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STAT5 signaling in normal and pathologic hematopoiesis

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1. ABSTRACT

Hematopoietic development is highly dependent upon cytokine/receptor initiated signaling pathways. Of those activated in hematopoietic cells, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway plays a major role. This review focuses on the key role of STAT5 activation in hematopoietic stem cells and early hematopoietic progenitor cells of normal and leukemic hematopoiesis. In normal hematopoietic stem cells STAT5 is required for robust competitive repopulation and proliferative responses to early acting cytokines. Activation of STAT5 by many activated receptor tyrosine kinases as well as by JAK2 and JAK3 has also been associated with hematologic malignancies and can result in cytokine-independent cell expansion. The biology of STAT5 function and its potential cooperation with other signaling pathways has become a key area of focus in the new era of molecularly targeted therapeutics for hematologic malignancy. In particular, interactions with Grb2-associated binding protein (Gab2) have linked STAT5 with the phosphatidylinositol-3-kinase pathway and its downstream signaling. Missing is a full understanding of the structure-function relationship of STAT5 activation, including functional targets and cooperating partners required to differentiate normal vs. leukemic STAT5 activation. This review summarizes the latest understanding of leukemogenesis and pathophysiology associated with constitutive STAT5 activation in hematologic malignancies.

2. OVERVIEW OF JAK/STAT SIGNALING COMPONENTS ASSOCIATED WITH HEMATOPOIETIC AND IMMUNE CELL FUNCTION

The Janus kinase (JAK) / Signal Transducer and Activator of Transcription (STAT) pathway links non-signaling cytokine receptors with the intracellular signaling molecules JAKs and STATs, both of which bind to SH2 domains on the receptor and are activated by tyrosine phosphorylation. STATs are latent transcription factors that continuously cycle between the nucleus and cytoplasm and upon phosphorylation by JAKs, form dimers that are retained in the nucleus, and regulate target gene expression. STATs consist of six conserved domains that include the N-terminal oligomerization domain, the coiled coil domain, the DNA binding domain, the linker domain, the SH2 domain, and the transcriptional activation domain (Figure 1). STATs interact through the SH2 domain and linker domain to dimerize and this is essential for directing DNA binding. The coiled coil domain and the N-domain of STATs is involved in further protein-protein interactions. Tetramerization of STATs also occurs through the N-domain and is believed to increase the repertoire of target genes expressed, possibly including cancer related genes(1). Since the main focus of this review is on STAT5 and its cooperative signaling, only a brief review of other STATs will be mentioned below.

Initial studies of interferon signaling identified the first STATs as part of this response pathway. STAT1

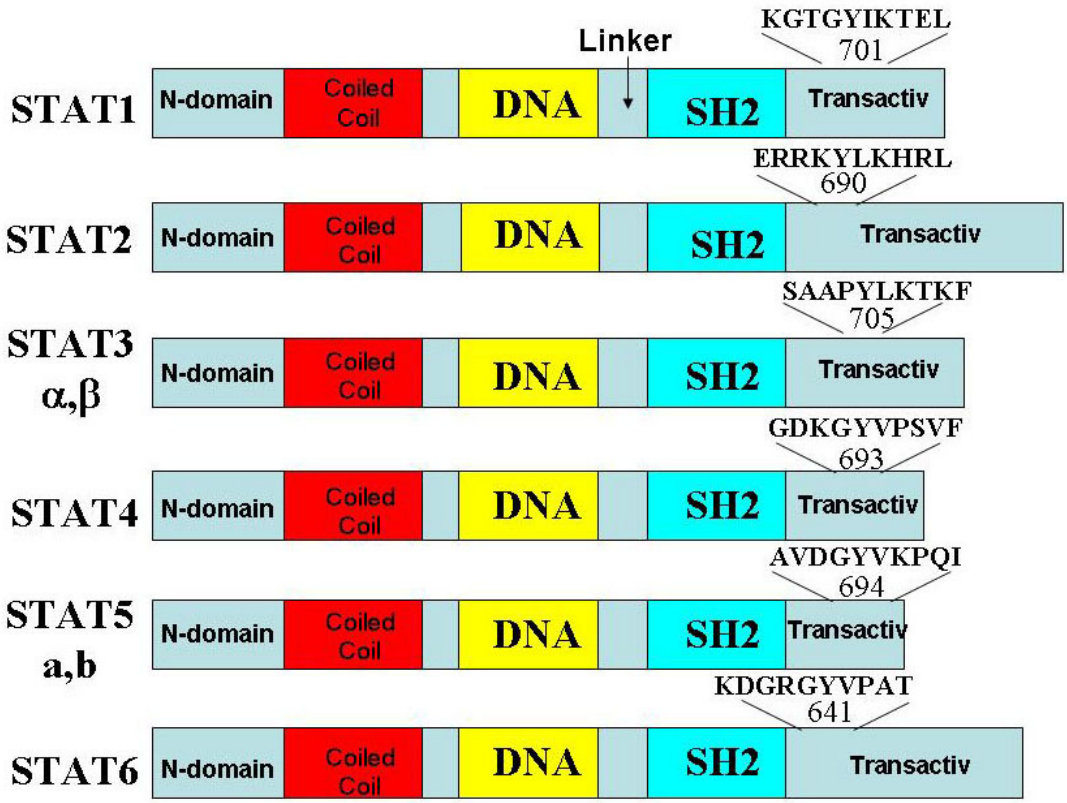

Figure 1. Structural domains of STAT molecules. There are 7 total STATs including STAT5a and STAT5b which are separate genes. All share conserved domains that play important roles in protein-protein interactions, DNA binding ability, and transactivation once bound to the promoter of target genes. All 6 conserved domains are shown in this figure, which include the N-domain, alpha helical region called the coiled coil domain, the critical DNA binding domain, a flexible linker region that connects the SH2 and transactivation domains to the rest of the molecule and bridges the interactions that occur when bound to DNA. Included in the transactivation domain (transactiv) are the critical tyrosine residues that require phosphorylation in order to promote conformational changes leading to dimerization and DNA binding capacity.

and STAT2 are critical for interferon response and mice lacking STAT1 have impaired innate immunity(2,3). The role of STAT2 in interferon signaling was also confirmed through generation of knockout mice(4). Double knockout of STAT1 and STAT2 has profound defects on interferon response and STAT2 requires sequence-specific contacts provided by STAT1(5). STAT4 is critical for IL-12 signaling and its deficiency causes defects in Th1/Th2 polarization(6,7). Complementary defects are observed in STAT6 knockout mice where IL-4 signaling is impaired(8,9,10). Early-acting cytokines activate primarily STAT3 and STAT5 with much broader functions throughout hematopoiesis. STAT3 is very pleiotropic, with multiple complex roles in hematopoiesis(11,12) and immune tolerance(13,14). STAT3-deficient mice develop colitis(15,16) and have increased T cell response, indicating that STAT1 binding to the same sites on gp130 can lead to complex effects and differential downstream SOCS expression.

In contrast to STAT3 activation by gp130, the common cytokine receptor gamma chain (gammaC) mediated activation of JAK3 by IL-2, 4, 7, 9, 15, and 21 is required for lymphocyte development in mice and humans. Downstream of JAK3 is STAT5 in the canonical JAK/STAT pathway. STAT5 is a duplicated gene encoding two similar isoforms termed STAT5a and STAT5b that differ in molecular weight of 94 and 92 kDa respectively. The variability between these two proteins is primarily in the 12 C-terminal amino acids. When referring to both STAT5a and STAT5b here, we will collectively call them STAT5. STAT5-deficient mice have been generated to further study this gene(17). Single knockout mice lacking STAT5a show defects in cell proliferation, mammary gland development, and lactogenesis(18). Mice lacking STAT5b show defects in sexual dimorphism of body growth rate and liver gene expression patterns(19). To eliminate any compensating function between the two STAT5 isoforms, homozygous mutant mice lacking STAT5a and STAT5b have been generated(20). These mice are viable, but the females are infertile. Heterozygous mice can breed normally, however, and give STAT5a/STAT5b double knockout mice. STAT5 appears critical for IL-2 signaling via gammaC and is required for T cell proliferation and for production of NK cells(21,22). In addition to immune function, STAT5 activation is also mediated by erythropoietin (EPO), thrombopoietin (TPO), and stem cell factor (SCF) signaling via JAK2.

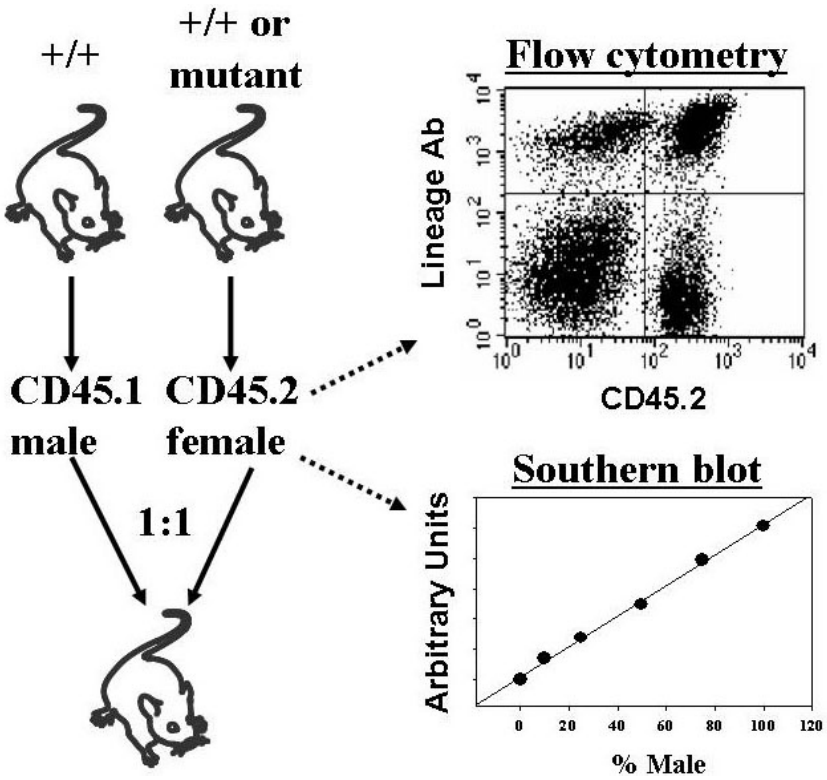

Figure 2. General design for competitive repopulation assays. Competitive repopulation assays are based on a principle of quantitating stem cell numbers or assessing stem cell function through competition with stem cells of known number or function. In principle, normal stem cells from mice will compete equally against normal stem cells from a congenic strain or an opposite sex mouse when transplanted into irradiated recipients. We have used these two approaches in prior studies to demonstrate that our mutant mouse (STAT5 knockout) was defective in repopulating function. Congenic strains such as the CD45.1/CD45.2 and the use of male/female competition allows for separation of donor graft contributions in the irradiated host. Readout of CD45 is done by flow cytometry using antibodies (Lineage Ab) specific for the cell surface CD45 (Ly5) polymorphisms. Readout of the male/female system is more labor intensive and requires isolation of hematopoietic cells from recipient mice, preparation of genomic DNA, and Southern blot analysis for male Y chromosome. Quantitation of the percentage of male engraftment has to be done relative to a standard curve prepared by mixing male and female DNA as shown.

In recent years it has been demonstrated that $CD4^+CD25^+FoxP3^+$ regulatory T cells are critical for maintenance of tolerance and prevention of autoreactivity(23,24,25). IL-2/STAT5 survival signaling is integral for this function and mice lacking either IL-2R(26) or STAT5a and STAT5b(23), demonstrate strikingly similar autoimmune defects affecting multiple tissues. IL-2 mediated activation of STAT5 in human T cells leads to transcriptional activation and direct binding to the FoxP3 promoter(27). It is important to point out that these homozygous mutant mice express variable levels of an N-terminally truncated STAT5 isoform (mice referred to as STAT5ab deltaN) in many tissues. A new true null STAT5 knockout mouse was derived by Lothar Hennighausen (NIH). These mice will be referred to here as $STAT5ab^{null/null}$ mice. STAT5ab deltaN mice have reduced numbers of $CD4^+CD25^+$ regulatory T cells but they still have gamma-delta T cells and CD4+ T cells present and these mice have a shortened life span due to an age dependent progression of autoimmunity(23). In contrast, $STAT5ab^{null/null}$ mice(28) have no gamma delta or $CD8^+$ T cells and much lower $CD4^+$ T cells than STAT5ab deltaN mice(29).

3. ROLE OF STAT5 ACTIVATION IN NORMAL HEMATOPOIETIC STEM CELL FUNCTION

Since $STAT5ab^{null/null}$ mice have only recently become available, the vast majority of work in the literature has been obtained from STAT5ab deltaN mice. STAT5ab deltaN BM transplant results in engraftment with donor HSCs, although associated with T cell chimerism(30,31). The 30-50% donor T cell chimerism prevents development of the autoimmune disorder that plagues adult STAT5 knockout mice and these chimeras are healthy and live long-term. STAT5ab deltaN mice have normal HSC numbers and bone marrow (BM) and fetal liver (FL) HSCs from these mice can engraft into irradiated hosts(31) but are deficient in self-renewal capacity(32). To assess the quality of the stem cell compartment, competitive repopulation experiments using congenic competitor bone marrow cells was used for an experimental design as shown in Figure 2. The STAT5 mutant mice and most C57BL/6

mice are CD45.2 and stem cells from these mice can be transplanted into lethally-irradiated CD45.1 recipient mice, differing only by a polymorphism in the CD45 (Ly5) locus. In competitive repopulation studies, the STAT5ab deltaN CD45.2 positive grafts were greatly inferior to wild-type BM(23,30,31,32,33,34,35,36) indicating defects in HSC multilineage differentiation. To further define the nature of this deficiency, Sca-1$^+$c-Kit$^+$Lin$^-$ cells, which are enriched for HSC potential, were exposed to cytokine cocktails *in vitro*. The enriched HSC fraction from STAT5ab deltaN hosts was greatly defective in response to early-acting cytokines such as interleukin(IL)-3 and stem cell factor (SCF). Defective cytokine responsiveness of STAT5ab deltaN HSCs also led to reduced *in vivo* sensitivity to the S-phase active anticancer drug, 5-fluorouracil(33) as measured using a male/female competitive repopulation assay.

In addition to the general growth defects, others examined apoptosis phenotypes of the STAT5ab deltaN mice. Snow *et al.* reported increased BM apoptosis associated with an infiltration of T lymphocytes during progression of the autoimmune disease(23). This group used transgenic Bcl-2 expression from a beta-actin promoter and this was not found to be sufficient to rescue repopulating defects(36). It was also unclear whether stem cell phenotypes were due to indirect effects of T cells or cell intrinsic loss of STAT5 in HSCs. Studies in RAG2$^{-/-}$ STAT5ab deltaN mice were able to determine whether defects were truly independent of the autoreactive T cells. HSC repopulating deficiency was found to be cell intrinsic resulting from ineffective responsiveness to early-acting cytokines(33). Other phenotypes of these mice have been analyzed. Snow *et al.* showed that homing of donor STAT5ab deltaN BM cells into lethally-irradiated recipients was normal(35). However, STAT5ab deltaN HSCs failed to remain in the niche during steady-state hematopoiesis following injection of STAT5ab deltaN newborn mice with wild-type BM cells(30). These results indicate a defect in competition for the niche in the non-myeloablative setting.

Despite residual STAT5 activity, STAT5ab deltaN HSCs have severe competitive repopulation defects following BM transplant and that these defects are cell intrinsic, leading to deficient growth in response to early-acting cytokines(30,31,32,33). Furthermore, defects in multilineage repopulation were more severe than from c-Mpl$^{-/-}$ BM indicating that multipotent progenitors are particularly dependent upon STAT5 activation. A unique significance for STAT5 in steady-state engraftment of HSCs was found in adult BM chimeras generated following lethal irradiation and transplant with STAT5ab deltaN BM cells. The engrafted BM cells in these mice could be gradually replaced by a single injection of congenic wild-type HW80 or CD45.1 BM cells even 16 weeks later and without any ablative conditioning(33). Furthermore, green fluorescent protein (GFP) transgenic BM cells injected into these mice allowed tracking of donor engraftment and could correct hematopoietic function long-term. When wild-type GFP$^+$ BM cells were injected into STAT5ab deltaN BM chimeras, the hematopoietic system of injected mice became GFP$^+$ within 8-12 weeks. This approach was chosen to further test the engraftment defects in STAT5ab deltaN hosts. 2-3 day old newborns were used because at this time the mothers could be confirmed to be taking care of the pups and overall yield of surviving pups was improved. GFP transgenic BM was injected into newborn STAT5ab deltaN mice using an approach previously described in the context of a gene therapy model for JAK3-deficient severe combined immuno-deficiency(37). Following injection, the pups were kept with their mothers until weaning age. At weaning, they were then analyzed for GFP expression by flow cytometry. As expected, the levels of GFP expression were extremely low to undetectable in total blood leukocytes of wild-type recipients. The highest levels of HSC engraftment of GFP$^+$ donor cells were obtained in STAT5ab deltaN mice with lower levels in heterozygote mice, indicating a dose-dependent requirement for STAT5.

When STAT5ab deltaN HSCs were inversely injected into stem cell defective W/W$_v$ hosts in the absence of myeloablation, STAT5ab deltaN HSCs on the C57BL/6 background (Hb$_s$) were capable of high level of engraftment of 26/27 W/W$_v$ recipients when injected at a 1 donor to 5 recipient ratio. Based on the BM cellularity of STAT5ab deltaN mice, this was a cell dose of approximately 2 x 10^6 cells per W/W$_v$ recipient. This was documented by hemoglobin electrophoresis on peripheral blood and also by Southern blot analysis of BM and spleen tissues from mice that were euthanized 16 weeks following transplant. Surprisingly, when the same experiments were done using HW80 background STAT5ab deltaN BM cells (Hb$_d$), only 4 of 19 mice were engrafted. HW80 mice differ from C57BL/6 in only a small region of chromosome 7 derived from Balb/c mice. This region includes the hemoglobin and albino coat color loci, among 246 other genes which include Gab2. This novel method for assaying steady-state HSC seeding efficiency in the absence of myeloablation allowed seeded cells to contribute to hematopoiesis due to a competitive advantage against the W/W$_v$ competitor. Failure to engraft long-term thus reflected a seeding deficiency. Therefore, the potential for migration and defects in STAT5ab deltaN mice on the C57BL/6 background remain an area that has not been fully explored. Furthermore, the minor genetic differences between C57BL/6 and the congenic HW80 mouse strain may provide a useful model system for defining further the modifier gene(s) associated with this steady-state engraftment defect.

4. ROLE OF GAB2 ADAPTER FUNCTION IN ACTIVATION OF MULTIPLE SIGNALING PATHWAYS AND IN HEMATOPOIESIS

A prominent motif in signaling molecules is the Src homology-2 (SH2) domain of JAKs, STATs, Grb2, p85 (PI3-K pathway), Shc, and others. By binding and docking with the phosphorylated tyrosine residues these domains play important roles in signal transduction pathways. Multiple protein binding motifs characterize adapter molecules and their ability to form multimeric complexes with CrkL, PLC, SHIP, and SHP-2. The Grb2-associated

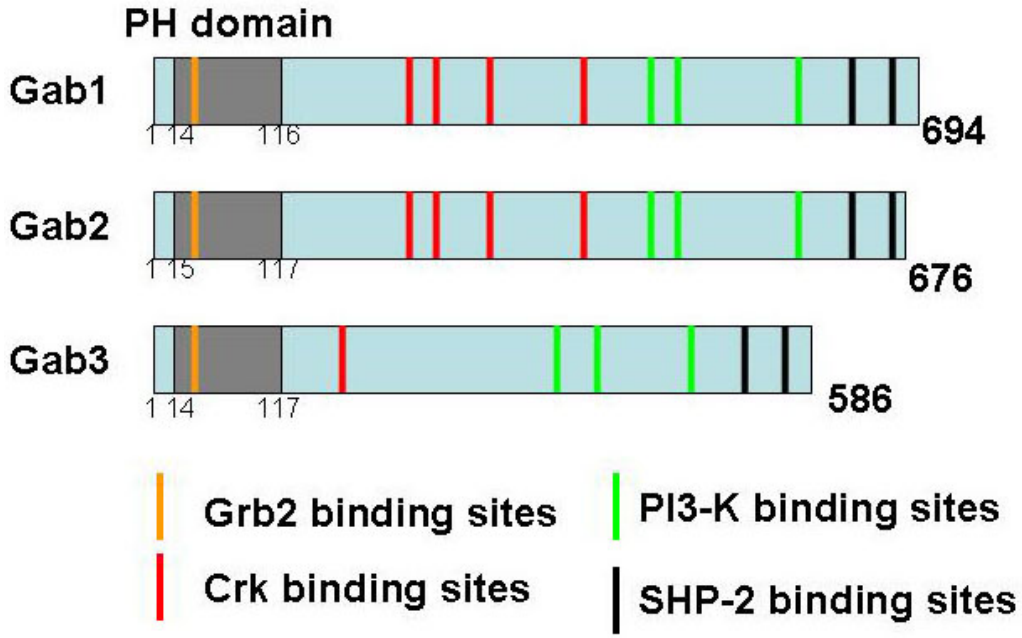

Figure 3. Gab structure and multiple binding sites. Gab molecules were originally identified as the mammalian homologs of the daughter of sevenless (DOS) drosophila adapter protein. Like STATs, Gab1, Gab2, and Gab3 share many common binding sites but they are quite distinct in their functions *in vivo*. All three Gabs have an pleckstrin homology (PH) domain in the amino-terminus that is believed to be essential for many functions. Gab2 knockout mice have been generated by deleting all or part of this PH domain. Gabs contain multiple binding sites and act as scaffolding molecules to support cytokine signaling. Binding sites for Grb2, Crk, PI3-K, and SHP-2 have been defined and extensively studied.

binding protein (Gab) family of adapter proteins (Gab1, Gab2, Gab3) regulate multiple signaling pathways including the phosphatidylinositol-3 kinase (PI3-K), and mitogen associated protein kinase (MAPK) pathways through multiple protein binding sites(38,39,40) (Figure 3). These proteins are tyrosine phosphorylated following cytokine stimulation and are able to interact with a large number of partners. The mechanisms that confer specificity in directing which interactions occur in any particular cell type upon cytokine stimulation remain to be determined. Gab1 deficiency results in embryonic lethality and conditional deletion of Gab1 shows a role for Gab1 in promoting Erk activation in hepatic function(41,42). Gab1 acts as an adapter protein to link gp130 signaling to the Erk pathway(43). In contrast, Gab3 knockout mice do not show major phenotypes(44).

Gab2 is tyrosine phosphorylated by several early acting cytokine receptors such as c-Mpl, Flt3, c-Kit, and IL-3R. Gab2 has both SH2 and SH3 domains that promote binding to signaling molecules(38,45,46). Gab2 activates the PI3-K and the MAPK pathways and can regulate hematopoietic cell migration functions(47). $Gab2^{-/-}$ mice are viable but lack allergic response(48) and suffer from osteopetrosis due to decreased osteoclast differentiation via RANK-induced progenitor differentiation(49). Gab2 deficiency has also been shown to alter mast cell development(50) in a manner similar to STAT5ab deltaN mice(51). Gab proteins also contain consensus serine/threonine sequences, suggesting that phosphorylation as a secondary mode of regulation may be possible. A recent study showed that Erk phosphorylated serine 623 of Gab2 promotes interaction between Gab2 and SHP-2, leading to prolonged STAT5 activation(52). SHP-2 is a negative regulator of STAT5 activation but positively regulates JAK2 activation and signaling via Gab2. Accordingly, STAT5 activation can be strongly enhanced by $Gab2^{Y614F}$, which blocks interaction with SHP-2(52).

Understanding of the balance between Gab2 recruitment of SHP-2 and modulation of STAT5 activation in cytokine signaling is still incomplete. However, potential evidence supports cooperation between STAT5 and Gab2. First, Gab2 is located on the chromosome 7 modifier locus described to modulate HSC engraftment during steady-state hematopoiesis(30). Although this locus could contain complex modifier interactions, Gab2 stands out as a potential top candidate. Second, *in vitro* studies have shown a role of the docking protein Gab2 in beta1-integrin signaling pathway-mediated hematopoietic cell adhesion and migration(47). Overexpression of the PH domain or a mutant Gab2 molecule lacking SHP-2 binding sites resulted in significant reductions in Ba/F3 cell adhesion and migration. Biochemical analyses revealed that enforced expression of a Gab2 deletion mutant lacking Y604 and Y633 (a.a. 1-603) dramatically reduced beta1-integrin ligation-triggered PI-3 kinase activation, whereas Erk kinase activation remained unaltered. However, it is possible that these *in vitro* studies overestimated the physiological role of Gab2 since they used an overexpression approach. Third, *in vitro* studies in Ba/F3 cells show that Gab2 associates indirectly with

constitutively active and wild-type STAT5, p85, Grb2, but not SHP-2 and promotes STAT5-mediated signaling through induction of PI3-K and MAPK pathways(53,54). Fourth, STAT5 mediated PI3-kinase activation and cyclin D2 induction results from a delayed cycloheximide-sensitive mechanism(55). Fifth, functional cooperation among STAT5 and signaling via Erk and p85(56) and SHP-2/Gab2(57) has been described in Bcr/Abl induced oncogenic activities. However it remains unclear what the physiological role of Gab2-mediated Erk and p85 signaling is in HSC biology and in respect to wild-type STAT5-mediated functions in HSCs.

5. ROLE OF STAT5 AND GAB2 IN LEUKEMOGENESIS

The accepted two-hit model for leukemogenesis places JAK/STAT signaling as a potential proliferation and/or survival hit. An additional hit that blocks differentiation is required for leukemogenesis and this can be via altered cytogenetics or mutation of differentiation promoting transcription factors(58). Overexpression studies using retroviral vectors providing constitutive expression in all hematopoietic lineages have clearly shown that many early-acting cytokines such as IL-3(59), IL-6(60,61), IL-11(62,63), and Flt3L(64,65)can cause abnormal myeloproliferation and leukemia. Therefore, the transformation process can involve signal transduction pathways affecting cell proliferation or apoptosis. STAT5 is activated normally by a wide range of factors including many early acting hematopoietic cytokines including IL-3, SCF, and TPO. Activation of STAT5 via oncogenic Flt3 internal tandem duplication (Flt3-ITD) is very efficient, although wild-type Flt3 activation of STAT5 is minimal. $JAK2^{V617F}$ and Flt3-ITD activate STATs, p85/Akt, and Erk pathways. It is currently unclear whether these are redundant or whether a particular pathway is most prominent in the pathogenesis of these mutations.

Data comparing Flt3-ITD to Flt3-TKD has shown a unique requirement for STAT5 activity in the resulting disease output(66,67), suggesting that STAT5 activation is a major component of the transforming potential of Flt3 mutants. Additionally dual Flt3-ITD-TKD mutants induce hyperactivation of STAT5 and overexpression of Bcl-XL(68). In a recent study Y589 and Y591 STAT5-binding sites were shown essential for Flt3-ITD induced MPD(69). Flt3-ITD has also been shown to be expressed on the human SCID mouse repopulating cell(70) and to require Akt(71,72) and Pim1(73,74) activation for optimal survival. Therefore, STAT5 activation is highly associated with MPD as a contributing factor leading to leukemia. STAT5 has also been reported as activated via the MPD-inducing $JAK2^{V617F}$ and required for Tel-JAK2(75) and Bcr-Abl(29,76) induced myelo- and lympho-proliferative disease. STAT5-mediated association with and activation of p85 has most recently been shown to be mediated by an indirect interaction with Gab2(53), most likely also involving a delayed secondary phase of kinase activation other than Jak2(55). Gab2 has been reported as important for disease mediated by Bcr-Abl(77), SHP-2 (Ptpn11) hyperactivated mutant (E76K)(78), and short form (SF)-Stk(79) *in vitro* for myeloid and erythroid transformation. Interestingly, Tel-Jak2 which still requires STAT5 does not require Gab2(77). It is likely that different fusion tyrosine kinase or mutant kinase interactions leading to PI3-K activation are distinct(80). Interestingly, recent studies have uncovered an unexpected connection between phosphorylated STAT5 and Gab2 in the cytoplasm which is associated with myeloid leukemias(81).

Oncogenic tyrosine kinases commonly activate mitogenic signaling(82,83) through STAT5, Gab2, PI3-K, and MAPK to regulate cancer-related genes such as Bcl-XL, Cyclin D2, and Myc. Interestingly human leukemia patients rarely show mutations in STATs, but proteolytic C-terminal truncations may contribute to a block in hematopoietic differentiation(84,85,86). Rather, STAT5 activating mutations are found in receptors and JAKs. Unlike JAK2 expression which is limited to myeloid lineages, STAT5 is more ubiquitously expressed and could impact upon lymphoid and myeloid hyperproliferation. Insertional mutagenesis using the *Sleeping Beauty* retro-transposon has also recently reported insertion sites in STAT5b(87). In lymphoid malignancies, overexpression of wild-type STAT5 is a predisposing factor(88,89,90). Despite the lack of clinical evidence of STAT5 mutations promoting leukemogenesis, mutations of STAT5 in mouse models are sufficient to drive multilineage disease and mastocytosis. Retroviral-mediated gene transfer of a constitutively active mutant of STAT5(91) or of OsM(92) caused myeloproliferative disease, splenomegaly, and early death in transplanted mice(75).

Although STATs are found constitutively activated in samples of peripheral blood from patients with hematologic malignancies(85,93,94), the role of the C-terminal truncated STAT5 isoforms is not clear(84,85,86). These truncated STAT5 molecules act in a dominant-negative manner(95) and suppress STAT5 target gene expression in model cell lines *in vitro*(96,97). However, why are these isoforms preferentially present at relapse in AML and CML patients? It is possible that they confer a growth or survival advantage on leukemic cells and some studies have suggested that this may be by blocking differentiation(96) or by promoting survival(98). Further characterization of these C-terminally deleted STAT5 isoforms in a $STAT5ab^{null/null}$ background may be particularly informative regarding their true function. These isoforms are generated by proteolytic cleavage of STAT5(99,100), in a variety of hematopoietic cell types, ranging from early hematopoietic progenitors(101), immature macrophages(102), multipotential myeloid FDCP-1 cells(103,104), and human neutrophils(105). It also cannot be excluded that these molecules also function in a transcription independent manner to promote leukemogenesis.

6. PATHOPHYSIOLOGY RESULTING FROM CONSTITUTIVELY ACTIVATED JAK2/STAT5

In addition to the early onset of leukemic expansion and proliferation, mouse models of MPD have been developed based on TPO overexpression and these

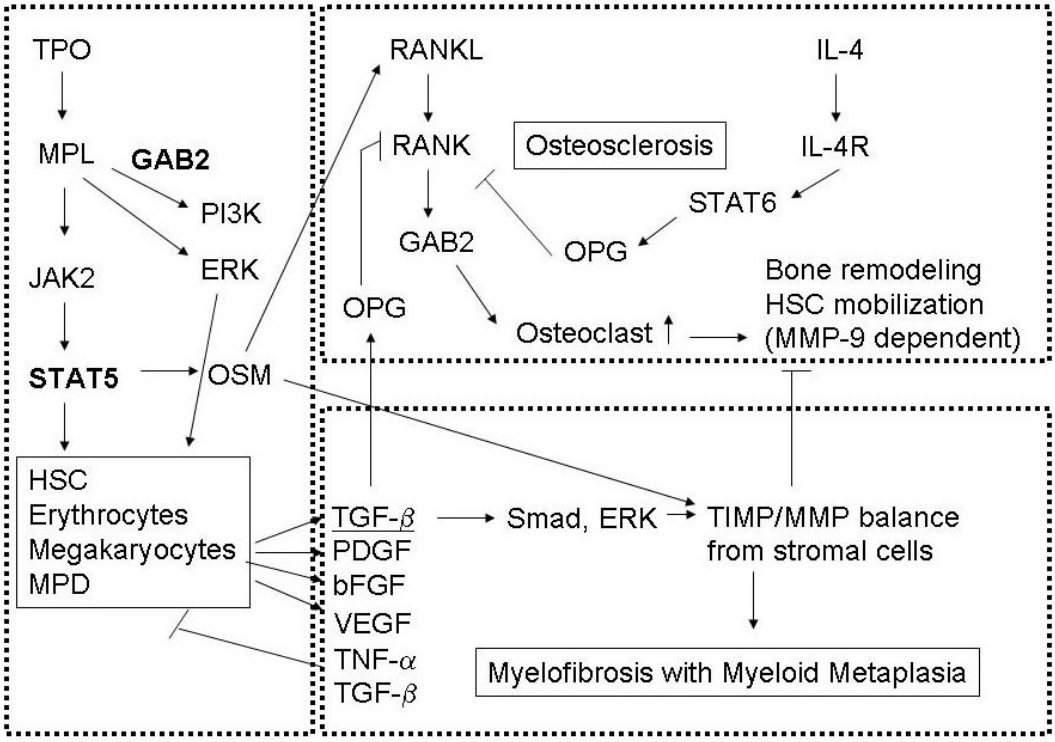

Figure 4. Role of JAK/STAT signaling in myeloid malignancy and pathophysiology. The variable pathophysiology of MPD remains an important obstacle to treatment. Major issues in the field involve the definition of target cells involved in the generation of MPD and understanding modifiers that cooperate in the development of disease pathology. Animal model studies promise to lead to a greater understanding of the abnormal HSC niche and its evolution. Presented is a simplified overview of TPO mediated signaling, leading to JAK2 and STAT5 activation and potential modifiers of MPD progression into myelofibrosis and osteosclerosis. OSM, oncostatin M; OPG, osteoprotegrin; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TNF-alpha, tumor necrosis factor-alpha.

recapitulate a number of features common to human MPD characterized by increased megakaryocytopoiesis, myelofibrosis, and osteosclerosis(106,107,108), correctable by BM transplant. Since TPO strongly activates JAK2 and STAT5 signaling, its overexpression has effects on early hematopoiesis. Other mouse models for megakaryocytopoiesis and myelofibrosis have been described which share common activation of transforming growth factor (TGF)-beta(109). Mice that are GATA-1^{low} (110,111) , c-Myb^{low} (112,113), or NF-$E2^{-/-}$ (114,115) have blocks in megakaryocyte-erythrocyte development recapitulating some aspects of MPD. A very recent model has been based on $JAK2^{V617F}$ mutation which nicely recapitulates a Polycythemia vera (PV) phenotype in mice(116,117,118) is unique from the others in that the effects require type I cytokine receptors(119) and directs more erythroid differentiation when highly expressed. On the C57BL/6 background, $JAK2^{V617F}$ expressing mice showed little leukocytosis and myelofibrosis may depend on JAK2 expression level and/or megakaryocyte expansion(118). On the Balb/c background, myelofibrosis was more prominent. As a whole, these mouse models provide tools for study of the basic processes and biology of these diseases, despite the limitation that some of the mutations such as Gata-1 or Myb are underrepresented in human patients. The C57BL/6 background JAK2 model is useful for studying how other signals direct myelofibrosis and reciprocally the TPO model is useful for studying how to attenuate the predominant myelofibrosis and osteosclerosis. Megakaryocyte and platelet expansions are closely linked to adverse BM pathology and produce numerous factors that could modulate hematopoietic and non-hematopoietic BM cells (Figure 4). However, it is important to note that the strength of JAK2 activation seems to correlate with myelofibrosis and when sufficiently robust, as induced by TEL-JAK2, myelofibrosis can occur independent of megakaryocytic expansion(120).

Pro-fibrotic TGF-beta is believed to be a central figure in the pathogenesis of MPD and is necessary for the myelofibrosis phenotype resulting from TPO overexpression in the mouse model using a TPO expressing retroviral vector(121). It has also been shown that osteoprotegrin (OPG) production is increased following TPO overexpression and that it is essential for the osteosclerosis phenotype(122). Despite the major role of TGF-beta in this disease progression, the mechanism for pro-fibrotic response is unknown(123). Because it affects both cells of the osteoblast and osteoclast lineage, TGF-beta is one of the most important factors in the bone

environment. Alterations in the osteoblastic HSC niche(124,125) through parathyroid hormone(125) or osteopontin expression(126) can have direct impact upon HSC pool size. Production of OPG in osteosclerosis associated with MPD(122,127,128) combined with megakaryocyte-osteoblast interactions(129,130,131) may also be associated with increase of N-cadherin positive osteoblasts in the setting of MPD.

The TIMP/MMP balance is an important determinant of the normal turnover of tissue matrix components. A wide range of disease states can be attributed to alterations in ECM turnover such as arthritis, liver fibrosis, cancer, and hematological disorders(132). Tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 have been identified in various myeloid cell types including platelets, megakaryocytes, and BM fibrobasts(133). The TIMPs are natural inhibitors of matrix metalloproteinases (MMPs) and act by tightly binding the MMP in a 1:1 stoichiometric ratio. MMP substrates include many components of the microenvironment of HSCs and progenitors, such as collagens, laminins, and fibronectin(134). In addition to stimulating collagen synthesis, TGF-beta increases TIMP-1 expression through AP1(135,136), Erk1/2(137), and Smad3/4(138,139) that in a conditional animal model leads to reversible intermediary liver fibrosis(140). However, MMPs are also coordinately upregulated with TIMPs and it is currently unclear whether TIMPs or MMPs are causative for myelofibrosis. Patients with agnogenic myeloid metaplasia (AMM) or idiopathic myelofibrosis (IM) have elevated TIMP-1 expression(141). Additional clinical studies have found that the ratio of total TIMP-1/MMP-9 was significantly higher in patients with chronic MPD and myelofibrosis(142). Although the cause/effect relationship could not be examined in the clinical studies, the data show that TIMP-1 is increased in a range of chronic MPD and a recent review has hypothesized that plasma TIMP levels are important for development of fibrosis in AMM(143). However, a recent study showed that mRNA for TIMP-1, TIMP-2, MMP-2 are constitutive and not increased in IM but that increased MMP-13, MMP-14 are associated with advance stages(144). Furthermore, constitutive mobilization of progenitors is associated with increased gelatinase and neutrophil elastase release in IM(145,146).The degree to which the HSC niche is modified through osteoclast dependent MMP production or increased TIMP and effects on HSC mobilization(147) and fibrosis remains unclear. However, the linkage between constitutive STAT5 activation and numerous events associated with disease pathophysiology may provide important new therapeutic routes.

7. SUMMARY AND PERSPECTIVE

The JAK/STAT signaling field has recently undergone a major expansion due to the increased interest in the $JAK2^{V617F}$ mutation associated with human chronic myeloproliferative diseases. The critical role that STAT5 plays in signaling by many aberrantly activated receptors and JAKs in hematologic malignancies makes it critically important to understand at the molecular level how STAT5 functions as an oncogene. Clearly much work needs to be done to dissect the functional protein-protein interactions, target genes, and other mechanisms. If one thing can be certain about this field, it is that surprises will be made. The need to dissect signaling in leukemogenesis and in MPD pathophysiology remains a high priority in light of the development of new targeted therapeutics with potential to interrupt critical signaling nodes and target the synergistic effects of cooperative cytokine signaling. Although the complexity of leukemic cells is continually being uncovered and their ability to become resistant to targeted therapeutics such as Gleevec is established in the clinic, this approach still holds much promise for customized therapies on a patient by patient basis. These new therapies may not only be guided toward starving the leukemic stem cell but also may help to ameliorate the aberrant bone marrow pathology associated with myeloproliferative disease.

8. ACKNOWLEDGMENT

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Abbreviations: signal transducer and activator of transcription (STAT), janus kinase (JAK), grb2-associated binding protein (Gab2), thrombopoietin (TPO), stem cell factor (SCF), bone marrow (BM), fetal liver (FL), phosphatidylinositol-3-kinase (PI3-K), mitogen-associated protein kinase (MAPK), flt3-internal tandem duplication (Flt3-ITD), myeloproliferative disease (MPD)

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