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The Jak-STAT Signal Transduction Pathway in Melanoma

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1. Introduction

The Janus-activated kinase (Jak)-signal transducer and activator of transcription (STAT) pathway is thought to play a central role in melanoma cell biology. Membrane-associated Jak proteins can couple with a variety of receptors to transmit signals into the cytoplasm. Immediately downstream, are the STAT family of transcription factors, which can be influenced by a variety of post-translational modifications and localize to the cell nucleus, directly binding DNA. In response to extracellular stimuli or through dysregulated activation of other signaling molecules, STAT proteins are often constitutively phosphorylated or 'activated' in melanoma cells. The balance between the activation of individual STAT proteins influences unique gene expression profiles within melanoma cells. For example, gene expression driven via the STAT1 transcription factor is associated with growth inhibition, resistance to apoptosis and reduced angiogenesis. Conversely, the STAT3 transcription factor mediates a gene expression profile associated with cell growth, anti-apoptosis, angiogenesis, metastasis and immune suppression. Altered activity of STAT proteins has been demonstrated in both melanoma cells, and interestingly, in the immune system of patients with melanoma. Recent data suggest that targeting the aberrant activation of this signal transduction pathway could have therapeutic effects via both modulating tumor cell and host immune function. In this chapter, we aim to summarize the role of the Jak-STAT pathway in melanoma, with a focus on the seemingly divergent effect of the STAT1 and STAT3 transcription factors. We will also highlight recent data suggesting that inhibition of signal transduction via the STAT3 pathway may be of interest as a future therapeutic approach for melanoma.

2. Melanoma

Melanoma represents a complex and heterogeneous form of cancer that relies on altered molecular pathways within the tumor cell and cooperation from the host immune system to permit its growth and progression. The incidence of malignant melanoma is rising faster than any other cancer. In the United States alone, more than 68,000 new cases of melanoma were diagnosed in the year 2009 (Jemal, A. et al. 2010). The median survival of patients with

metastatic melanoma is approximately 10 months even with aggressive systemic therapy (Atkins, M. B. 1998). Limited treatment options are available to patients with metastatic disease, and standard chemotherapeutic and immunologic agents are ineffective in a majority of patients. Therefore, there remains an urgent need to understand melanoma biology so that novel targets for therapeutic intervention can be identified and evaluated. Over the past several years, an increased understanding of the molecular pathways driving melanoma have allowed for some advances to be made in our ability to treat this disease. Namely, discovery of an activating mutation of the B-Raf protein at the V600 locus has led to the development of small molecule inhibitors of this mutated protein or its downstream pathways, such as MAPK, ERK and MEK (Ball, N. J. et al. 1994; Davies, H. et al. 2002; Pollock, P. M. et al. 2003; Smalley, K. S. et al. 2008). The agents targeting mutated B-Raf have produced impressive regression of established melanoma lesions, leading to their FDA-approval. However, these dramatic responses do not typically persist for more than several months before new melanoma lesions arise and many patients progress (Aplin, A. E. et al. 2011). Clearly there remains a need for identifying novel pathways in melanoma that could serve as therapeutic targets and be utilized to improve both single-agent or combination treatment options for this disease.

In addition to aberrant molecular pathways within malignant cells, there is strong evidence that the immune system plays a role in regulating the development and progression of melanoma. For example, melanoma incidence is increased in patients who are immunosuppressed (Vajdic, C. M. et al. 2009) and circulating, tumor-antigen specific T cells are present in patients with melanoma (Kawakami, Y. et al. 1994). Melanoma is also considered a tumor in which immune-based therapies can elicit dramatic clinical responses in a subset of patients. Recombinant cytokines such as interleukin-2 (IL-2), interferon-alpha (IFN- α) and granulocyte macrophage colony stimulating factor (GM-CSF) have been clinically utilized for treatment for melanoma patients at various disease stages ranging from metastatic disease to adjuvant therapy. In addition, the use of Ipilimumab, an antibody targeting the negative regulatory CTLA-4 receptor on T cells has recently gained FDA-approval for the treatment of metastatic melanoma (Traynor, K. 2011). In those melanoma patients who respond to immune-based therapies, vitiligo and autoimmune phenomena are often observed, further supporting a role for the immune system in regulating this malignancy (Bouwhuis, M. G. et al. 2010; Gogas, H. et al. 2006). As we gain experience with using new molecular-targeted agents for melanoma therapy, it will also be important to assess how these compounds modulate immune function of patients, as it relates to cancer progression or anti-tumor immune responses.

3. The Jak-STAT signal transduction pathway

The Janus-activated kinase (Jak)-signal transducer and activator of transcription (STAT) pathway is a signal transduction pathway that has been well-characterized in basic biologic study (Darnell, J. E., Jr. et al. 1994; Haque, S. J. et al. 1998). This pathway is thought to play a major role in regulating both the development and elimination of cancer. The various protein components of the Jak-STAT pathway are expressed ubiquitously within a majority of somatic cells. Four individual members of the Jak family of proteins (Jak1, Jak2, Jak3, Tyk2) and seven unique STAT proteins (STAT1-STAT7) have been identified. The structural features of these proteins are presented in Figures 1 and 2 in this section.

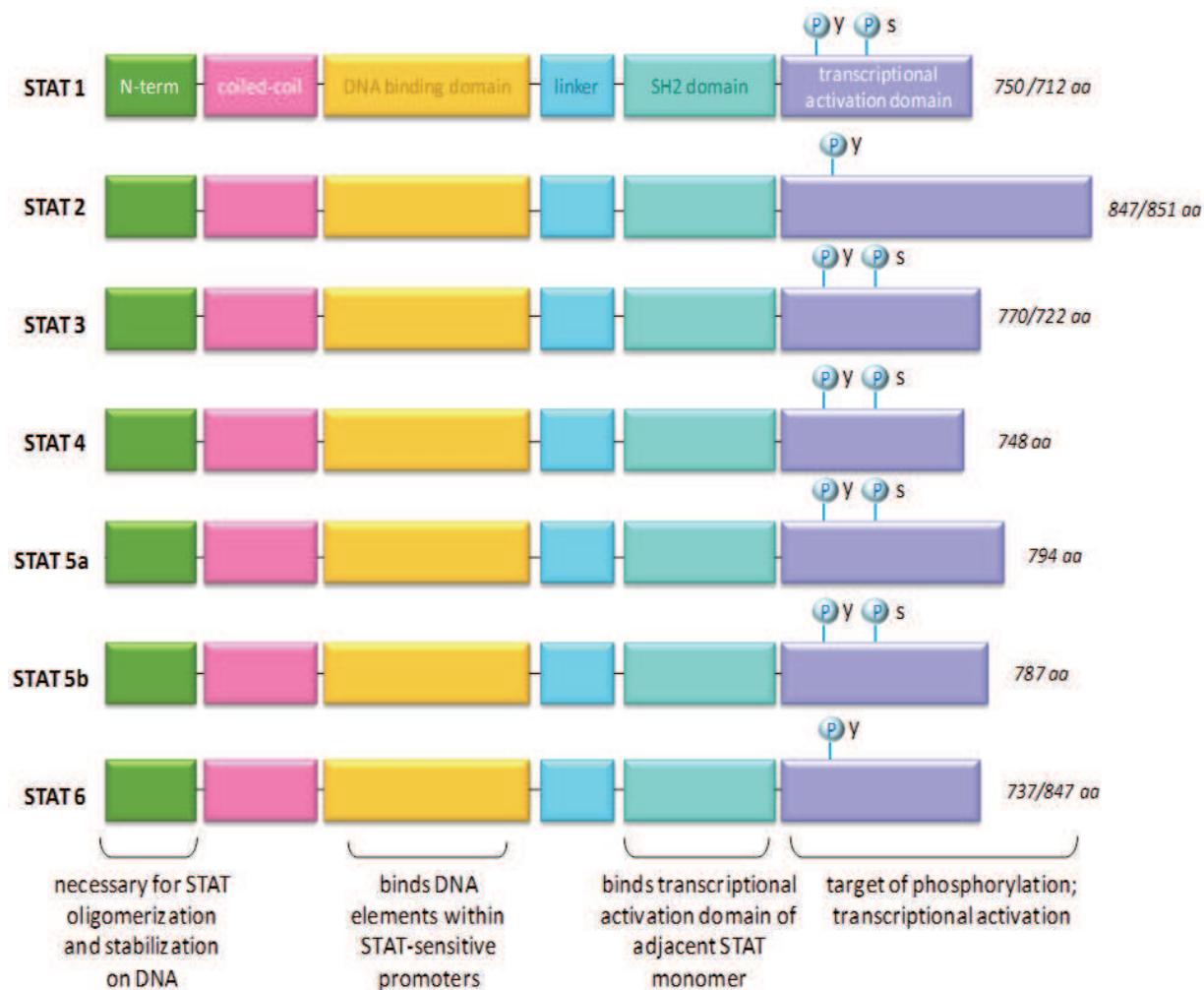


Fig. 1. Structural homology of the STAT family of transcription factors. P = phosphorylation, y = tyrosine, s = serine; isoforms are indicated by number of amino acids (aa). Adapted from (Jatiani, S. S. et al. 2010; Yue, P. et al. 2009)

A number of extracellular receptors can directly associate with Jak proteins residing in the cell membrane. The Jak proteins are typically activated upon binding of a ligand (typically a cytokine) to the receptor in normal cells, although they can be constitutively activated in malignant cells. Activation of Jak proteins leads to post-translational modifications of cytoplasmic STAT proteins that influence their subsequent cellular actions. Phosphorylation of STAT proteins is the most well-characterized of these modifications, an action which typically allows for STAT proteins to dimerize and translocate to the nucleus.

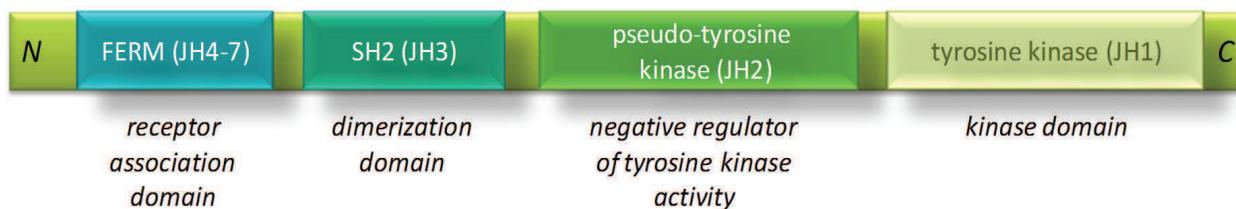


Fig. 2. Basic structure of Jak/Tyk kinases involved in STAT pathways. Adapted from (Jatiani, S. S. et al. 2010)

Other kinases such as Src or Abelson (Abl) kinases can also lead to STAT protein phosphorylation (Al Zaid Siddiquee, K. et al. 2008). Once in the nucleus, STAT proteins can cooperate with other co-factors, bind to promoters and activate the transcription of specific genes involved in a diverse array of cellular processes. The profile of individual STAT proteins that are activated has a profound influence upon the biologic response of the cell. For the purposes of this chapter, our discussion will focus primarily on the difference between biologic responses induced by STAT1 as compared to STAT3. These specific STAT proteins appear to play a major role in regulating melanoma biology and the response of this cancer to various types of therapy (Ransohoff, R. M. 1998). Under normal biologic conditions, activation of STAT proteins is rapid and transient. However, aberrant activation of the STAT proteins is associated with a variety of human malignancies (Al Zaid Siddiquee, K. et al. 2008) (Bowman, T. et al. 2000; Bromberg, J. F. 2001; Buettner, R. et al. 2002; Darnell, J. E., Jr. 2002; Darnell, J. E. 2005; Turkson, J. 2004; Turkson, J. et al. 2000; Yu, H. et al. 2004).

4. STAT1

The STAT1 transcription factor has been traditionally viewed as a 'tumor suppressor' (Bromberg, J. F. et al. 1996; Chin, Y. E. et al. 1997; Kaplan, D. H. et al. 1998). A great deal of knowledge regarding the function of this protein has been gained from analysis of its role in mediating signal transduction in response to cytokines such as the Type I and Type II interferons (Darnell, J. E., Jr. 1998; Darnell, J. E., Jr. et al. 1994; Haque, S. J. et al. 1998). These studies have established that transcriptionally active STAT1 is required for the anti-proliferative effects of interferons (Bromberg, J. F. et al. 1996). This is particularly relevant in the setting of melanoma, where aside from their immunostimulatory effects, interferons can bind their receptors and exert direct anti-proliferative effects against the tumor cell.

It has also been observed that some interferon-resistant human melanoma cell lines exhibited defects in specific Jak-STAT intermediates, which when reversed led to the recovery of *in vitro* sensitivity to interferons (Kaplan, D. H. et al. 1998; Pansky, A. et al. 2000; Huang, M. et al. 2002; Wong, L. H. et al. 1998). Interestingly, the most common defect appeared to be the loss of STAT1. These data support a role for STAT1 as a negative regulator of melanoma cell proliferation. In other cell types, STAT1 has been shown to negatively regulate the cell cycle by down-regulating the expression of cyclins, c-myc and by inducing the CDK inhibitors p21 and p27 (Regis, G. et al. 2008). A brief overview of STAT1 transcriptional targets can be found in Figure 3.

The STAT1 transcription factor is also important for mediating melanoma cell sensitivity to various pro-apoptotic stimuli. In a sentinel study by Kumar et al., it was shown that STAT1-deficient human fibrosarcoma cells were less susceptible to tumor necrosis factor alpha (TNF- α) induced cell death as compared to parental cells containing STAT1 (Kumar, A. et al. 1997). This STAT1 dependent regulation of cell death is largely dependent on a transcriptional mechanism such as the activation of death-promoting genes, or induction of the Interferon Regulatory Factor 1 (IRF1) (Bernabei, P. et al. 2001; Sato, T. et al. 1997; Regis, G. et al. 2005; Kim, H. S. et al. 2007). For example, STAT1 can regulate the expression of death-receptor-4 (DR4) expression on melanoma cells, which could affect TRAIL sensitivity (Meng, R. D. et al. 2001). Similarly, STAT1 has also been shown to upregulate the expression of caspases, Fas, and FasL, (Kumar, A. et al. 1997; Chin, Y. E. et al. 1996; Lee, C. K. et al. 2000; Ouchi, T. et al. 2000; Stephanou, A. et al. 2005). Non-transcriptional mechanisms such as the ability of STAT1 to interact with TRADD, p53 or HDAC have also been implicated in the

regulation of cell death by STAT1 (Kim, H. S. et al. 2007). The precise role for these STAT1-mediated factors in regulating apoptosis specifically in melanoma cells is an area of interest that could be exploited for further therapeutic benefit.

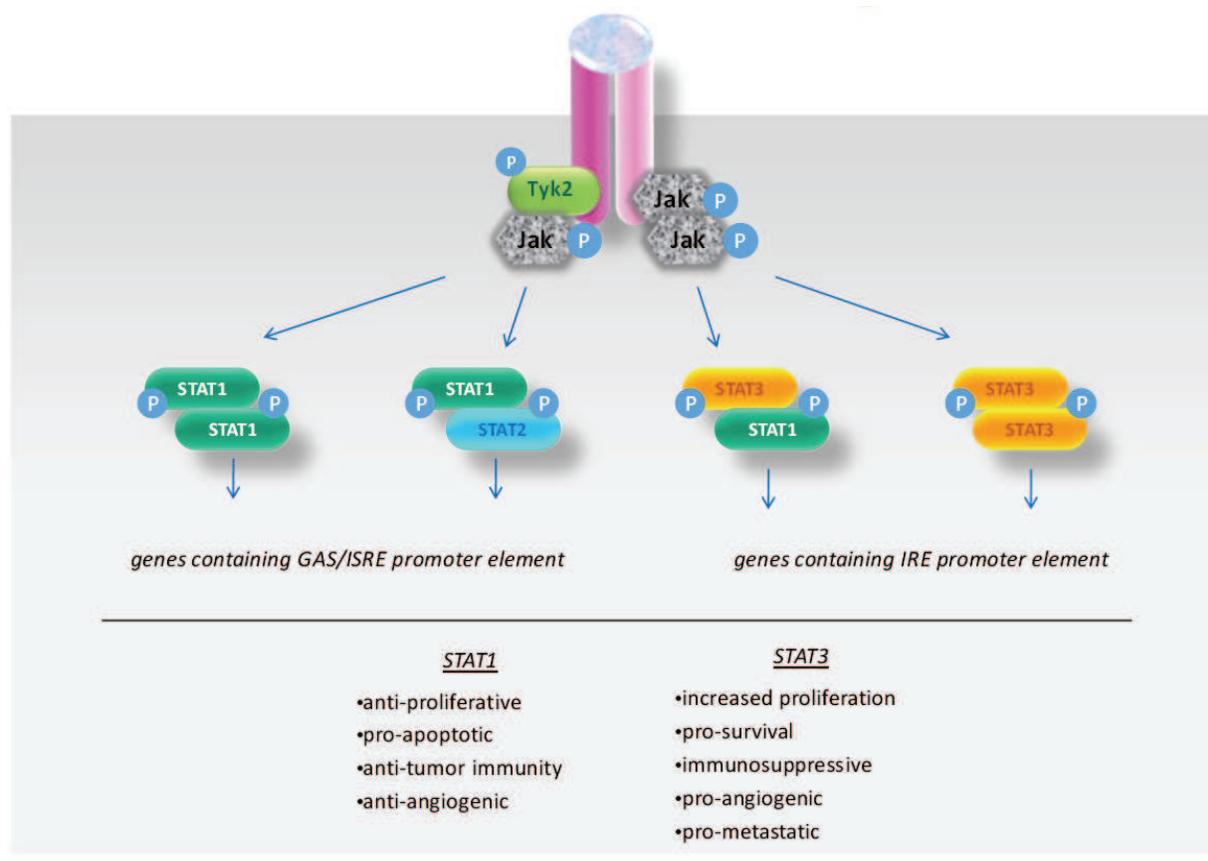


Fig. 3. Comparison of STAT1 and STAT3 signaling and their effects in cancer. Adapted from (Yu, H. et al. 2009)

Other evidence that supports a role for STAT1 as a tumor suppressor gene has come from STAT1-deficient mice. These animals were initially described by Durbin *et al.*, and found to be more susceptible to infection with microbial pathogens. It was discovered that these animals had defective immune responses, and more specifically in their lack of interferon-responsiveness (Durbin, J. E. et al. 1996). Later studies showed that these animals were also more susceptible to tumorigenesis induced by exposure to chemical carcinogens as compared to their wild-type counterparts (Kaplan, D. H. et al. 1998). Finally, when crossed on to a p53-deficient background, STAT1-deficient mice demonstrated a greater propensity for tumor development as compared to mice lacking either p53 or STAT1 alone (Kaplan, D. H. et al. 1998). Together these data support a role for STAT1 as a negative regulator of tumor development (Bromberg, J. F. 2001).

In addition to its putative role as a tumor suppressor, STAT1 signal transduction within non-malignant cells can also regulate the outgrowth of tumors and the response to therapy with exogenous IFN- α . Since STAT1 deficient (STAT1^{-/-}) mice manifested deficiencies in IFN-mediated anti-viral immunity, we postulated that the anti-tumor effects of IFN- α might proceed primarily via immunologic mechanisms (Durbin, J. E. et al. 1996). To explore the contribution of STAT1-mediated gene regulation within the tumor, our group generated a

STAT1-deficient murine melanoma cell line, AGS-1 (Badgwell, B. et al. 2004; Lesinski, G. B. et al. 2003). STAT1 was reconstituted within AGS-1 cells by retroviral gene transfer. The resulting cell line (AGS-1^{STAT1}) showed normal regulation of IFN- α -stimulated genes (H2k, ISG-54) as compared to AGS-1 cells infected with the empty vector (AGS-1^{MSCV}). However, mice challenged with the AGS-1, AGS-1^{STAT1}, and AGS-1^{MSCV} cell lines exhibited nearly identical survival in response to IFN- α treatment, indicating that restored STAT1 signaling within the tumor did not augment the anti-tumor activity of IFN- α . In contrast, STAT1^{-/-} mice could not utilize exogenous IFN- α to inhibit the growth of STAT1^{+/+} melanoma cells in either an intraperitoneal tumor model or in the adjuvant setting. The survival of tumor-bearing STAT1^{-/-} mice was identical regardless of whether they were received IFN- α or PBS. STAT1^{-/-} mice exhibited normal levels of circulating immune effector cells, but splenocytes from STAT1^{-/-} mice exhibited a 90-95% reduction in cytotoxic activity against the NK-sensitive YAC1 cell line in a ⁵¹Cr-release assay. Thus, STAT1-mediated gene regulation within immune effectors (but not tumor cells) was necessary for mediating the anti-tumor effects of IFN- α in this experimental system (Lesinski, G. B. et al. 2003). Several subsequent studies have indicated that signaling via the Jak-STAT1 pathway in other cellular compartments is altered in the setting of melanoma, and could contribute to the development or progression of this tumor in humans. For example, significant impairments in the phosphorylation of STAT1 has been observed in T cells or bulk peripheral blood mononuclear cells obtained from patients with metastatic melanoma (Lesinski, G. B. et al. 2004; Critchley-Thorne, R. J. et al. 2009; Mortarini, R. et al. 2009). Other evidence suggests that intratumoral expression of STAT1 does not correlate with effectiveness of IFN- α used as an adjuvant therapy for melanoma. In one study, a large cohort of high-risk patients that exhibited prolonged survival in response to adjuvant IFN- α was identified. In some of these patients, loss of STAT1 expression was noted within their tumor. In contrast, other patients, who had normal expression of Jak-STAT proteins, recurred after just a few months of IFN therapy (Lesinski, G. B. et al. 2005). These results have been confirmed by a second group, who showed that while phosphorylation of STAT1 at Tyr⁷⁰¹ and Ser⁷²⁷ was not inducible in 63% of patient tumors, STAT1 activation defects showed no correlation with disease outcome or response to IFN- α -2b immunotherapy as indicated by progression-free survival (Boudny, V. et al. 2003). Recent studies have further shown that patient melanoma cells exhibit negligible levels of STAT1 activation following IFN- α stimulation (as compared to immune cells from the same patient), even when all major components of the Jak-STAT signaling pathway were present (Lesinski, G. B. et al. 2007). These experiments provide compelling evidence that STAT1 signal transduction within non-transformed cellular compartments may be altered in patients with melanoma and required for effective anti-tumor immune responses.

Although STAT1 has traditionally been associated with anti-tumor effects mentioned above, more recent studies have suggested that in certain situations, STAT1 may play a paradoxical role in promoting melanoma progression. In murine tumor models, it has been shown that stable knockdown of STAT1 from B16F1 melanoma cells constitutively over-expressing STAT1 led to a less aggressive tumor phenotype and decreased colonization of tumor cells into the lung (Khodarev, N. N. et al. 2009). In another study, a large-scale gene expression analysis of melanoma metastases was conducted to identify genes involved in late-stage tumor progression. A comparison of differential gene expression between peripheral areas of the tumor (around the 'invasion front') with that of central tumor areas revealed that

STAT1 was highly expressed within peripheral, compared with central tumor areas. Furthermore, STAT1 knockdown reduced the metastatic behavior of melanoma cells in a murine model (Schultz, J. et al. 2010). These studies suggest that STAT1 might play a role in promoting the progression of certain late stage melanomas. These seemingly pro-tumor properties of activated STAT1 within a subset of melanoma cells could be due to the ability of STAT1 to serve as a central mediator of inflammation. Indeed, STAT1 is activated following stimulation with pro-inflammatory cytokines in other tumor such as tumor-associated macrophages. For example, STAT1 can regulate the expression of arginase and nitric oxide (NO), which in turn suppress tumor-specific T cells (Kusmartsev, S. et al. 2005). In addition, STAT1 can induce the expression of indoleamine 2,3-dioxygenase, an enzyme that is expressed in cancer that can block T cell activation (Uyttenhove, C. et al. 2003). However, the approach of STAT1 inhibition as a potential therapeutic strategy should be approached with caution. Prior studies have shown that in the absence of STAT1, IFN- γ stimulation leads to a strong and prolonged activation of STAT3, which could exacerbate tumorigenesis or metastasis (Qing, Y. et al. 2004).

5. STAT3

The STAT3 transcription factor plays a key role in the setting of melanoma and numerous other cancers. This protein induces unique transcriptional profiles in response to a variety of growth factors, cytokines, hormones and oncogenes (e.g. IL-6, leptin, IL-12, IFNs, IL-10, G-CSF, prolactin, growth hormone, EGF, HGF, bFGF, v-Src, v-Fps, v-Sis) (Dewilde, S. et al. 2008). A brief overview of STAT3 transcriptional targets can be found in Figure 3. STAT3 is considered an oncogene due to prior studies showing that over-expression of its constitutively active form could transform cultured cells or form tumors in nude mice (Bromberg, J. F. et al. 1999; Dechow, T. N. et al. 2004; Azare, J. et al. 2007). In the malignant cell, this protein is a key mediator which promotes cell proliferation and angiogenesis, inhibits apoptosis, and promotes the transcription of genes important for invasion and metastasis. Phosphorylation of STAT3 on specific tyrosine residues can modulate its propensity to dimerize with other STAT proteins (STAT1, STAT3, or STAT5), or to increase its affinity for DNA once it has dimerized and translocated to the nucleus. In contrast to other STAT proteins, loss of STAT3 in a whole organism is embryonic lethal (Takeda, K. et al. 1997). However, tissue specific knockout of STAT3 has been possible, and has led to a great appreciation as to the complexity of STAT3 in regulating various inflammatory, growth or physiologic processes (Dewilde, S. et al. 2008). In contrast to normal cells, where STAT3 activation is rapid and transient, neoplastic cells often display constitutive STAT3 activation (Fletcher, S. et al. 2008). Like other STAT proteins, STAT3 exists in two isoforms generated by alternative splicing, the full length STAT3 α and the truncated STAT3 β . These distinct isoforms add further to the complexity of this protein, as the STAT3 β isoform is thought to act as a dominant negative factor, and to be transcriptionally active (Dewilde, S. et al. 2008). Two additional STAT3 isoforms, gamma and delta are generated proteolytically in myeloid cells, however their role in these or other cells remains unclear (Hevehan, D. L. et al. 2002; Chakraborty, A. et al. 1996). STAT3 was initially identified as Acute Phase Response Factor (APRF), which was shown to activate promoters of acute phase genes following IL-6 (Wegenka, U. M. et al. 1993).

Constitutive activation of STAT3 is a common characteristic of multiple tumor types. This observation was first noted associated with oncogenic transformation by the viral Src

oncoprotein (Al Zaid Siddiquee, K. et al. 2008; Yu, C. L. et al. 1995). In a majority of melanoma cell lines and clinical specimens, constitutive phosphorylation of STAT3 at the Tyr705 residue has also been observed (Alas, S. et al. 2003; Buettner, R. et al. 2002; Duan, Z. et al. 2007; Real, P. J. et al. 2002; Shen, Y. et al. 2001; Wang, T. et al. 2004; Yu, H. et al. 2004). This post-translational modification can occur in response to receptor-ligand interactions such as cytokines (interleukin-6), growth factors (vascular endothelial growth factor) or other soluble mediators (oncostatin M) that may be secreted in an autocrine fashion by melanoma cells or in a paracrine manner by other cells present in the microenvironment. In addition to soluble mediators, constitutive activation of other intracellular proteins such as Src within melanoma cells can also promote STAT3 phosphorylation and its downstream effects. Recent studies have led to further insight as to the mechanism by which STAT3 is persistently activated in tumors. In a study by Lee *et al.*, it was shown that expression of the sphingosine-1-phosphate receptor-1 (S1PR1), a G protein-coupled receptor for the lysophospholipid sphingosine-1-phosphate (S1P), is elevated in STAT3 positive tumors, including melanoma (Lee, H. et al. 2010). In this report, the authors further showed that STAT3 was a transcription factor for the *S1pr1* gene, and that enhanced *S1pr1* expression led to STAT3 activation and upregulated IL-6 expression. This reciprocal relationship led to accelerated tumor growth and metastasis in a STAT3 dependent manner. Together these data identify that STAT3 and S1PR1 participate in a positive feedback loop to sustain STAT3 activation in cancer cells (Lee, H. et al. 2010).

Constitutive activation of STAT3 in melanoma tumors is associated with a poor prognosis (Kortylewski, M. et al. 2005; Xie, T. X. et al. 2006; Niu, G. et al. 2002). Besides melanoma, STAT3 is thought to play a critical role in promoting an oncogenic, metastatic and drug-resistant phenotype in several types of cancer including breast, lung, and pancreatic among others (Alas, S. et al. 2003; Buettner, R. et al. 2002; Duan, Z. et al. 2007; Real, P. J. et al. 2002; Shen, Y. et al. 2001; Wang, T. et al. 2004; Yu, H. et al. 2004). The transcriptional signature mediated by STAT3 activation consists of numerous genes that contribute to retaining a malignant phenotype. For example, the oncogenic role of STAT3 is highlighted through its ability to regulate the expression of genes which mediate proliferation (c-myc, cyclin D1), inhibit apoptosis (Bcl-xL, survivin), and promote metastasis (e.g. matrix metalloproteinases) (Bromberg, J. 2002; Bromberg, J. F. et al. 1996; Fletcher, S. et al. 2008). STAT3 is also associated with regulating apoptosis by the noncanonical nuclear factor- κ B (NF- κ B) (Barre, B. et al. 2007) and induces VEGF or HIF1- α expression to promote angiogenesis (Barre, B. et al. 2007; Chauhan, D. et al. 2001; Xi, S. et al. 2005; Niu, G. et al. 2002; Xu, Q. et al. 2005; Xie, T. X. et al. 2006). STAT3 can also indirectly repress pro-apoptotic factors via its ability to repress the expression of the p53 tumor suppressor gene. Finally, studies in preclinical models of melanoma have also shown that STAT3 stimulates invasion and metastasis by inducing matrix metalloproteinase-2 (MMP-2) *in vitro* and *in vivo* (Xie, T. X. et al. 2006).

Unphosphorylated STAT3 has also been shown to regulate a specific subset of genes in fibroblast cell lines. Some of the genes present in the transcriptional profile induced by unphosphorylated STAT3 included well-known oncoproteins (e.g. MRas, MET, CDC2, Cyclin B1, E2F1) (Yang, J. et al. 2005). High levels of unphosphorylated STAT3 typically occur via a transcriptional mechanism following IL-6 stimulation. However to the best of our knowledge, the role of this mechanism has not been investigated specifically in melanoma.

6. Balance between STAT1 and STAT3 in Melanoma

The balance between phosphorylated STAT1 and STAT3 may be important in melanoma cell biology. Since IFN- α is a clinically-relevant therapy for melanoma, much investigation has occurred into differential regulation of STAT1 and STAT3 by this cytokine. Studies have shown that the differential phosphorylation of STAT1 and STAT3 regulates the cellular response to IFNs, and influences their anti-tumor activity (Ho, H. H. et al. 2006; Lesinski, G. B. et al. 2007; Wang, W. et al. 2007). STAT3 may act as a molecular “sink” for phosphorylation events upon ligand binding to a receptor. This functional property of STAT3 can negatively regulate the transcriptional response mediated via other STAT proteins.

The concept that the ratio of phosphorylated STAT1 to STAT3 could serve as a biomarker for melanoma progression has recently gained attention. For example, in a study by Wang et al., the level of pSTAT1 and pSTAT3 was evaluated in biopsies of atypical nevi from patients receiving IFN- α (Wang, W. et al. 2008). Results from these studies showed that the percentage of pSTAT3-positive melanocytes was positively associated with the atypical degree of nevi. These data showed that the relative balance of pSTAT1/pSTAT3 may be associated with melanocyte differentiation *in vivo*. Studies from our group have also shown that the level of pSTAT3 present in melanoma cell lines was inversely correlated with IFN- α induced STAT1 phosphorylation (Lesinski, G.B. et al. 2007). The therapeutic utility of this inverse relationship between STAT1 and STAT3 is currently being evaluated in pre-clinical studies of melanoma utilizing combinations of STAT3 pathway inhibitors with cytokines that activate STAT1 (Kong, L. Y. et al. 2010). These studies are described in detail below in section 9.

7. Rationale for STAT3 as a molecular target for therapy

In melanoma and numerous other cancers, constitutively active STAT3 is a poor prognostic indicator and potential therapeutic target. Indeed constitutive phosphorylation of STAT3 can afford melanoma cells a survival advantage and promote an aggressive phenotype. Constitutive STAT3 phosphorylation is mediated by several kinases including Jak2, members of the Src family (hck, src), Erb B1, Erb B2, anaplastic lymphoma kinase, protein kinase C (PKC)- δ , c-fos, gp130, and epithelial growth factor (EGF) receptor (Jain, N. et al. 1999; Nelson, K. L. et al. 1998; Ren, Z. et al. 2002; Sellers, L. A. et al. 1999; Smithgall, T. E. et al. 2000; Zhang, Y. et al. 2000). Interestingly, despite its necessity in early embryogenesis, STAT3 appears to be largely dispensable in most normal adult cell and tissue types (Akira, S. 2000; Takeda, K. et al. 1997). In addition to the role of STAT3 in tumor cells, recent studies have demonstrated that STAT3 also promotes immune tolerance in the setting of cancer (reviewed in (Yu, H. et al. 2007)). Together these data suggest that inhibition of STAT3 represents a rational approach to cancer therapy as it could have both an effect directly on the tumor cell while also promoting the ability of immune cells to recognize and eliminate cancer. Interestingly, recent studies from our group and others have demonstrated that the presence of constitutively active STAT3 can inhibit the response to cytokines which act via STAT1 signal transduction (Lesinski, G. B. et al. 2007). These data suggest that the balance between pSTAT1 and pSTAT3 may influence the responsiveness of cells to immunostimulatory cytokines and ultimately immune-mediated tumor regression (Lesinski, G. B. et al. 2007; Wang, W. et al. 2007). Based on these data, it is rational to suggest that

inhibition of STAT3 could augment responsiveness to standard or experimental immune-based therapies that act via the STAT1 transcription factor.

Notably, administration of IFN- α 2b is currently the standard of care and only FDA-approved agent for use as an adjuvant therapy in melanoma patients after surgical resection of high-risk cutaneous lesions (Balch, C. M. 1998). Therefore, STAT3 inhibition could enhance the anti-tumor properties of these existing therapies for melanoma patients.

8. Relevant structural features of STAT3 for inhibitor development

The structure of STAT3 includes a STAT dimerization domain at the amino-terminus, a coiled-coil domain important in protein interactions, a DNA binding domain, a SH2 domain, and a carboxy terminus that controls transcriptional activation (Frank, D. A. 2007; Levy, D. E. et al. 2002; Turkson, J. et al. 2000). Disrupting SH2 homodimerization of STAT3 can be achieved through inhibitor competition with the phosphoryl tyrosine 705 (Y705) binding site. These two SH2 domains are hinged together by a loop segment from each monomer. The pY705 critical for the biological function of STAT3 locates right on this loop segment, and binds, together with several adjacent amino acid residues (leucine 706, threonine 708, and phenylalanine 710), to a cavity on the SH2 domain of the other monomer.

9. Experimental strategies for STAT3 inhibition

Several strategies have been used to inhibit the STAT3 pathway as a therapeutic approach for treating various types of cancer including malignant melanoma. Direct STAT3 inhibitors can be categorized into different classes of compounds: peptides, peptidomimetics, small molecules, platinum complexes, siRNA and plant polyphenols (Fletcher, S. et al. 2008). Indeed, a number of peptides, peptidomimetics and peptide aptamers have been reported to inhibit STAT3 via their interaction with the DNA binding domain or the dimerization domain of the protein (Alas, S. et al. 2003; Yu, H. et al. 2007). These various strategies of STAT3 inhibition are outlined briefly in Figure 4. Each of these strategies for STAT3 inhibition have their strengths and weaknesses. For example, although there is a high degree of specificity using peptide-based approaches, these agents traditionally suffer from limited cell permeability and *in vivo* stability. Unfortunately, these properties have restricted their practical application *in vivo* (Fletcher, S. et al. 2008). More recently, however, advances have been made in designing cell-permeable, STAT3 SH2 domain mimetics. These mimetics have shown promising anti-tumor effects *in vitro* against a variety of solid tumor types (Zhao, W. et al. 2010). Moreover, siRNA specific for the SH2 coding region of STAT3 could induce apoptosis in prostate cancer cells *in vitro* and in nude mice bearing human xenograft tumors (Bromberg, J. F. et al. 1996). Recent studies have also shown that platinum complexes promote anti-tumor activity by their ability to inhibit STAT3 (Turkson, J. et al. 2004). A variety of plant polyphenols (e.g. resveratrol, flavopiridol, indirubin, magnolol, picetannol, parthenolide, EGCG, curcubitacin Q and curcumin) have also been shown to down-regulate the activity of STAT3 and other targets in malignant cells (Bharti, A. C. et al. 2003; Blaskovich, M. A. et al. 2003; Chakravarti, N. et al. 2006; Chen, S. C. et al. 2006; Lee, Y. K. et al. 2006; Masuda, M. et al. 2001; Nam, S. et al. 2005; Sobota, R. et al. 2000; Su, L. et al. 2000; Wung, B. S. et al. 2005). Collectively, these studies provide precedent for targeting STAT3 as a means of inducing apoptosis of tumor cells. However, the specificity of many existing inhibitory strategies for STAT3 and not other STAT proteins (e.g. STAT1) or

oncogenic pathways is still being validated in biological systems. Arguably, numerous early generation small molecule STAT3 inhibitors (e.g. Stattic, STA-21, S32-M2001, S3I-201) have shown particular promise, due to their ability induce apoptosis (Fletcher, S. et al. 2008). In the past, our ability to identify novel small molecule inhibitors that disrupt SH2 domain phosphotyrosine interactions has been limited due to the very high degree of hydrophobic interactions present within this portion of the molecule. However, recent advances from a number of groups have shown that this is indeed possible. For example, Zhang *et al.* have recently described a novel small molecule S3I-201.1066, a structural derivative of S3I-201 that can disrupt phosphotyrosine interactions at the STAT3 SH2 domain (Zhang, X. et al. 2010). Similarly, other derivatives of the early generation STAT3 inhibitor LLL-3 (designated LLL-12) (Ball, S. et al. 2011; Lin, L. et al. 2011; Onimoe, G. I. et al. 2011; Wei, C. C. et al. 2011; Lin, L. et al. 2010) or purine scaffolds (Shahani, V. M. et al. 2011) show similar activity in a variety of tumor models. Finally, recent studies have described that FLLL32, a structural analog of curcumin, can preferentially target STAT3 while retaining STAT1 mediated signal transduction within melanoma and immune effector cells. Treatment of human melanoma cell lines, and early passage primary melanoma tumors with FLLL32 led to caspase-dependent apoptosis via its inhibition of STAT3 (Bill, M. A. et al. 2010).

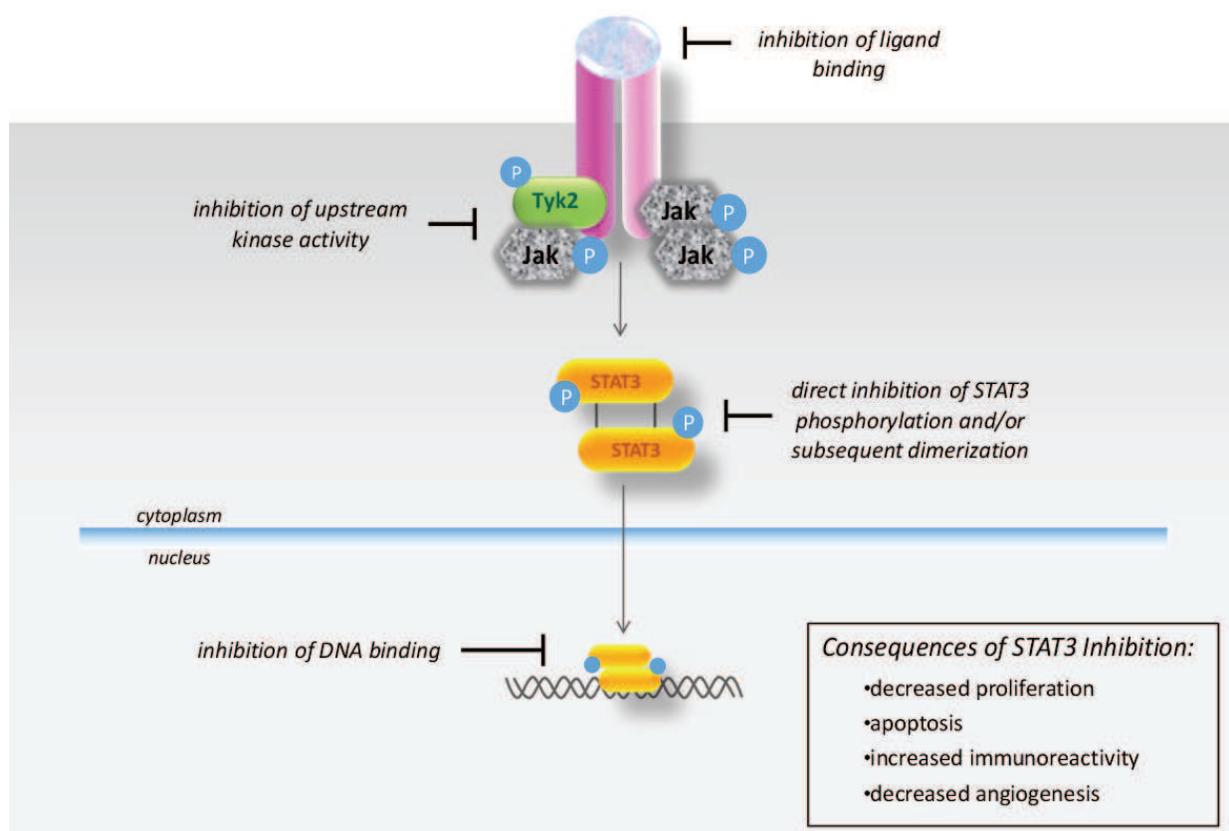


Fig. 4. Strategies of STAT3 pathway inhibition

Because STAT3 can promote immune tolerance in the setting of cancer, this pathway represents an attractive target to enhance immunotherapy (Reviewed in (Yu, H. et al. 2007)). Recent studies have demonstrated that the presence of constitutively phosphorylated STAT3 in melanoma cells is correlated with reduced responsiveness to cytokines which act via STAT1 signal transduction (Lesinski, G. B. et al. 2007). These data suggest that the balance

between pSTAT1 and pSTAT3 may influence cellular responsiveness to immunostimulatory cytokines and ultimately immune-mediated tumor regression (Lesinski, G. B. et al. 2007; Wang, W. et al. 2007).

It is apparent that STAT3 inhibition may serve as a means to sensitize melanoma cells, or the immune system of a tumor bearing host to interferon-alpha, a cytokine that exerts its anti-tumor effects in a manner dependent upon STAT1 signaling. For example, Kong *et al.* demonstrated that combined therapy with the Jak2/STAT3 pathway inhibitor WP1193 (a third generation AG490 analog) and IFN- α could enhance innate and adaptive cytotoxic T cell activity and elicit impressive anti-tumor activity in an intracerebral murine tumor model of melanoma (Kong, L. Y. et al. 2010). Further supporting the concept that STAT3 inhibition may augment immunotherapy with IFN- α was a study by Ito *et al.* performed in patients with renal cell carcinoma. In this report, linkage disequilibrium mapping revealed that a SNP in the 5' region of STAT3, rs4796793, leads to reduced STAT3 protein expression. Importantly this SNP was a significant predictor of clinical response to IFN- α . The authors of this study concluded that patients with a minor rs4796793 allele had better intrinsic immunosurveillance. Although immunomodulation was the focus of these prior studies, it remains possible that this strategy could also enhance the direct anti-proliferative or pro-apoptotic effects of IFN- α on melanoma cells (Ito, N. et al. 2007).

With these studies in mind, recent data indicate that inhibition of STAT3 with the FLLL32 small molecule does not alter production of granzyme b or IFN- γ by NK cells from normal donors when cultured with K562 targets, or their viability when cultured with IL-2 (Bill, M. A. et al. 2010). These properties are of importance based on recent murine studies showing the Jak2 inhibitor WP1193 can augment immunotherapy with IFN- α (Kong, L. Y. et al. 2010), and STAT3 siRNA-CpG oligodeoxynucleotides can elicit anti-tumor immune responses (Kortylewski, M. et al. 2009). Together these data suggest that STAT3 inhibition could be investigated further as a potential means by which to overcome immune tolerance and augment responsiveness to standard or experimental immune-based therapies (Bill, M. A. et al. 2010).

10. Other post-translational modifications of STAT proteins: An opportunity for therapeutic intervention

In addition to phosphorylation, other post-translational modifications can regulate the activity of STAT proteins and deserve mention in this chapter. Among these modifications are methylation, ubiquitination, sumoylation, isgylation and acetylation (O'Shea, J. J. et al. 2005). Although we will not provide an exhaustive review of these processes, the balance of these modifications is thought to have a profound influence on the gene expression that occurs within the cell. The relevance of each of these post-translational modifications is a rapidly emerging area of research, and is not completely understood in most cellular systems including melanoma. These various post-translational modifications of STAT proteins are a clear opportunity for further study. Below we will highlight a few brief examples that provide great insight into how these events might regulate cellular function.

First, in an eloquent study by Yuan *et al.*, it was demonstrated that STAT proteins undergo acetylation of a single amino acid residue, lysine 685, in response to cytokine stimulation. The authors were able to conclude that this modification was essential for STAT proteins to dimerize and initiate gene transcription (O'Shea, J. J. et al. 2005; Yuan, Z. L. et al. 2005). These initial data were instrumental in demonstrating that acetylation of STAT proteins is a

cytokine-induced post-translational modification that is critical for initiating the downstream gene expression characteristic of these transcription factors.

Acetylation has also been shown to be critical for crosstalk between STAT proteins and other signal transduction pathways. For example, a study by Kramer *et al.* demonstrated that only acetylated STAT1 was able to interact with NF- κ B p65. As a consequence, p65 DNA binding, nuclear localization, and expression of anti-apoptotic NF- κ B target genes decreased (Kramer, O. H. et al. 2006). In addition, STAT1 protein acetylation is a process that is tightly controlled by histone deacetylases (HDAC) and may play a negative regulatory role by counteracting interferon-stimulated gene expression (Kramer, O. H. et al. 2010).

The importance of these post-translational modifications to melanoma cell biology represents an area of great interest and importance. A greater understanding of their role in melanoma could be utilized to develop novel therapeutic agents that alter cell survival and response to therapy.

11. Negative regulation of Jak-STAT signal transduction

Negative regulation of cytokine-induced STAT protein phosphorylation can be mediated by a variety of mechanisms. Proteins involved in the negative regulation of this pathway are cytokine receptors, kinases, phosphatases (PTPs), suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STAT (PIAS). Accurate 'tuning' of the Jak-STAT pathway is instrumental in regulating the cellular response to various cytokines or growth factors to maintain physiological processes in normal and malignant cells. Thus, altering negative regulatory mediators involved in Jak-STAT signal transduction can have dramatic consequences on cellular function and survival. Because many STAT proteins are constitutively active in melanoma cells, these various negative regulatory proteins are currently under investigation as novel therapeutic targets, primarily in pre-clinical models.

The negative regulatory SOCS proteins (briefly illustrated in Figure 5) are a particularly promising target for melanoma (Alexander, W. S. 2002). The SOCS family of proteins has eight members, all of which have a central SH2 domain, an amino terminal domain, and a 40-amino acid motif at the carboxy terminus that is known as the SOCS box. In unstimulated cells, signaling molecules (such as the Jaks and STATs) are typically inactive, and SOCS genes are generally not expressed. The induction of SOCS proteins following cytokine stimulation defines a negative feedback loop, and serves to regulate cytokine-induced signal transduction. SOCS1 possesses a kinase inhibitory region (KIR) that binds to the catalytic groove of Jak2 and inhibits its activity (Nicholson, S. E. et al. 1999; Yasukawa, H. et al. 1999). SOCS3 has significant sequence homology to SOCS1 and appears to inhibit Jak catalytic activity via its KIR region. SOCS3 does not bind directly to Jaks but instead utilizes direct receptor binding to access to the Jak activation loop (Belardelli, F. et al. 2002).

Since melanoma is traditionally viewed as an immune-reactive tumor, targeting SOCS proteins in immune effector cells is attracting interest as a potential therapeutic approach. This concept is founded on the fact that SOCS proteins are important for regulating multiple cytokine-induced processes in immune cells. Several lines of evidence point to SOCS proteins as important regulators of T cell development and function. For example, previous studies have demonstrated that over-expression of SOCS3 inhibits the proliferation of T cells following TCR ligation and exposure to cytokines. Mice with a targeted deficiency of SOCS1 within the T cell compartment exhibit an increased ratio of CD8/CD4 mature thymic cells and a significant increase in the prevalence of CD44^{hi} CD8⁺ memory T cells within the

periphery (Davey, G. M. et al. 2005). SOCS1-deficiency has also been shown to promote the processing of tumor antigens by DC and their recognition by effector T cells (Hanada, T. et al. 2005; Shen, L. et al. 2004). One recent study has also demonstrated that vaccination of mice with melanoma antigen-primed, SOCS1-deficient DC elicited protection against lethal melanoma tumors (Shen, L. et al. 2004). Other studies have demonstrated that mice lacking both SOCS1 and IFN- γ can be cured of otherwise lethal melanoma tumors through exogenous administration of IFN- α (Zimmerer, J. M. et al. 2007; Guenterberg, K. D. et al. 2011). Finally, studies from Hashimoto *et al.* have demonstrated that silencing SOCS1 in macrophages enhances the survival of mice bearing B16 melanoma tumors (Hashimoto, K. et al. 2009). Together these data suggest that SOCS proteins within the adaptive immune system play an instrumental role in regulating the anti-tumor response against melanoma.

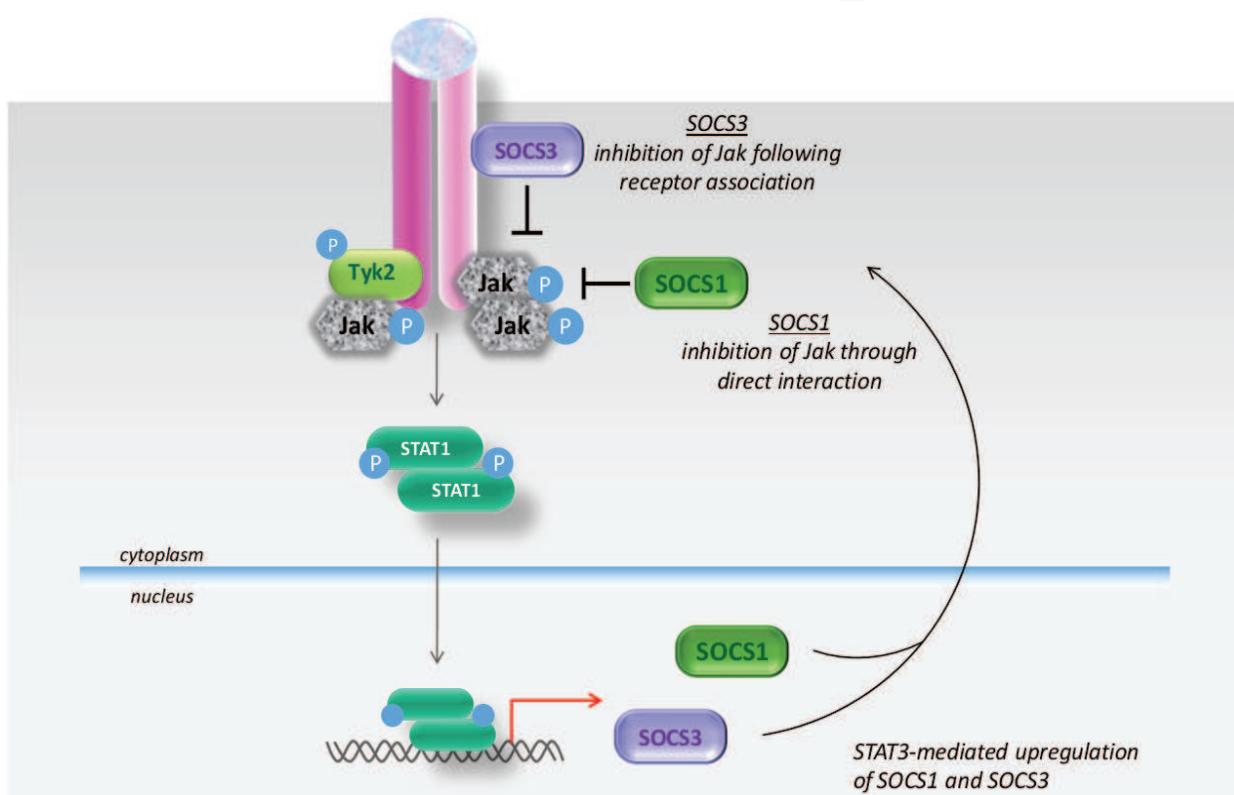


Fig. 5. Negative feedback inhibition of the STAT pathway through SOCS1 and SOCS3 (Alexander, W. S. 2002)

Jak-STAT activation is also negatively regulated by other mechanisms including protein inhibitors of activated STATs (PIAS), SHP-1 and SHP-2 (which de-phosphorylate activated receptors), truncated STAT isoforms (Brassard, D. L. et al. 2002; Liu, B. et al. 1998; Sakai, I. et al. 2002; You, M. et al. 1999) and acetylation, as mentioned previously in this chapter (Kramer, O. H. et al. 2010; O'Shea, J. J. et al. 2005). Moreover, other post-translational modifications such as sumoylation of STAT1 can influence IFN-induced signal transduction (Liu, B. et al. 2008; Lim, C. P. et al. 2006; Kim, H. S. et al. 2007). These data highlight the diversity of intracellular mechanisms available to limit Jak-STAT signal transduction and suggest numerous potential molecular targets for future study in melanoma. Despite the various data demonstrating upregulated negative regulatory proteins such as SOCS1 and SOCS3 in melanoma, it remains somewhat unclear as to how constitutive STAT protein

phosphorylation can occur in a highly prevalent manner along with consistent upregulation of the negative regulatory factors.

12. Conclusions

The Jak-STAT pathway plays an instrumental role in mediating the biology of malignant melanoma cells. This signal transduction pathway also holds great opportunity for therapeutic targeting of melanoma. In particular, the STAT3 transcription factor represents an area of increasing interest for development of small molecule inhibitors or other approaches for therapeutic use. Finally, there lies a tremendous opportunity to better understand melanoma biology through our investigation into novel post-translational modifications and negative regulation of signal transduction via the Jak-STAT pathway.

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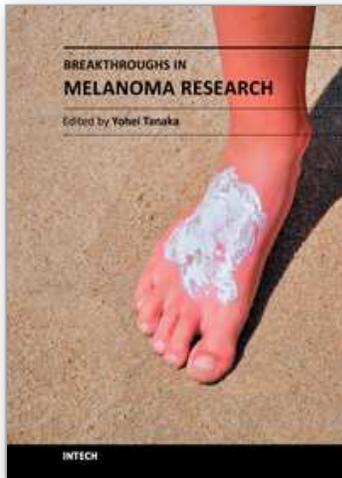
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Breakthroughs in Melanoma Research

Edited by Dr Yohei Tanaka

ISBN 978-953-307-291-3

Hard cover, 628 pages

Publisher InTech

Published online 30, June, 2011

Published in print edition June, 2011

Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

How to reference

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Courtney Nicholas and Gregory B. Lesinski (2011). The Jak-STAT Signal Transduction Pathway in Melanoma, Breakthroughs in Melanoma Research, Dr Yohei Tanaka (Ed.), ISBN: 978-953-307-291-3, InTech, Available from: <http://www.intechopen.com/books/breakthroughs-in-melanoma-research/the-jak-stat-signal-transduction-pathway-in-melanoma>

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