Regulation of Metastases by Signal Transducer and Activator of Transcription 3 Signaling Pathway: Clinical Implications

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Abstract Numerous cytokines, growth factors, and oncogenic proteins activate signal transducer and activator of transcription 3 (Stat3), which has been recognized as one of the common pathways in cancer cells. Stat3 signaling affects the expression and function of a variety of genes that are critical to cell survival, cell proliferation, invasion, angiogenesis, and immune evasion. Evidently, the Stat3 signaling pathway regulates cancer metastasis and constitutes a potential preventive and therapeutic target for cancer metastasis.

Signal transducer and activator of transcription 3 (Stat3) is a member of the Janus-activated kinase/STAT signaling pathway (1). STAT proteins are a family of latent cytoplasmic transcription factors that contain the DNA-binding domain in the middle region as well as a src-homology 2 domain and a phosphotyrosine-binding domain in the COOH terminus. Upon activation, STAT is phosphorylated on a tyrosine residue by activated Janus-activated kinases in receptor complexes, leading to the formation of homodimers and heterodimers and their migration to the nucleus, where they regulate transcription (Fig. 1). The STAT src-homology 2 domain interacts with sites of tyrosine phosphorylation for the recruitment of STAT receptor complexes. Tyrosine phosphorylation is essential for the dimerization of STATs and the concomitant nuclear translocation of the dimer. The DNA-binding domain makes direct contact with STAT-binding sites in gene promoters.

Stat3 was first identified as a DNA-binding factor that selectively binds to the interleukin-6 (IL-6)–responsive element in the promoter of acute-phase genes from IL-6–stimulated hepatocytes (2). Stat3 was also independently identified as a DNA-binding protein, in response to epidermal growth factor (3). The gene that encodes Stat3 is located on chromosome 17q21, and it encodes a 92-kDa protein. Stat3 is activated by many cytokines and growth factors, including epidermal growth factor, platelet-derived growth factor, and IL-6 as well as by oncogenic proteins, such as Src and Ras (1, 4, 5). Conversely, Stat3 activation is negatively regulated by several proteins, including the suppressors of cytokine signaling family and protein inhibitor of activated STAT3 proteins. The suppressors of cytokine signaling proteins was shown to bind to the Janus-activated kinase activation loop as pseudosubstrate inhibitors through their src-homology 2 domain, thereby blocking subsequent signaling that requires phosphorylation and activation of Stat3 (6). The protein inhibitor of activated STAT3 is a nuclear factor that is able to interact with phosphorylated Stat3 and block transcription (7).

The biological functions of Stat3 are very broad. Stat3 plays a crucial role in the regulation of cell proliferation, survival, apoptosis, and differentiation. In fact, *in vivo* functional analyses using knockout mice indicate that Stat3 is required for embryogenesis because homozygous deletion of Stat3 results in early embryonic lethality. Furthermore, conditional knockout mice have shown the pleiotropic roles of Stat3 in many organs and cell types, including the heart, skin, T lymphocytes, monocytes/neutrophils, mammary epithelium, liver, and neurons (8). For example, cardiomyocyte-specific conditional Stat3 knockout mice showed enhanced susceptibility to cardiac injury caused by either myocardial ischemia, systemic inflammation, or drug toxicity. These mice were also more prone to the pathogenesis of age-related heart failure. Stat3-deficient T cells displayed a severely impaired proliferative response to IL-6 due to a defect in IL-6–mediated suppression of apoptosis. However, despite the roles of Stat3 in a wide variety of physiologic processes, it has been shown that Stat3 activation in normal cells is transient and tightly controlled. In contrast, the Stat3 protein is persistently activated in many cancer cell lines and tumors. In particular, constitutively activated Stat3 protein has been found in various types of tumors, including leukemia and cancers of the breast, head and neck, melanoma, prostate, and pancreas. Recent studies have also revealed that altered Stat3 activation can contribute to oncogenesis. For example, activation of Stat3 is required for cell transformation by oncogenic Src (9) and by a constitutively active form of $G\alpha_o$, a heterotrimeric G-protein subunit (10). In addition, overexpression of the constitutively activated Stat3 mutant (Stat3-C) into immortalized 3T3 cells and immortalized human mammary epithelial cells induces cellular transformation and tumor formation in nude mice (11, 12). Moreover, blockade of constitutive Stat3 signaling results in

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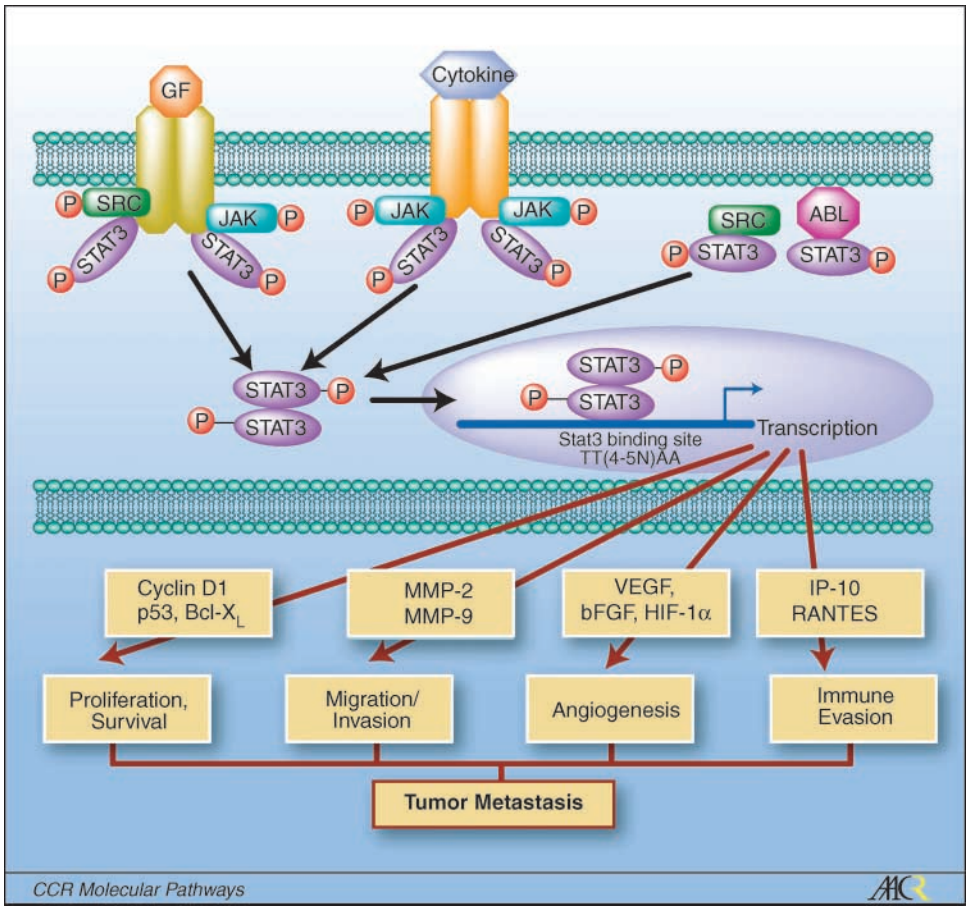

Fig. 1. Contribution of Stat3 signaling pathway to cancer metastasis. Stat3 in the cytoplasm of unstimulated cells becomes activated by recruitment to phosphotyrosine motifs within complexes of growth factor receptors (e.g., epidermal growth factor receptor), cytokine receptors (e.g., IL-6 receptor), or non-receptor tyrosine kinases (e.g., Src and BCR-ABL) through their SH2 domain. Stat3 is then phosphorylated on a tyrosine residue by activated tyrosine kinases in receptor complexes. Phosphorylated Stat3 forms homodimers and heterodimers and translocates to the nucleus. In the nucleus, Stat3 dimers bind to specific promoter elements of target genes and regulate gene expression. The Stat3 signaling pathway regulates cancer metastasis by regulating the expression of genes that are critical to cell survival, cell proliferation, invasion, angiogenesis, and tumor immune evasion. Abbreviations: GF, growth factor; JAK, Janus-activated kinase; P, phosphorylated tyrosine residue; Bcl-xL, Bcl-2-like 1; bFGF, basic fibroblast growth factor; HIF-1α, hypoxia-inducible factor-1α; RANTES, regulated upon activation/normal T-cell expressed and secreted; IP-10, IFN-γ–inducible protein-10.

the growth inhibition of tumor cells with activated Stat3 *in vitro* and *in vivo*. These results, therefore, suggest that Stat3 functions as an oncogene and plays a critical role in transformation and tumor progression.

Downstream Signaling of Stat3 and Cancer Biology

Numerous downstream genes of Stat3 signaling have been identified. Altered expression and regulation of those genes plays important roles in pathogenesis of diverse types of diseases, including inflammation and cancer (1, 4, 5, 7). The effects of Stat3 on cancer biology are mediated, at least in part, through the expression of Stat3 target genes, which are essential to the regulation of multiple aspects of tumor cell survival, growth, angiogenesis, and invasion and evasion of immune surveillance. Activated Stat3 has been shown to protect tumor cells from apoptosis and promote cell proliferation by regulating genes encoding antiapoptotic and proliferation-associated proteins, such as *Bcl-x_L*, *Mcl-1*, *Bcl-2*, *Fas*, *cyclin D1*, *survivin*, and *c-Myc* (4, 5, 11, 13–15). Activated Stat3 can also induce nuclear factor-κB p100 processing to p52, which subsequently inhibits apoptosis of cancer cells (16). Moreover, it has been shown that Stat3 represses the *p53* gene transcription rate by binding to the p53 promoter and subsequently affecting p53-mediated tumor cell apoptosis (17).

The role of Stat3 in angiogenesis was first evidenced when vascular endothelial growth factor (VEGF) was found to be a direct target of Stat3 in mouse melanoma cells (18) and then confirmed by a study in a human pancreatic cancer system (19). Stat3 also regulates the transcription of VEGF indirectly by controlling the expression of hypoxia-inducible factor-1α, a key inducible transcription factor for the *VEGF* gene (20). In addition, our recent study found that Stat3 regulates the expression of other angiogenic molecules, such as basic fibroblast growth factor (21), which participates in angiogenesis by inducing the migration, proliferation, and differentiation of endothelial cells and by regulating VEGF expression in tumor cells. Furthermore, it has been shown that Stat3 can be activated by basic fibroblast growth factor and VEGF upon binding of these molecules to their receptors and transducing the receptor signals in endothelial cells (22–24). Consequently, Stat3 activation is necessary for endothelial cell proliferation, migration, and microvascular tube formation (22–24).

There is substantial evidence of Stat3 involvement in tumor cell migration and invasion (25). Stat3 has been shown to transcriptionally activate the expression of genes that promote tumor cell migration and invasion. Specifically, activated Stat3 regulates tumor invasion of melanoma cells by regulating the gene transcription of matrix metalloproteinase 2 (MMP-2; refs. 21, 26). Stat3 activation increases migration and invasion of bladder cancer cells by induction of MMP-1 and MMP-10 (27) and has also been required for the induction of MMP-9 in transformed human mammary epithelial cells (12). Moreover, Stat3 may contribute to tumor cell migration and invasion through transcription-independent pathways. For example,

Stat3 has been shown to interact directly with the central machinery for cell motility, such as microtubules and focal adhesion. In fact, Stat3 protein in ovarian cancer cells has been found to localize to focal adhesions along with focal adhesion kinases and paxillin, and it interacts with phosphorylated focal adhesion kinase and paxillin, subsequently modulating the invasiveness of the cells (28).

Finally, recent studies show unambiguously that Stat3 signaling plays a pivotal role in the regulation of tumor immunity. Stat3 has been shown to be constitutively activated in both tumor cells and tumor-associated immune cells (29, 30). Stat3 signaling in tumors inhibits the expression of inflammatory cytokines and chemokines that activate anti-tumor innate and adaptive immunity, such as IFN-β, tumor necrosis factor-α, IL-6, regulated upon activation/normal T-cell expressed and secreted, and IFN-γ–inducible protein-10. Stat3 signaling in tumors also promotes the expression of factors, such as VEGF and IL-10, that inhibit dendritic cell maturation and T-cell activation, giving rise to immune tolerance. Blockade of Stat3 signaling in tumors reverses these negative effects, resulting in enhanced innate immunity and activation of dendritic cells, leading to tumor-specific T-cell responses. Furthermore, studies in mice with immune cell lineage–specific Stat3 deficiency have revealed that activated Stat3 in immune cells plays a critical role in tumor immune evasion (29, 30). Ablation of Stat3 in the immune cells of tumor-bearing hosts elicits multicomponent antitumor immunity that inhibits tumor growth, including dendritic cells, T cells, natural killer cells, and neutrophils (30). Blocking of Stat3 in mice with a small-molecule inhibitor induces T cell– and natural killer cell–dependent antitumor immune responses (30).

Roles of Stat3 in Cancer Metastasis

Metastasis is the major cause of death in most cancer patients. The process of metastasis is complex and requires multiple steps. These steps include invasion of tumor cells into tissue surrounding the primary tumor, entering either the lymphatic vessels or the bloodstream, survival of circulatory cells, arrest in a new organ, extravasation into the tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor (31, 32). In general, the molecular mechanisms that control the steps of metastasis are related to alterations of various oncogenes, tumor suppressor genes, metastasis suppressor genes, and growth factors and their receptors (31–33). Stat3 is known to be activated by numerous cytokines, growth factors, and oncogenic proteins, including epidermal growth factor, platelet-derived growth factor, VEGF, basic fibroblast growth factor, IL-6, Src, and Ras, suggesting that Stat3 signaling may be one of the common pathways involved in regulating cancer metastasis.

Several lines of evidence indicate that the constitutive activation of Stat3 influences metastasis. Specifically, the level of activated Stat3 protein has been found to be associated with metastases in various types of tumors. For example, activation of Stat3 in thymic epithelial tumors, colorectal adenocarcinoma, and cutaneous squamous cell carcinoma correlates with lymph node metastasis (34–36). Furthermore, activation of Stat3 in renal cell carcinoma is associated with distant metastatic disease (37). More recently, we have analyzed Stat3 activity in human melanoma specimens and detected higher levels of activated Stat3 protein in melanoma brain metastases than in a cohort of primary melanomas (21). Moreover, the roles and mechanisms of activated Stat3 in metastases have been shown using *in vivo* model systems of liver, lung, and brain metastases. For example, a study using an orthotropic pancreatic tumor animal model showed that blockade of activated Stat3 via ectopic expression of dominant-negative Stat3 significantly suppressed VEGF expression, angiogenesis, and liver metastasis of pancreatic tumor cells (19). Blocking of Stat3 signaling in highly metastatic melanoma cells significantly suppressed the expression of the *MMP-2* gene and invasiveness of the tumor cells and prevented lung metastasis in a mouse melanoma model (26). Blocking of Stat3 activation also suppressed brain metastasis of human melanoma cells in a brain metastasis animal model (21). Conversely, enforcing the expression of a constitutively activated Stat3 protein converted poorly metastatic melanoma cells into highly metastatic brain tumor cells in the model. The mechanisms that promote brain metastasis by Stat3 could be attributable to the overexpression of basic fibroblast growth factor, MMP-2, and VEGF caused by Stat3 activation, hence increasing tumor invasion and angiogenesis (21). Further implicating Stat3 in tumor metastases is the finding that the elimination of Stat3 protein by RNA interference in mouse breast cancer cells can block the expression of metastatic regulator Twist and prevent metastases of the breast cancer cells (38). Collectively, these studies provide evidence that the Stat3 signaling pathway may affect tumor metastasis via regulation of the multiple steps of its process (Fig. 1).

Clinical-Translational Advances

Recent advances in investigating the role of Stat3 in tumor metastasis indicate that Stat3 signaling might be a common molecular target for blocking metastasis in human tumors. Several strategies have been pursued for developing inhibitors of Stat3, based on the complex nature of its gene expression and activity. Strategies to abolish Stat3 expression include the delivery of antisense oligonucleotides and small interfering RNA, whereas approaches for inhibition of Stat3 activity include the use of tyrosine kinase inhibitors, phosphopeptides, G-quartet oligodeoxynucleotides, decoy oligonucleotides, and small-molecule compounds that inhibit DNA-binding (39–47). Among these strategies against Stat3 is the use of small-molecule inhibitors, such as JSI-124, WP1066, STA-21, and IS3 295, which have the most immediate therapeutic potential. JSI-124 (cucurbitacin I) has been identified in the National Cancer Institute Diversity Set as an inhibitor of Janus-activated kinase-2/Stat3, with no apparent effect on the Akt, extracellular signal-regulated kinase, or c-Jun NH_2-terminal kinase pathways (39). JSI-124 is reported to inhibit cellular proliferation and induce apoptosis of multiple cancer cell lines *in vitro* as well as to inhibit tumor growth of A549 lung adenocarcinoma and MDA-MB-468 breast cancer cells in nude mice. WP1066, another Janus-activated kinase-2 inhibitor, has been shown to suppress the growth of malignant glioma U87-MG and U373-MG cells *in vitro* and *in vivo* (40). STA-21 was discovered via virtual database screening of the nearly 429,000 compounds for Stat3 inhibitors (45). STA-21 showed remarkable inhibition of Stat3 dimerization, DNA binding, Stat3-dependent luciferase activity, and nucleus translocation. Moreover, STA-21 reduces

the growth and survival of breast cancer cells with constitutively activated Stat3 *in vitro*. IS3 295, a novel platinum(IV) compound, has also been identified in the National Cancer Institute Diversity Set as a selective Stat3 inhibitor (46). It interacts with Stat3 directly and inhibits Stat3 binding to specific DNA response elements. Unlike its prototype (cisplatin), IS3 295 has no effects on Akt and mitogen-activated protein kinase family pathways. IS3 295 inhibits Stat3-regulated genes *cyclin D1*, *Bcl-X_L*, and *VEGF*. It also blocks cell cycle progression and proliferation and mediates apoptosis of malignant cells *in vitro*. Another novel platinum(IV) compound (CPA-7) has been shown to inhibit tumor growth and metastases in mouse models (30, 47). These studies represent a significant advance in the development of small molecules that target Stat3 as therapeutic against tumors and metastasis with important clinical implications.

Stat3-specific inhibitors have not yet been entered into clinical trials, although other drugs that can indirectly inhibit Stat3 activation, such as Atiprimod and gefitinib, have undergone clinical testing. At this point, based on the evidence from studies using relevant animal models, it might be expected that Stat3 inhibitors would have limited toxicity in normal cells because Stat3 activation in normal cells is transient and tightly controlled, and normal cells are not dependent on aberrant Stat3 activation for growth and survival. Indeed, primary embryo fibroblasts from conditional knockout mice lacking Stat3 proliferated similarly to their wild-type counterparts and displayed similar survival (48). Moreover, despite the pivotal role of Stat3 during embryonic development, the effects of conditional ablation of Stat3 in adult tissues are mild (8). Furthermore, Stat3 inhibitors reduce the survival of tumor cells with constitutive Stat3 signaling but have minimal effect on the cells in which constitutive Stat3 signaling is absent. However, in spite of these optimistic data, it is noteworthy that recent studies have indicated that Stat3 activation promotes cardiomyocyte survival and cardiac angiogenesis, in response to various pathophysiologic stimuli, thereby protecting the heart from injury and failure (49). In this regard, Stat3 inhibitors may have side effects in patients with cardiac problems. Consequently, the toxicity and side effects of Stat3 inhibitors must be thoroughly investigated in animal models and ultimately in clinical trials, with careful assessment as to whether the potential benefits far outweigh the associated risks to patients. Nevertheless, given the critical role of Stat3 activity in regulating the multiple steps of the metastasis process, targeting of Stat3 activation may prove to be a more effective approach to controlling metastasis than merely targeting individual molecules, such as VEGF and MMP-2. Thus, targeting of Stat3 signaling may represent a novel approach to controlling cancer metastasis.

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