



STAT proteins: novel molecular targets for cancer drug discovery

James Turkson^{1,2} and Richard Jove^{*1,2,3,4}

¹Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA; ²Department of Oncology, University of South Florida College of Medicine, Tampa, Florida, USA; ³Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, Florida, USA; ⁴Department of Pathology, University of South Florida College of Medicine, Tampa, Florida, USA

Signal Transducers and Activators of Transcription (STATs) are a family of cytoplasmic proteins with roles as signal messengers and transcription factors that participate in normal cellular responses to cytokines and growth factors. Frequently, however, abnormal activity of certain STAT family members, particularly Stat3 and Stat5, is associated with a wide variety of human malignancies, including hematologic, breast, head and neck, and prostate cancers. Application of molecular biology and pharmacology tools in disease-relevant models has confirmed Stat3 as having a causal role in oncogenesis, and provided validation of Stat3 as a target for cancer drug discovery and therapeutic intervention. Furthermore, a constitutively-active mutant form of Stat3 is sufficient to induce oncogenic transformation of cells, which form tumors *in vivo*. Constitutive activation of Stat3 signaling is accompanied by upregulation of cyclin D1, c-Myc, and Bcl-x, changes consistent with subversion of normal cellular growth and survival control mechanisms. Block of constitutive Stat3 signaling results in growth inhibition and apoptosis of Stat3-positive tumor cells *in vitro* and *in vivo*. The observed dependence of certain tumors on constitutive Stat3 signaling for growth and survival has wide implications for cancer therapy, offering the potential for preferential tumor cell killing. This review evaluates constitutive Stat3 activation as a 'cancer-causing' factor, and proposes a number of molecular strategies for targeting Stat3 signaling for therapeutic intervention. *Oncogene* (2000) 19, 6613–6626.

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STAT proteins: an overview

STATs were originally discovered as latent cytoplasmic transcription factors that mediate cellular responses to diverse cytokines and growth factors (Darnell, 1997; Darnell *et al.*, 1994; Horvath and Darnell, 1997; Ihle and Kerr, 1995; Schindler and Darnell, 1995; Stark *et al.*, 1998). STATs are activated by tyrosine phosphorylation following the binding of cytokines or growth factors to cognate receptors on the cell surface (Figure 1). Tyrosine kinases that mediate STAT activation include growth factor receptors and cytoplasmic tyrosine kinases, particularly Janus kinase (JAK) and Src kinase families. Once tyrosine phosphorylated, two

STAT monomers form dimers through reciprocal phosphotyrosine-SH2 interactions, translocate to the nucleus, and bind to STAT-specific DNA-response elements of target genes to induce gene transcription. To date, there are seven STAT family members identified in mammals, designated Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. STATs have diverse normal biological functions, which include roles in cell differentiation, proliferation, development, apoptosis, and inflammation (Akira, 2000; Bromberg *et al.*, 1996; Cressman *et al.*, 1996; Fukada *et al.*, 1996; Hirano *et al.*, 2000; Kaplan *et al.*, 1996a,b; Planas *et al.*, 1997; Smithgall *et al.*, 2000; Stephens *et al.*, 1996; Takeda *et al.*, 1997; Zhong *et al.*, 1994). Gene knockout studies have defined the biological importance of STAT members in normal cells (Akira, 2000). In particular, Stat2 or Stat3 null mice are embryonic lethal, consistent with a fundamental role for these STAT proteins in development. Mice with targeted Stat1 gene disruption are viable, have impaired response to interferons and show high susceptibility to bacterial and viral infections (Durbin *et al.*, 1996; Meraz *et al.*, 1996). Furthermore, Stat1 null mice have higher incidence of tumors than normal in response to methylcholanthrene, consistent with Stat1's anti-proliferative activity (Bromberg *et al.*, 1996; Kaplan *et al.*, 1998). Stat5 knockout mice are viable with phenotypic defects that are tissue-specific, including defects in mammary gland development and lactation during pregnancy (Liu *et al.*, 1997), as well as sexually dimorphic pattern of liver gene expression (Udy *et al.*, 1997), infertility and immune dysfunction (Teglund *et al.*, 1998). Because of their diverse biological functions, aberrations in STAT signaling are predicted to have a wide variety of consequences.

STATs and oncogenesis

Requirement for constitutively-active Stat3 in Src oncogenesis

Studies of the molecular basis of oncogenesis by oncoproteins like v-Src have provided insights into changes in intracellular signaling proteins that participate in malignant transformation. The initial finding that Stat3 is constitutively activated in v-Src transformation (Cao *et al.*, 1996; Yu *et al.*, 1995) suggested that aberrant STATs may have key roles in oncogenesis, a view validated by more recent observations that constitutive Stat3 activation is required for oncogenic transformation by v-Src (Bromberg *et al.*, 1998; Turkson *et al.*, 1998). Moreover, a constitutively-

*Correspondence: R Jove, Molecular Oncology Program, Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, Florida, FL33612, USA

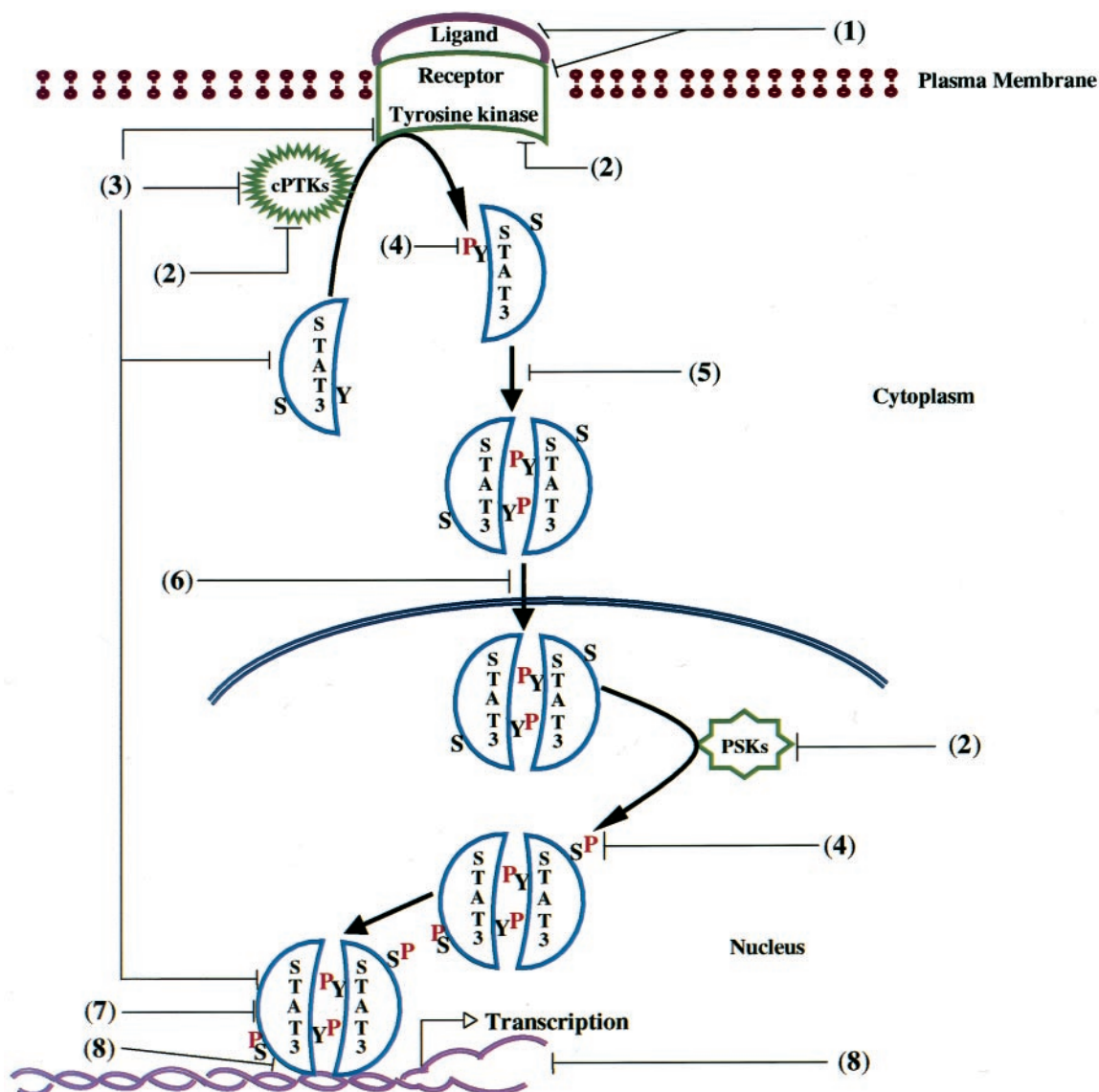


Figure 1 Schematic representation of STAT signaling from a cell surface receptor to the nucleus: molecular targets for disruption of Stat3 signaling. STAT activation is initiated by tyrosine phosphorylation that is mediated by growth factor receptors and/or cytoplasmic protein tyrosine kinases (cPTKs), such as JAKs and Src. Phosphorylation of STATs induces dimerization, which allows STATs to translocate to the nucleus where they bind to consensus STAT binding sequences of target genes and thereby activate gene transcription. Serine phosphorylation of some STATs, including Stat3, by protein serine kinases (PSKs) allows maximal transcriptional activity. Possible sites of disruption of STAT signaling that can be targeted for drug discovery are numbered in parenthesis. (1) The receptor-ligand interaction is the target of ligand/receptor antagonists, such as IL-6 'superantagonists', and receptor-neutralizing antibodies against EGF-R. (2) Protein tyrosine or serine kinases can be targeted by inhibitors, including inhibitors of the tyrosine kinases, EGF-R, JAKs and Src, and the various serine kinases that can phosphorylate Stat3. (3) Physiological protein modulators of STAT activation, including the biological protein inhibitors of STAT activity, such as SOCS or PIAS, and Stat3-interacting protein, STIP1, can be manipulated to alter STAT function. (4) Dephosphorylation of phospho-STATs can be regulated by modulation of protein tyrosine or serine phosphatases, which will in turn diminish the levels of active STATs and the extent of their transcriptional activity. (5) Dimerization of STATs, a crucial event in their activation, can be targeted to interfere with STAT function. (6) Represents STAT translocation to the nucleus, blocking of which can interfere with their presence and transcriptional activity in the nucleus. (7) STAT-coactivator interactions, events that are necessary for STAT transcriptional activity, can be targeted. (8) Represents areas where antisense or DNA-binding decoy oligodeoxynucleotide sequences and dominant-negative mutants block STAT transcriptional function. See text for further details.

activated Stat3 mutant alone is sufficient to induce transformation, and cells transformed this way can form tumors in nude mice (Bromberg *et al.*, 1999a), providing genetic evidence that Stat3 has oncogenic potential. These findings together underscore the ability of abnormal Stat3 activity to induce permanent changes in gene expression programs that ultimately lead to the malignant phenotype. Hence, constitutive Stat3 signaling contributes to transformation by oncogenic tyrosine kinases.

Constitutive STAT activation in transformation by diverse oncoproteins

In addition to v-Src, other transforming tyrosine kinases, such as v-Eyk (Besser *et al.*, 1999), v-Ros (Zong *et al.*, 1998), v-Fps (Garcia *et al.*, 1997), Etk/BMX (Wen *et al.*, 1999), and Lck (Lund *et al.*, 1999; Yu *et al.*, 1997), all activate Stat3 in the context of oncogenesis (for reviews see Bowman *et al.*, 2000b; Catlett-Falcone *et al.*, 1999a; Garcia and Jove, 1998).

Of notable importance, constitutive Stat3 activation is also associated with transformation by tumor viruses, including HTLV-1 (Migone *et al.*, 1995), polyomavirus middle T antigen (Garcia *et al.*, 1997), EBV (Weber-Nordt *et al.*, 1996), and herpesvirus saimiri (Lund *et al.*, 1997a,b, 1999), that directly or indirectly activate JAKs or Src family tyrosine kinases. Because of its central position downstream of the signaling pathways from protein tyrosine kinases, aberrant Stat3 activity is a key mediator in the transforming process induced by oncogenic tyrosine kinases. In contrast, Stat3-independent mechanisms mediate transformation by oncoproteins that are not tyrosine kinases themselves or do not activate tyrosine kinase signaling pathways, including v-Ras and v-Raf (Garcia *et al.*, 1997).

With regard to other STATs, constitutive activation of both Stat1 and Stat5 accompanies transformation of pre-B lymphocytes by the v-Abl tyrosine kinase (Danial *et al.*, 1995; Danial and Rothman, 2000). The transforming BCR-Abl fusion protein also activates Stat1 and/or Stat5 (Carlesso *et al.*, 1996; Chai *et al.*, 1997; Danial and Rothman, 2000; Frank and Varticovski, 1996; Shuai *et al.*, 1996a) and constitutive Stat5 activity is essential for BCR-Abl-induced transformation (de Groot *et al.*, 1999; Nieborowska-Skorska *et al.*, 1999; Sillaber *et al.*, 2000). Consistent with these findings, mutationally activated forms of Stat5 are sufficient to induce certain properties of transformed cells (Yamada *et al.*, 2000). Thus, Stat3 and Stat5 are the STAT family members with intrinsic oncogenic potential and most strongly associated with human cancer.

Stat3 activation and human diseases

Profile of human tumors that harbor constitutively-active Stat3

Mounting evidence gives credence to Stat3 as a bona fide mediator of oncogenesis that participates in human malignancies. In the context of human cancer, there is a high frequency of activation of Stat1, Stat3 and Stat5 (Table 1), with higher incidence of abnormal Stat3 activation in almost all the tumors studied. As the list of human tumors that harbor constitutive Stat3 activity keeps growing, there is increasing chance that many more cases of human cancers will be identified in which Stat3 has prominent role in the induction and/or maintenance of the oncogenic phenotype. Constitutive Stat3 tyrosine or serine phosphorylation has been detected in breast carcinomas (Garcia *et al.*, 1997, 2000; Watson and Miller, 1995), head and neck squamous cell carcinomas (Grandis *et al.*, 1998, 2000a; Song and Grandis, 2000), as well as lymphomas and leukemias (Catlett-Falcone *et al.*, 1999a,b, 2000; Coffer *et al.*, 2000; Frank *et al.*, 1997; Gouilleux-Gruart *et al.*, 1996; Weber-Nordt *et al.*, 1996; Zhang *et al.*, 1996c). Other cases of tumors with constitutive Stat3 activity include prostate, melanoma, pancreas, ovarian and brain (Cattaneo *et al.*, 1998; Florenes *et al.*, 1999; Kirkwood *et al.*, 1999; Lou *et al.*, 2000; Magrassi *et al.*, 1999; Pansky *et al.*, 2000; Schrell *et al.*, 1998; L Mora, R Garcia, J Seigne, T Bowman, G Niu, J Pow-Sang, J Diaz, C Muro-Cacho, D Coppola, T Yeatman, J Cheng, S Nicosia, S Shivers, T Landowski,

D Reintgen, W Dalton, H Yu and R Jove, unpublished results). These observations make it compelling to examine the role of Stat3 signaling in malignant progression to establish whether the constitutive Stat3 activation present in human tumors is essential for the malignancy. The critical question is whether the association of constitutive Stat3 with human cancers makes it a suitable target for disease intervention.

Other human disease models with constitutive STAT activation

Evidence suggests key roles for abnormal or defective activation of STAT signaling pathways in other human diseases. Abnormal STAT signaling associated with defects in activation of JAKs underlies the human immunodeficiency syndromes, human X-linked combined immunodeficiency (XCID), X-linked severe combined immunodeficiency (XSCID), as well as the less severe form, SCID (Candotti *et al.*, 1997; Heim, 1999; Miyazaki *et al.*, 1994; Russell *et al.*, 1994, 1995). Furthermore, constitutive STAT activity is observed in rheumatoid arthritis, where it participates in the early and late stages of the disease, as well as in the inhibitory immune mechanisms in rheumatoid arthritis synovium (Muller-Ladner *et al.*, 2000). Moreover, links have been established between constitutive STAT signaling and asthma (Sampath *et al.*, 1999), and with human immunodeficiency virus (HIV) infection where elevated STAT activities are detected in CD4(+) T lymphocytes from HIV patients (Bovolenta *et al.*, 1999). While these studies present some preliminary observations implicating abnormal STAT signaling in the pathogenesis of these diseases, it is essential to validate the key role of constitutive STAT signaling in order to assess the therapeutic usefulness of anti-Stat3 intervention for each disease type. Moreover, the important role of JAK-STAT signaling in many fundamental biological processes provides basis for evaluating the target validity in human disease cases where abnormal JAKs or STATs are observed (Frank, 1999; Lin *et al.*, 2000; Seidel *et al.*, 2000).

Validation of Stat3 as target for cancer drug discovery

In considering a 'target' for drug discovery, one of the most important issues to be addressed is whether a target hypothetically associated with a disease necessarily represents an appropriate point for new drug intervention. It is vital to ascertain the credibility of the hypothetical target as it relates to its activity in the context of disease and its mechanism of action, and also to establish a cause and effect relationship. The idea is to present a clear definition of the position of the target in relation to the disease phenotype (Drews, 2000; Gibbs, 2000). Consideration of Stat3 as target for cancer drug discovery ideally requires: (i) evidence in cell culture and whole animal models that constitutive Stat3 alone is sufficient to induce relevant disease phenotype, and (ii) definitive proof from studies in disease-relevant models of cell culture, whole animals, and clinical situations that blocking aberrant Stat3 signaling reverses disease phenotype. Moreover, the molecular mechanism of oncogenesis by Stat3 must be clearly defined.

Table 1 STAT activation in human tumors and cell lines

<i>Tumor type</i>	<i>Activated STATs</i>	<i>References</i>
<i>Breast cancer</i>		
Tumors	Stats 1,3	(Garcia <i>et al.</i> , 2000; Watson and Miller, 1995) (J Bromberg and JE Darnell, unpublished results; P Chaturvedi and EP Reddy, unpublished results; R Garcia, C Muro-Cacho, S Minton, C Cox, N Ku, R Falcone, T Bowman and R Jove, unpublished results) (Garcia <i>et al.</i> , 1997; Sartor <i>et al.</i> , 1997)
Cell lines	Stat 3	
<i>Head and neck cancer</i>		
Cell lines and tumors	Stats 1,3	(Grandis <i>et al.</i> , 1998, 2000a)
<i>Malignant melanoma</i>		
Cell lines and tumors	Stats 1,3	(Florenes <i>et al.</i> , 1999; Kirkwood <i>et al.</i> , 1999; Pansky <i>et al.</i> , 2000)
<i>Pituitary tumors</i>		
Cell lines	Stat 1	(Ray <i>et al.</i> , 1998)
<i>Brain tumors (primary tumors)</i>		
Gliomas	Stats 1,3	(Cattaneo <i>et al.</i> , 1998)
Medulloblastomas	Stat 3	(Cattaneo <i>et al.</i> , 1998)
Cerebral meningiomas	Stats 1,3,5	(Magrassi <i>et al.</i> , 1999; Schrell <i>et al.</i> , 1998)
<i>Multiple myeloma</i>		
Cell lines and tumors	Stats 1,3	(Catlett-Falcone <i>et al.</i> , 1999b)
<i>Lymphomas (tumors and cell lines)</i>		
Anaplastic large T cell lymphoma	Stats 3,5	(Zhang <i>et al.</i> , 1996c)
Sezary syndrome	Stats 3,5	(Zhang <i>et al.</i> , 1996c)
EBV-related Burkitt's lymphoma	Stat 3	(Weber-Nordt <i>et al.</i> , 1996)
HSV Saimiri-dependent (T cell)	Stat 3	(Lund <i>et al.</i> , 1997b, 1999)
Cutaneous T cell lymphoma	Stat 3	(Sun <i>et al.</i> , 1998)
LSTRA T cell lymphoma (mouse)	Stat 5	(Yu <i>et al.</i> , 1997)
Mycosis fungoides	Stat 3	(Nielsen <i>et al.</i> , 1997)
<i>Leukemias (tumors and cell lines)</i>		
HTLV-I-dependent	Stats 3,5	(Migone <i>et al.</i> , 1995; Takemoto <i>et al.</i> , 1997)
Erythroleukemia	Stats 1,5	(Carlesso <i>et al.</i> , 1996)
Acute lymphocytic leukemia (ALL)	Stats 1,5	(Gouilleux-Gruart <i>et al.</i> , 1996; Weber-Nordt <i>et al.</i> , 1996)
Chronic lymphocytic leukemia (CLL)	Stats 1,3	(Frank <i>et al.</i> , 1997)
Acute myelogenous leukemia (AML)	Stats 1,3,5	(Chai <i>et al.</i> , 1997; Gouilleux-Gruart <i>et al.</i> , 1996; Weber- Nordt <i>et al.</i> , 1996)
Chronic myelogenous leukemia (CML)	Stat 5	(Carlesso <i>et al.</i> , 1996; Chai <i>et al.</i> , 1997; Shuai <i>et al.</i> , 1996a)
Megakaryocytic leukemia	Stats 1,3,5	(Liu <i>et al.</i> , 1999)
Large granular lymphocyte (LGL) leukemia	Stat 3	(Epling-Burnette <i>et al.</i> , 2000)
<i>Other cancers (tumors and cell lines)</i>		
Prostate	Stat 3	L Mora, R Garcia, J Seigne, T Bowman, M Huang, G Niu, J Pow-Sang, J Diaz, C Muro-Cacho, D Coppola, T Yeatman, J Cheng, S Nicosia, S Shivers, T Landowski, D Reintgen, W Dalton, H Yu and R Jove, unpublished results
Renal cell carcinoma	Stat 3	
Pancreatic adenocarcinoma	Stat 3	
Ovarian carcinoma	Stat 3	
Melanoma		

Evaluation of the credibility of Stat3 as a valid target on the basis of present data reveals that the minimum criteria have been exceeded. Application of molecular biology tools, including use of dominant-negative and activated mutant forms of Stat3, and antisense oligonucleotides, in relevant cell culture, animal models and patient samples has provided a high degree of validation for Stat3 as a target for cancer drug intervention. As already pointed out above, an artificially-engineered, constitutively-active Stat3 mutant alone induces transformation, and cells transformed by this active Stat3 mutant form tumors *in vivo* (Bromberg *et al.*, 1999b). In other key studies that highlight the oncogenic importance of Stat3 and establish a direct link to tumor progression, aberrant Stat3 signaling is obligatory for growth and survival of various human tumor cells, including multiple myelomas (MM), breast carcinomas, head and neck squamous cell carcinomas (HNSCC), the T cell lymphoma mycosis fungoides, and large granular lymphocyte (LGL) leukemia (Catlett-Falcone *et al.*, 1999b; Epling-Burnette *et al.*, 2000; Garcia *et al.*, 2000; Grandis *et al.*, 1998, 2000a; Nielsen *et al.*, 1997, 1999).

Significantly, pharmacological or genetic interruption of constitutive Stat3 signaling inhibits expression of the anti-apoptotic Bcl family members, Bcl-xL, Bcl-2, or Mcl-1, in MM (Catlett-Falcone *et al.*, 1999b), HNSCC xenograft model (Grandis *et al.*, 2000a), mycosis fungoides (Nielsen *et al.*, 1999), and LGL leukemia cells (Epling-Burnette *et al.*, 2000). Inhibition of Stat3 signaling also sensitizes MM to chemotherapy-induced apoptosis (Oshiro *et al.*, 2000), and increases the expression of the pro-apoptotic Bax protein (Nielsen *et al.*, 1999). Importantly, using *in vivo* tumor models, murine B16 melanoma tumors regress on inhibition of constitutive Stat3 activity by gene therapy with a dominant-negative Stat3 variant (Niu *et al.*, 1999), making the compelling argument that targeting Stat3 for therapeutic intervention in certain types of human cancers can block tumor growth. Moreover, a number of the Stat3 target genes have been identified, including cyclin D1 (Bromberg *et al.*, 1999b; Sinibaldi *et al.*, 2000), p21 (Chin *et al.*, 1996; Sinibaldi *et al.*, 2000), c-Myc (Bowman *et al.*, 2000a; Kiuchi *et al.*, 1999; Odajima *et al.*, 2000), and the Bcl-family members (as already mentioned), which have critical roles in regulating cell proliferation and survival. Indeed, cells

devoid of cyclin D1 or c-Myc genes are refractory to Stat3-mediated transformation (Bowman *et al.*, 2000a; Bromberg *et al.*, 1999a). Thus, it seems appropriate to infer that by subversion of genetic programs that control normal cell cycle progression and survival signals, constitutive Stat3 induces changes that lead to initiation and/or maintenance of tumorigenesis.

The 'cancer-causing' propensity of constitutively-activated Stat3 protein, and the evidence of potential clinical benefits of blocking constitutive Stat3 signaling, make strong arguments for target validity of Stat3 for drug intervention in cancer therapy. The obvious final question is whether oncogenesis can be induced in a Stat3 null genetic background by oncoproteins, such as v-Src, that induce Stat3 signaling. Gene knockout approaches do not lend themselves readily to biological studies of Stat3 signaling for the reason that early attempts to create Stat3 knockout mice led to embryonic lethality at day 6.5-7.5 (Akira, 1999; Takeda *et al.*, 1997), an observation consistent with a biological role for Stat3 as mediator of self-renewal (Matsuda *et al.*, 1999; Niwa *et al.*, 1998), and its absolute requirement for development, growth and survival. Recent efforts have generated conditional Stat3 knockouts (Takeda *et al.*, 1998), which will allow addressing the question of whether Stat3 null cells are indeed resistant to transformation by the Src oncoprotein.

Signaling pathways that induce constitutive STAT activation as molecular target points for drug discovery

Concurrent induction of multiple signaling pathways occurs in response to stimulation by growth factors, cytokines, or oncoproteins, allowing the possibility of complex regulation by cross-talk among signaling pathways. Since there has, as yet, not been identification of a naturally-occurring dominant-positive mutation in the *stat3* gene that confers constitutive Stat3 activity and oncogenic ability, it is reasonable to infer that aberrant upstream signaling events are the major driving force for the induction of dysregulated Stat3 signaling that is observed in oncogenesis. Dysregulation of Stat3 signaling can result from persistent input signals from hyperactive ligands or their receptors, constitutive receptor-ligand complexes, aberrant functional properties of specific proteins, such as activated c-Src, and tumor viruses that activate Stat3 directly or indirectly. Indeed, studies have confirmed that aberrant signaling pathways upstream from Stat3 participate in the constitutive Stat3 activation in the context of human cancers (Catlett-Falcone *et al.*, 1999b; Garcia *et al.*, 2000; Grandis *et al.*, 1998, 2000a,b; Turkson *et al.*, 1999). Equally important are downstream effectors of Stat3 activity, which are target gene products with critical functions in oncogenesis. The interplay of these multiple abnormal signaling pathways provides the needed hyperactivity for the induction and/or maintenance of Stat3-mediated oncogenesis. In the context of oncogenesis, current evidence is consistent with Stat3 being a point of convergence for tyrosine and serine kinase signals (Beadling *et al.*, 1996; Ng and Cantrell, 1997; Turkson *et al.*, 1999; Wen *et al.*, 1995) (Figure 1).

Tyrosine phosphorylation and constitutive STATs activation

Tyrosine phosphorylation of STATs constitutes an early event in the activation of these transcription factors that is required for their dimerization and DNA-binding activity. Aberrant tyrosine kinase activities of mutant JAKs, growth factor receptors, Src, and tumor viruses that activate tyrosine kinase signals consequentially induce hyperactive Stat3. Indeed, constitutive tyrosine phosphorylation of Stat3 is a necessary requirement for the oncogenic transforming property of this transcription factor (Bromberg *et al.*, 1998, 1999a; Turkson *et al.*, 1998). This is a highly significant observation that suggests that constitutive upstream tyrosine kinase signals, aberrant Stat3 activity and transformation are interrelated events.

Details of the biochemical mechanisms of induction of constitutive STAT activation are beginning to emerge, and are revealing a cooperation of proteins (with and without tyrosine kinase activity) to induce STATs phosphorylation. In proposed models of activation that are observed for Stat3 and/or Stat1, direct tyrosine phosphorylation by growth factor receptors (Vignais and Gilman, 1999), Src or JAKs (Cao *et al.*, 1996; Chaturvedi *et al.*, 1998; Garcia *et al.*, 2000; Reddy *et al.*, 2000; Zhang *et al.*, 2000), or a conjunction of all three proteins (Garcia *et al.*, 2000; Zhang *et al.*, 2000), has been observed. That protein tyrosine kinase inhibitors (PTKIs) block Stat3-mediated oncogenesis, sensitize chemoresistant tumor cells, and induce apoptosis (Catlett-Falcone *et al.*, 1999b; Garcia *et al.*, 2000; Oshiro *et al.*, 2000), suggests that targeting tyrosine kinases that feed into Stat3 signaling is functionally important in the context of aberrant Stat3 signaling and oncogenesis, and that PTKIs may be clinically useful. This approach for drug discovery is discussed in later sections below.

Serine phosphorylation of STATs

Phosphorylation of a serine residue in the C-terminal transcriptional activation domain of some STATs, corresponding to Ser-727 in Stat3, enhances their transcriptional activity (Decker and Kovarik, 2000; Wen and Darnell, 1997; Wen *et al.*, 1995). On the identities of the serine kinases, members of the mitogen-activated protein kinases (MAPK) family (Schaefer *et al.*, 1999), extracellular signal-regulated kinases (ERKs) (Chung *et al.*, 1997; David *et al.*, 1995; Kuroki and O'Flaherty, 1999; Ng and Cantrell, 1997), c-Jun N-terminal kinase (JNK) (Lim and Cao, 1999; Turkson *et al.*, 1999) and p38mapk (p38) (Gollob *et al.*, 1999; Turkson *et al.*, 1999), are strong candidate kinases that participate in the serine phosphorylation of Stat1 and Stat3. Given that Stat3 serine phosphorylation is required for maximal activation of its signaling, it is predicted that tyrosine phosphorylation alone is not sufficient for the obligatory role of Stat3 signaling in oncogenesis. Indeed, serine phosphorylation is evidently required for the full transforming property of constitutively active Stat3 (Bromberg *et al.*, 1998; Turkson *et al.*, 1999).

Understanding the signaling pathways that are required for aberrant Stat3 serine phosphorylation

identifies the specific signaling components that are necessary for Stat3-mediated transformation. For example, in line with the requirements for constitutive Stat3 (Bromberg *et al.*, 1998; Turkson *et al.*, 1998) and Ras signaling (Stacey *et al.*, 1991) in Src transformation, studies demonstrated cross-talk between Stat3 and Ras signaling pathways (Turkson *et al.*, 1999). Furthermore, recent studies have confirmed the concept of obligate cooperation of multiple signaling pathways involving Stat3 and Ras for Src oncogenesis (Odajima *et al.*, 2000). Application of both genetic and pharmacological tools led to the findings that p38, JNK, and their upstream MAPK kinases are key mediators of Stat3 serine phosphorylation required for Src transformation (Turkson *et al.*, 1999), identifying these kinases and Ras as possible targets for intervention in constitutive Stat3-mediated oncogenesis. Because serine phosphorylation is important for full STAT activity, the observations that Stat3 is constitutively serine phosphorylated in lymphomas (Frank *et al.*, 1997) and tumor cell lines (Nielsen *et al.*, 1997; Zhang *et al.*, 1995) are significant as they suggest that Stat3 serine phosphorylation has a critical biological role in the pathogenesis of these conditions. Similar to the wide-spread involvement of Stat3 tyrosine phosphorylation in a variety of human tumors, it is likely that future investigation will reveal a wider role of constitutive Stat3 serine phosphorylation in malignant transformation (Gollob *et al.*, 1999), and provide support for targeting STATs serine phosphorylation signaling for therapeutic intervention.

Interleukin-6/Stat3/Bcl-xL model in human multiple myeloma

In the human MM cell line, U266, Stat3 DNA-binding and transcriptional activities are constitutive (Catlett-Falcone *et al.*, 1999b) as a result of aberrant upstream signals. These events are induced by IL-6 and blocked by JAK inhibitors (Catlett-Falcone *et al.*, 1999b), consistent with a participatory role for the gp130 signaling subunit of IL-6 receptor and a requirement for activities of JAK family kinases in Stat3 signaling (Hirano *et al.*, 2000). These observations provide a rationale for targeting IL6/gp130 and JAKs for disrupting constitutive Stat3 signaling in the context of MM. Moreover, the finding that constitutive Stat3 activity upregulates expression of the *bcl-x* gene crucial for survival of myeloma cells (Catlett-Falcone *et al.*, 1999b), provides a direct connection between Stat3 and survival factors like IL-6. Similar links have also been confirmed for constitutive Stat5 in other tumor models (Dumon *et al.*, 1999; Horita *et al.*, 2000; Nosaka *et al.*, 1999; Silva *et al.*, 1999; Socolovsky *et al.*, 1999). Furthermore, U266 cells are resistant to chemotherapy- or Fas-mediated apoptosis, and become sensitized to drug-induced cell death following inhibition of constitutive Stat3 activity and consequent downregulation of Bcl-xL expression (Catlett-Falcone *et al.*, 1999b; Oshiro *et al.*, 2000). These studies together describe an anti-apoptotic pathway triggered by protein tyrosine kinase-STAT signaling that contributes to tumor progression and resistance of malignant cells to therapy and provide a basis for targeting Stat3 for certain types of cancer therapy.

Breast carcinoma model

Breast carcinoma cells, including primary tumors and cell lines, have elevated Stat3 activities (Garcia *et al.*, 1997, 2000; Sartor *et al.*, 1997; Watson and Miller, 1995; Xie *et al.*, 1997), and in some cases, also show elevated EGF receptor (EGF-R) (Sartor *et al.*, 1997) as well as c-Src kinase activities (Garcia *et al.*, 1997). Studies to define how Stat3 is abnormally induced in breast carcinomas reveal cooperative interactions of Src with JAK tyrosine kinase activities that are required for Stat3 activation (Garcia *et al.*, 2000). These interactions have been confirmed in model fibroblast cells that overexpress c-Src and/or EGF-R, and suggest a paradigm of cooperation between Src, JAKs and EGF-R to induce Stat3 activation (Garcia *et al.*, 2000). The elevated protein expressions or kinase activities of Src, JAKs and/or EGF-R, may increase the chance of constitutive STAT activation. Hence, Src and EGF-R overexpressing breast carcinoma cells are susceptible to growth inhibition and cell death induced by Src or JAK inhibitors (Garcia *et al.*, 2000). It can be concluded that signaling pathways that lead to constitutive Stat3 activity are required for the survival of breast cancer cells, and this supports the view that drugs that target growth factor receptor/Src/JAKs/Stat3 signaling may be clinically beneficial to patients who are positive for Stat3 activities.

Strategies for targeting Stat3 for drug discovery

Knowledge of the mechanisms of constitutive STAT activation and transcriptional activity from the cell surface receptor to the nucleus provides the framework for strategies to target constitutive STAT signaling and their functions. Figure 1 is a schematic representation of signaling pathways leading to STAT activation and transcriptional activity, as well as potential points of interception of STAT function. In focusing on signal transduction mechanisms for drug targeting, the objective is to determine the contribution of each key module in the pathway that participates in the aberrant STAT function. Conceptually, the targeting of upstream modules in signal transduction pathways leading to constitutive STAT activation would impair STAT function and, for certain types of cancers, provide the avenue for intervention to halt the disease. Some of the strategies discussed below are already being developed and are at different stages of evaluation. The numbers of the following subheadings correspond to the numbers indicating specific sites of intervention in Figure 1.

(1) Receptor/ligand antagonists and receptor-neutralizing antibodies

Receptor overexpression is frequently associated with many types of cancers, including breast, head and neck, lung, stomach, and ovarian cancers that show elevated expression of the EGF-R family (Aderem, 1993; Hynes, 1993; Reddy *et al.*, 1992). Where receptor overexpression is connected to disease, use of receptor antagonists could in principle have promising results by blocking receptor activation. Moreover, the functional importance of the ligand-receptor-STAT signal-

ing module in biological processes, such as immune responses, provides a rationale for drug targeting of ligands and/or receptors in diseases with causal roles for abnormal STAT activities (see Lin *et al.*, 2000; Seidel *et al.*, 2000 for reviews). Conceptually, physiological receptors or ligands that activate STAT signaling pathways provide a means to block STAT signaling if it is proven that abnormal receptor or ligand activity is the stimulus for the constitutive STAT activation. Receptor or ligand antagonists include molecules that are structurally related to but lack the intrinsic activating property of the physiological ligand and possess higher affinity for receptor. The potential of this approach has been demonstrated using the IL-6 'superantagonist', Sant7, that blocks constitutive Stat3 activation in U266 myeloma cells (Catlett-Falcone *et al.*, 1999b) and inhibits cell growth (Demartis *et al.*, 1996; Petrucci *et al.*, 1999). Moreover, current clinical applications of this approach in certain types of human disease conditions, including the use of recombinant tissue necrosis factor receptor in treatment of rheumatoid arthritis (Moreland *et al.*, 1997; for review, see Seidel *et al.*, 2000), make a compelling argument for evaluating its usefulness for cancer therapy. However, considerably more studies remain to be done to show the correlation of growth inhibition of tumors in whole animals with block of constitutive Stat3 activity using receptor or ligand antagonists.

The application of monoclonal antibodies for the treatment of diseases, including human cancers, has received widespread recognition with the advent of recombinant antibody technology. In principle, the binding of neutralizing antibodies to their respective epitopes on receptors disrupts receptor interaction with its physiological ligand, and provides a means of targeted therapy that focuses on modulating the function of receptors that may be overexpressed on cancer cells. Therapeutic antibodies can be adopted as a single treatment, or as a combined therapy with traditional chemotherapy or novel compounds that inhibit various steps of signal transduction pathways (Fan and Mendelsohn, 1998). Where a link is established between receptor activity and disease, anti-receptor monoclonal antibodies are effective against disease conditions, such as growth inhibition by anti-EGF-R antibody of transplanted human tumors (Azemar *et al.*, 2000; Baselga *et al.*, 1998; Pegram *et al.*, 1999; Pietras *et al.*, 1998), and metastatic cancer (Baselga *et al.*, 1996; Pegram *et al.*, 1998).

As discussed in preceding sections, certain key components of signal transduction pathways upstream of Stat3 are critical for constitutive Stat3 activation (Catlett-Falcone *et al.*, 1999b; Garcia *et al.*, 2000; Grandis *et al.*, 1998, 2000b; Turkson *et al.*, 1999; Zhang *et al.*, 2000). In principle, similar to ligand antagonists, antibody targeting of cell surface receptors that are determined to be sources of signaling for Stat3 activation would be expected to impede binding of ligand, impair induction of the signaling, and block constitutive activation of Stat3 and Stat3-mediated oncogenesis. Use of receptor neutralizing antibody, for example, C225, anti-EGF-R antibodies, and herceptin, anti-HER2 antibodies, as therapeutic approaches is currently being exploited in clinical trials (Baselga *et al.*, 1996, 1998, 2000). Given that EGF-R mediates Stat3 activation in HNSCC (Grandis *et al.*, 1998,

2000a; Song and Grandis, 2000), it would be presumed that clinical responses in HNSCC to therapeutic C225 is a function of inhibition of EGF-R-mediated constitutive activation of downstream signaling components, including Stat3. However, the validity of this view must be critically evaluated by determining the degree of Stat3 activation before and after antibody therapy.

(2) Tyrosine or serine kinase inhibitors

Protein kinases as targets represent one of the most exploited approaches for therapeutic intervention, and the degree of success in this area is demonstrated in the number of ongoing clinical trials (Ben-Bassat and Klein, 2000; Fry, 2000; Levitzki, 1999; Miknyoczki *et al.*, 1999). Considerable efforts have gone into designing compounds that inhibit activities of tyrosine kinases that participate in STAT signaling. Targeting kinases that participate in STAT signaling for intervention of tumor progression can be very effective if it is shown that aberrant STAT signaling is the result of dysregulated upstream tyrosine kinases. Inhibition of tyrosine kinase activity inhibits growth of breast cancer cells (Garcia *et al.*, 2000; Reddy *et al.*, 1992) and it correlates with inhibition of constitutive Stat3 activity in cultured tumor cells (Garcia *et al.*, 2000). Furthermore, inhibition of Src, JAKs or Bcr-Abl kinase-mediated constitutive Stat3 or Stat5 signaling induces apoptosis in MM or CML cells (Catlett-Falcone *et al.*, 1999a,b, 2000; Horita *et al.*, 2000; Huang *et al.*, 2000; Sillaber *et al.*, 2000), and inhibits growth of prostate cancer cells (Ni *et al.*, 2000; L Mora, R Garcia, J Seigne, J Pow-Sang, J Diaz, A Kraker and R Jove, unpublished results). Antitumor activity against human tumor xenografts using an EGF-R tyrosine kinase inhibitor, PD 0169414 (Vincent *et al.*, 2000), provides compelling support for targeting tyrosine kinases for therapeutic intervention in tumor patients. Other protein tyrosine kinases already discussed above that are involved in STAT signaling and are potential targets for drug discovery include Src, Etk and Btk families. The challenge is to determine which of these kinases is suitable for targeting that would offer the most promising clinical benefits.

Because of the key role of serine phosphorylation for maximal transcriptional activity of some STATs (for review, see Decker and Kovarik, 2000), it is predicted that inhibition of this modification would be sufficient to impair STAT activity. Variants of STATs that are mutated at the specific serine residues show significant inhibitory activity consistent with dominant-negative effects against transcriptional activity of some STATs (Bromberg *et al.*, 1996, 1998; Caldenhoven *et al.*, 1996; Turkson *et al.*, 1998). That such dominant-negative mutant forms of STATs block the biological effects of wild-type counterparts (Bromberg *et al.*, 1996, 1998; Turkson *et al.*, 1998), underscores the functional importance of serine phosphorylation. Moreover, modification of the STAT serine phosphorylation signals via pharmacological agents that block STAT-specific serine kinases has shown promising effects. For example, pharmacological inhibition of p38 activity that is required for Stat3 serine phosphorylation and transcriptional activity blocks transformation induced by v-Src (Turkson *et al.*, 1999).

The application of protein kinase inhibitors has significant implications for chemotherapy for those cancers that are positive for Stat3 activity. Currently, one prototype drug that has been widely used is the tyrphostin, AG490, a JAK inhibitor (Levitzki, 1999). This compound not only blocks Stat3 signaling, but also inhibits Stat3-mediated Bcl-xL induction, induces apoptosis, and possesses antitumor activity (Burdelya *et al.*, 2000; Catlett-Falcone *et al.*, 1999b). Further structure-activity relationship studies should yield more effective and selective inhibitors of STAT protein tyrosine or serine kinases that can be therapeutically useful for Stat3 positive cancers.

(3) Physiological protein modulators of STAT activation

Physiological protein inhibitors of STAT signaling comprise endogenous proteins that directly or indirectly downregulate STAT activation. This family of inhibitors includes the cytokine-inducible SH2-containing (CIS) proteins (same as suppressor of cytokine signaling (SOCS), JAK binding protein (JAB) and STAT-induced STAT inhibitor (SSI)), as well as the protein inhibitors of activated STATs (PIAS) (Endo *et al.*, 1997; Hilton, 1999; Kovanen and Leonard, 1999; Naka *et al.*, 1997; Shuai, 2000; Starr and Hilton, 1999). The CIS family regulates STAT signaling at the level of cytokine receptors or JAKs, although the specificity of each family member may not be limited only to these targets. On the other hand, the PIAS family directly interacts with activated STATs, interfering with their DNA-binding activity, and inhibiting gene transcription (Shuai, 2000). Knowledge from the mechanisms of action of these biological inhibitors could help in the design of molecules that can mimic their actions and inhibit STAT function. Pharmacological or small molecule mimics of SOCS or PIAS that would effectively 'turn-off' the signaling pathways involved in the constitutive STAT activation, and thus downregulate Stat3 signaling, would have wide applicability to Stat3-sensitive tumors.

A newly identified cytoplasmic Stat3-interacting protein, StIP1, acts as a scaffold protein that regulates ligand-dependent Stat3 activation (Collum *et al.*, 2000). Unlike the others in this class of proteins that interact with STATs, StIP1 binds both inactive Stat3 and JAKs in a hypothetical Stat3-StIP1-JAKs trimolecular complex proposed to facilitate Stat3 activation. However, initial studies show that the expression of StIP1 mutant forms that encompass only the STAT-binding domain inhibit ligand-induced Stat3 tyrosine phosphorylation, DNA-binding and transcriptional activities (Collum *et al.*, 2000). Thus, critical biochemical analyses of the interaction of Stat3 with StIP1 may provide key structural information necessary for the design of peptides or mimics that can directly interact with Stat3 in a high affinity manner and inhibit its activation.

(4) Modulation of phosphatases

Protein phosphorylation is fundamentally important in cellular processes as it regulates the functional activities of signaling proteins. Overall, tyrosine phosphorylation states of proteins reflect the relative ratio of activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPase). Similarly, protein serine phos-

phorylation and dephosphorylation by kinases and phosphatases, respectively, maintain physiological balance of phosphoserine states of proteins. Activities of PTPase and phosphoserine phosphatase counterparts represent an essential regulatory mechanism that can be exploited for therapeutic benefits. With respect to STATs, studies with inhibitors of protein phosphatases reveal the importance of protein dephosphorylation in modulating STAT functions (David *et al.*, 1993; Haque *et al.*, 1995; Woetmann *et al.*, 1999). Two PTPases, SHP-1 and SHP-2, as well as a protein serine/threonine phosphatase, PP2A, are strongly implicated in STAT signaling, including Stat1, Stat3 and Stat5 (Ram and Waxman, 1997; Schaper *et al.*, 1998; Shuai *et al.*, 1996b; Stofega *et al.*, 1998; Strehlow and Schindler, 1998; Woetmann *et al.*, 1999; Yu *et al.*, 2000). Among others, SHP-1 associates with activated Stat5 in the nucleus in a ligand-dependent manner (Ram and Waxman, 1997), and SHP-2 enhances cell growth and development by downregulating IFN-activated Stat1 (You *et al.*, 1999). Moreover, modulation of activities of PTPases with non-specific inhibitors, such as vanadate, are sufficient to upregulate active STAT levels (Zhang *et al.*, 2000), and PP2A participates in induction of constitutive Stat3 serine phosphorylation and subcellular distribution in cutaneous T cell lymphoma (Woetmann *et al.*, 1999).

In principle, it is possible to alter STAT function at the level of protein phosphatases. In the context of constitutive Stat3 activity in oncogenesis, the intent is to design compounds that promote protein phosphatase activities that are capable of downregulating phospho-Stat3. The concern of this approach, however, is potential non-specific effects from regulation of activities of other phosphatase substrates. If it can be established that there are specific phosphatases that preferentially dephosphorylate active Stat3, it would be easier to achieve selectivity among the different phosphatases.

(5) Disrupters of STAT dimerization

Dimerization via reciprocal phosphotyrosine-SH2 interactions is a key event in the activation of STATs. Active STATs bind to DNA as dimers (Chen *et al.*, 1998), demonstrating the importance of this step for STAT function. Moreover, dimerization of STATs is also critical for their nuclear translocation and gene transcriptional activity (Becker *et al.*, 1998; Chen *et al.*, 1998; Horvath *et al.*, 1995; Schindler *et al.*, 1992; Shuai *et al.*, 1992). Thus, manipulations that do not allow dimerization, such as use of STAT mutants with a tyrosine to phenylalanine substitution, render the protein incapable of forming dimers, binding DNA and inducing gene transcription (Bromberg *et al.*, 1998). Disruption of Stat3 dimer formation therefore provides an effective approach of targeting this protein for blocking its signaling activity and functional effects. To interfere with STAT dimerization, ideal compounds should possess certain characteristics, including a stronger affinity for STAT monomer for a more favorable and cohesive interaction that generates a heterocomplex of STAT-compound over STAT-STAT dimers.

Concerning the ability of artificial compounds to disrupt preformed STAT dimers, in principle a much

more difficult step, it would be predicted that a 'super' high affinity for STAT monomer might allow the artificial compound to associate with pre-existing dimers of STAT. When this happens, it can be speculated that one or both of these events can take place: (i) the association might facilitate the dissociation of STAT dimers and the preferential formation of a heterodimeric complex involving STAT and compound; or (ii) the association would generate a heterotrimeric complex that would interfere with the ability of STAT dimer to effectively interact with co-activators and bind DNA. In terms of chemical design, disrupters of STAT dimerization could be SH2-like peptides with high affinity for the phosphotyrosine region of STATs, peptides with phosphotyrosine sequences, or small peptide mimetics that are specific for the SH2 sequence of STATs. Differences in sequences for the individual STAT members can be exploited to yield compounds that have selectivity for specific STATs. Information on the crystal structure of STAT dimers bound to DNA (Becker *et al.*, 1998; Chen *et al.*, 1998) should assist in the design of small molecules that can disrupt dimers of STATs. Such small molecules would provide leads for design of therapeutically useful compounds for clinical purposes.

(6) Inhibitors of STAT translocation

Following dimerization, STATs translocate into the nucleus and bind to specific regulatory sequences of responsive genes to induce gene transcription. The mechanisms of STAT translocation are not fully understood. Initial evidence of how the structure of STAT proteins might affect translocation suggests that the N-terminal region contains a nuclear translocation signal (Strehlow and Schindler, 1998), and the critical tyrosine residue of some STATs is required for (Sekimoto *et al.*, 1996) but does not guarantee nuclear translocation (Ali and Ali, 1998). STAT tyrosine phosphorylation is therefore not sufficient for translocation. On the question of STATs possessing authentic nuclear localization sequences (NLS), this is not widely believed to be the case (Johnson *et al.*, 1998b), raising the possibility of a distinct nuclear translocation mechanism whose induction signal originates from STAT proteins. There is some evidence that the phosphorylation of a critical tyrosine residue of the activating receptor is essential in recruiting intracellular components that regulate interactions of STAT dimers with cellular proteins in the cytoplasm, events that may modulate STAT nuclear translocation (Ali and Ali, 1998). Furthermore, it is also postulated based on preliminary evidence that certain cytokines or their receptors possess functional NLS, and that complexes of the cytokine-receptor might act as chaperones that associate with and transport STATs into the nucleus (Johnson *et al.*, 1998a,b). For example, it has been reported that interferon- γ (IFN γ) possesses a NLS whose function is required for the biological role of IFN γ , possibly through Stat1 transcriptional activity (Johnson *et al.*, 1998a,b). Although this intriguing observation remains to be confirmed in other systems, it suggests that blocking components that confer translocation of STATs into the nucleus can attenuate their biological effects.

Thus, understanding the details of translocation mechanisms of individual STATs, including defining the key points of interaction between STATs and molecular components of the translocation machinery complex, would provide the necessary information for design of selective compounds that interfere with one of the critical events of STAT function. Small molecule mimics of essential structural regions of key components of the translocation machinery could sufficiently disrupt STAT translocation and effectively block STAT function. Distinct mechanisms of translocation for individual STATs could allow for selective targeting of specific STAT members. In the context of human diseases associated with causal roles for constitutive STATs, the approach of blocking STAT nuclear translocation would provide another avenue for blocking STAT function as the cause of disease.

(7) Direct blocking of STAT DNA-binding and transcriptional activity

Interactions with specific sequences in the promoter regions of responsive genes allow STATs to induce gene expression as transcription factors. The transcriptional activity of STATs requires that they physically interact with the promoter sequences. Knowledge of the crystal structure of STAT dimer bound to its cognate DNA sequence would reveal the critical amino acid residues that are required for physical interaction with DNA. This information could be the basis for rational design of artificial competitors of STAT DNA-binding activity. Specific cooperative contacts of STATs with transcription coactivators, p48 (Bluyssen and Levy, 1997; Martinez-Moczygemba *et al.*, 1997), CBP/p300 (Paulson *et al.*, 1999; Zhang *et al.*, 1996b), Sp1 (Cantwell *et al.*, 1998), or with nuclear adaptor proteins (Rhodes *et al.*, 2000), are also essential for their transcriptional activity (Bromberg and Darnell, 2000). Regions of STATs identified to participate in cooperative interactions with other proteins include the coiled-coil domain and the C-terminal region (Paulson *et al.*, 1999) distant from the DNA contact sites (Zhang *et al.*, 1999). The conserved N-terminal regions of some STATs are also required for cooperative DNA binding of two STAT dimers (Xu *et al.*, 1996), giving STATs the ability to induce genes with variations of the consensus binding site in promoters even though the affinity for binding to such sequences may be weak.

Mutational analysis of amino acids in the N-terminal region of STATs should define the residues that are obligatory for cooperative interactions between STATs and coactivators for their functional effects. The requirement of STAT transcriptional activity for coactivators, or nuclear adaptors in some cases, suggests it is possible to modulate the transcriptional activity and biological functions of some STATs by using small molecule mimics of coactivators that would interfere with the cooperative interactions. The mimics of natural coactivators that can interact with STATs would lack the positive modulation effect. These 'pseudo-coactivators' could be engineered to possess higher affinity for Stat3 since they must compete with natural coactivator molecules. In principle, pseudo-coactivators would block the biological effects of STATs by substituting for natural coactivators and suppressing the transcriptional activities of Stat3,

analogous to the block of IFN- γ -Stat1-mediated gene activation and biological effects by a mutant adenovirus E1A protein (Look *et al.*, 1998). The competitive pseudo-coactivators could be designed taking advantage of the uniqueness of the point of interaction of these molecules with the specific STAT member that would allow preferential high-affinity interaction that confers specificity. For example, in the interaction of Stat1 with CBP/p300, or Stat3 with c-Jun, two contact regions have been identified that confer specificity of the interaction between each group of two proteins (Shuai, 2000; Zhang *et al.*, 1996b, 1999). Requirement for these cooperative interactions for stable complexes of STATs with DNA (Bluyssen and Levy, 1997) suggests that it is possible to annul the effects of constitutive Stat3 activity by ectopically expressing modified coactivators that no longer confer the necessary interactions with Stat3 for effective binding to DNA and gene transcription.

(8) Stat3 antisense, DNA-binding site decoy oligodeoxynucleotides and dominant-negative protein approaches

Antisense oligonucleotides can be effective molecular biological tools to investigate the function of a protein in a cell. The application of antisense is a simple, efficient and target-selective approach, as antisense sequence-specificity modulates gene expression and discriminately targets the biological functions of the gene product (Agrawal and Kandimalla, 2000). The application of antisense technology is already forging ahead in the number of existing clinical trial cases (Cunningham *et al.*, 2000; Green *et al.*, 2000; Marcusson *et al.*, 1999; Nemunaitis *et al.*, 2000; Yuen *et al.*, 1999). Experimentally, antisense oligodeoxynucleotide (ODN) against HER2/neu inhibits human tumor xenografts implanted in nude mice, and when used in combination with conventional chemotherapy shows enhanced growth inhibitory effects on tumor cell lines that overexpress HER2/neu (Roh *et al.*, 1999). With regard to Stat3 signaling, antisense targeting of Stat3 significantly inhibited growth of squamous epithelial cells (Grandis *et al.*, 1998, 2000a) and B lymphoma cells (Karras *et al.*, 2000). Although this is still at the experimental stage, it makes compelling argument for examining the therapeutic benefits of antisense against Stat3 in cases where constitutive Stat3 activity is linked to disease pathogenesis.

Similar to the application of antisense, use of decoy ODNs harboring protein DNA-binding sites is in principle an approach worthy of consideration. Evidence shows that activated STAT proteins bind to sequence-specific regions in the promoters of genes in the nucleus. In some cases, sequences are unique for binding by specific STAT members, such as the sequence present in the promoter region of the acute phase response protein, C-reactive protein, which is specific for Stat3 (Zhang *et al.*, 1996a). The ectopically expressed excess high-affinity STAT-specific ODNs binding sites in cells would theoretically compete with physiological gene promoters that are STAT target genes. The prediction is that because of the higher affinity and molar excess of ODNs in cells, the exogenous DNA sequence would effectively sequester active STATs and significantly reduce *in vivo* available

levels that can bind natural promoter sequences. In theory, this phenomenon of 'soaking-up' active STAT proteins by decoy ODNs would result in suppression of gene transcription and diminished biological effects. The decoy ODNs approach has been tested in cell culture as well as whole animal studies with respect to other systems (Amoah-Apraku *et al.*, 2000; Griesenbach *et al.*, 2000; Kuratsukuri *et al.*, 1999; Tomita *et al.*, 1999). With regard to STAT signaling, the decoy ODNs approach has been utilized to effectively block cellular responses (Huang *et al.*, 1999; Wang *et al.*, 2000), suggesting its potential practical usefulness.

Analogous to the preceding two approaches, use of dominant-negative interfering mutant forms of proteins to block functional effects of normal proteins has also shown considerable promise. Significantly, the introduction of a dominant-negative Stat3 gene blocks growth and induces regression of Stat3-dependent murine melanoma tumors in nude mice compared to control expression vector alone (Niu *et al.*, 1999). The anti-tumor effects of this dominant-negative Stat3 gene in B16 melanoma tumors establish the potential clinical usefulness of this gene therapy approach in cancers that are positive for constitutive Stat3 activity. Overall, gene-directed targeting is highly target-selective and has the advantage of being more specific than conventional drugs. The major challenge of this approach is the relative inefficiency of introducing genes encoding dominant-negative proteins into cells. However, in the case of Stat3 dominant-negative gene therapy, a transfection efficiency of 10–15% is sufficient to induce massive tumor cell killing involving more than 90% tumor cell death (Niu *et al.*, 1999). This potent 'bystander' effect may be the key to successful dominant-negative Stat3 gene therapy of human tumors. The mechanistic basis for this bystander effect is currently under investigation.

Conclusions

Analyses of human tumors are revealing increasing numbers that are positive for constitutive Stat3 activity. This is a trend that is likely to continue as we expand the tumor types that are assayed for constitutive Stat3 activation. Moreover, initial assessment of the extent of Stat3 activation in different stages of human malignancies suggests a correlation between the level of Stat3 activation and tumor progression. The key is to validate these patterns to provide a basis for classifying tumors relative to levels of Stat3 activity. In particular, the intent is to evaluate Stat3 as a molecular marker for early detection of certain types of cancers, and also as a prognostic indicator for determining the aggressiveness of cancer types and their response to different treatment modalities. Reliable data on Stat3 activity in tumors that were previously Stat3-negative and responsive to chemotherapy but eventually fail to respond to therapy on relapse would provide any correlation there might be between onset of constitutive Stat3 activation and aggressiveness of tumors on relapse, as well as chemosensitivity. These objectives call for screening of constitutive Stat3 activity in tumors prior to selection of treatment type and following the progress of treatment in patients. Ultimately, 'customized' therapies may be devised

based on the molecular STAT profile. Such custom therapy approaches would take into account the stage of the tumor and appropriately factor in the level of constitutive Stat3 activity as it relates to the disease progression. Lastly, where details of the mechanism of constitutive Stat3 activation are defined in the context of tumor type, novel therapeutic approaches based on molecular and/or pharmacological modulation of the components that are critically involved in the constitutive Stat3 signaling pathway would substantially

broaden the available effective treatment modalities for cancer.

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