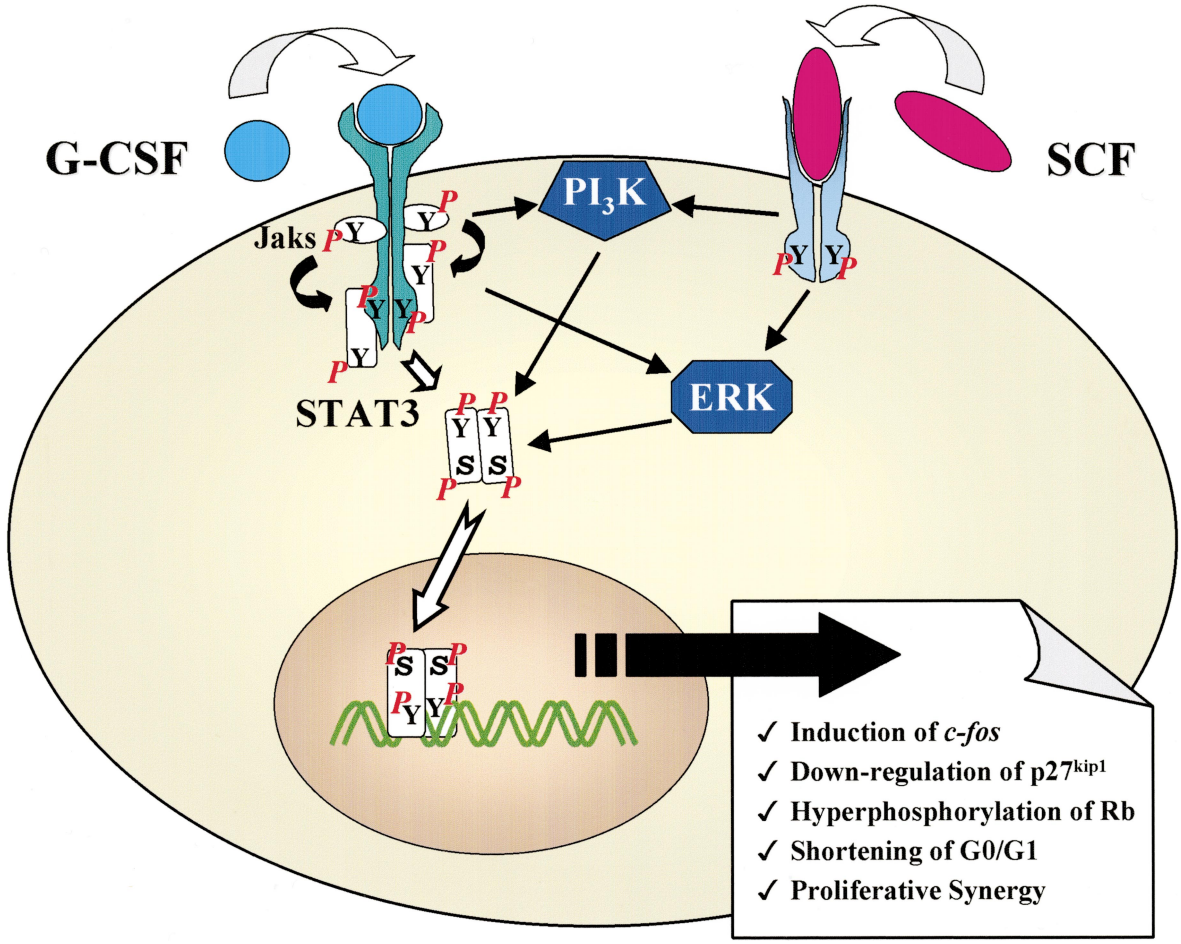

FIGURE 2 A model for the integration of SCF and G-CSF signal transduction. Binding of SCF to its receptor, which possesses intrinsic tyrosine kinase activity, induces kinase activation and transphosphorylation of the receptor chains. Phosphotyrosine residues in the receptor then function as docking sites for proteins that link c-kit activation to several signaling pathways including MAPK and PI3K. By contrast, the G-CSF receptor is a single-chain member of the cytokine receptor superfamily, which lacks tyrosine kinase activity. Binding of G-CSF to its receptor induces activation of receptor associated kinases and the activation of signaling pathways including Jak–STAT, MAPK and PI3K. The signaling cascades emanating from the G-CSF and SCF receptors converge on STAT-3, which becomes phosphorylated on tyrosine and serine residues, and mediates subsequent biological effects.

serine residue can be phosphorylated by several MAP kinase family members in addition to the ERKs (reviewed in Ref. [93]). Therefore, STAT3 is in a pivotal position to integrate complementary signaling pathways downstream of the receptors for SCF and G-CSF, which could mediate their synergistic effects on proliferation and gene expression. The identity of the serine/threonine kinase that phosphorylates this conserved carboxy-terminus residue of STAT3 in the MO7e-G model system in response to SCF and/or G-CSF is unknown. Therefore, experiments were designed to explore the signaling events induced by these cytokines which are necessary for the phosphorylation of STAT3 on serine727. It was found that SCF and G-CSF induce phosphorylation of this residue through different pathways. SCF-induced phosphorylation of STAT3 in serine727 is insensitive to H7, whereas G-CSF is highly sensitive to this kinase inhibitor. Furthermore, using pharmacological inhibitors, it was found that both phosphatidylinositol 3 kinase (PI3K) and ERK are upstream of serine727 phosphorylation of STAT3 in MO7e-G cells [22]. There is clear evidence in other systems that ERK is a bona fide STAT3 serine727 kinase (reviewed in Ref. [93]). However, the fact that complete ERK inhibition does not fully block STAT3 serine727 phosphorylation is compelling evidence that even if ERK can phosphorylate serine727, other kinases must be involved in this phosphorylation in the MO7e-G system. Although MAPK pathways other than ERK, such as p38MAPK play an important role in several systems in the phosphorylation of the PMSP motif in which serine727 is found [93], p38MAPK has been found to play no role in the phosphorylation of STAT3 on serine727 in the MO7e-G model. PI3K can be activated by both SCF and G-CSF (reviewed in Ref. [72]), and has also been found to mediate serine727 phosphorylation of STAT1 in response to antigen receptor triggering [94]. PI3K activation plays a key role in signal transduction from cytokine and growth factor receptors. For example, activation of PI3K is important in the transformation of hematopoietic cells induced by a constitutively activated form of the SCF receptor c-kit [95]. Thus, it is not surprising that both

the ERK and PI3K pathways are upstream of STAT3 serine phosphorylation.

Because PI3K and ERK-dependent pathways are necessary for the synergistic phosphorylation of STAT3 on serine727, it was important to determine whether inhibition of these pathways would affect gene activation or proliferation elicited by co-treatment with SCF and G-CSF. Simultaneous inhibition of these two pathways led to complete abolition of the synergistic induction of *c-fos*. Furthermore, the proliferative synergy between SCF and G-CSF was also completely dependent on PI3K and ERK, because the synergistic growth of MO7e-G cells in response to SCF and G-CSF was reduced to that seen with G-CSF alone in the presence of inhibitors of these pathways (Fig. 2). Consistent with this finding, PI3K and ERK have been recently shown to mediate synergistic induction of *c-fos* by SCF and GM-CSF in wild type MO7e cells, and to do so in part through activation of $p90^{RSK}$ [96]. However, whether transcription factors such as STAT3 mediated this effect on *c-fos* activation was not examined. Moreover, PI3K and ERK have been shown to be essential for SCF-dependent spermatogonial cell cycle progression and proliferation but not survival [97]. Again, the effector elements downstream of these two pathways remain undefined.

CONCLUSIONS

SCF is a potent co-stimulatory growth factor in hematopoiesis. In combination with G-CSF, SCF has important biologic and clinical effects. A novel system has recently been described in which to explore the signaling events underlying this synergy. MO7e-G cells recapitulate the proliferative synergy between SCF and G-CSF and have allowed the identification of a number of intracellular pathways which may mediate their biologic actions. The network of signaling events downstream of c-kit and the G-CSF receptor are complex, which makes it challenging to dissect their modes of interaction and to elucidate the biologic relevance of these interactions. For example, additional kinases involved in induction of proliferation by G-CSF, such as MEK5/ERK5 have been identified recently [98]. Molecules known to regulate serine727 phosphorylation of STAT3, such as Rac1 [88], have been found to do so through novel interactions [99]. Newly described post-translational regulatory mechanisms, such as arginine methylation, have been shown to control the function of STATs [100]. Our understanding of STAT serine de-phosphorylation, though rudimentary, has already revealed that phosphatases, such as PP2A, are critical for keeping an adequate balance with activating signals [101]. New mechanisms by which STATs phosphorylated on serine727 enhance their transcriptional activity are being revealed, such as association with the MCM5 member of the minichromosome maintenance family involved in DNA replication [102]. Therefore, it is possible that other pathways play a role in the biologic and clinical effects of SCF and G-CSF. While it is necessary to develop confirmatory systems in which to study these interactions, MO7e-G cells are likely to be useful in dissecting the pathways involved in many aspects of the biologically and clinically important synergism between SCF and G-CSF. Delineation of these signaling mechanisms will permit the design of a variety of cell-based and biochemical screens for the development of agents which can therapeutically target these clinically important pathways.

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