Cancer Therapy: Preclinical

STAT5A-Mediated SOCS2 Expression Regulates Jak2 and STAT3 Activity Following c-Src Inhibition in Head and Neck Squamous Carcinoma

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Abstract

Purpose: The inhibition of c-Src results in a striking reduction in cancer cell invasion, but the effect on cell survival is modest. Defining mechanisms that limit apoptosis following c-Src inhibition could result in an ideal therapeutic approach that both inhibits invasion and leads to apoptosis. In this regard, we discovered a novel feedback loop that results in STAT3 reactivation following sustained c-Src inhibition. Here we define the mechanism underlying this feedback loop and examine the effect of inhibiting it in vivo.

Experimental Design: We measured levels and activity of pathway components using PCR, Western blotting, and kinase assays following their manipulation using both molecular and pharmacologic approaches. We used a heterotransplant animal model in which human oral squamous cancer is maintained exclusively in vivo.

Results: Following c-Src inhibition, STAT5 is durably inhibited. The inhibition of STAT5A, but not STAT5B, subsequently reduces the expression of suppressors of cytokine signaling 2 (SOCS2). SOCS2 inhibits Janus kinase 2 (Jak2) activity and Jak2–STAT3 binding. SOCS2 expression is necessary for STAT3 inhibition by c-Src inhibitors. Overexpression of SOCS2 is adequate to prevent STAT3 reactivation and to enhance the cytotoxic effects of c-Src inhibition. Likewise, the combination of Jak and c-Src inhibitors led to significantly more apoptosis than either agent alone in vivo.

Conclusions: To our knowledge, ours is the first study that fully defines the mechanism underlying this feedback loop, in which sustained c-Src inhibition leads to diminished SOCS2 expression via sustained inhibition of STAT5A, allowing activation of Jak2 and STAT3, Jak2–STAT3 binding, and survival signals.

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Introduction

One potential and promising therapeutic cancer target is c-Src, given its well-defined roles in promoting cell migration and metastasis as well as regulating proliferation, survival, and angiogenesis. The Src family kinases (SFK) are nonreceptor tyrosine kinases involved in signal transduction in both normal and cancer cells (1). c-Src is the SFK that is most often implicated in cancer progression. Inhibition of c-Src results in a nearly universal reduction in invasion of cancers in vitro and in vivo (2, 3). However, despite c-Src expression and activation in epithelial tumors and c-Src’s robust inhibition by clinically relevant agents, the effect of c-Src inhibition on epithelial cancer cell survival and proliferation has been modest (3). A clinical trial of the SFK inhibitor dasatinib as a single agent in head and neck squamous cell carcinoma (HNSCC) did not show significant activity (4).

Current treatment for HNSCC includes a combination of cytotoxic chemotherapy, radiotherapy, and surgery. Cetuximab enhances the efficacy of chemotherapy and radiotherapy, but no kinase inhibitors are currently a standard of care for HNSCC. Although invasion is important in the pathophysiology of many cancers, local invasion is a critical determinant of both morbidity and mortality for HNSCC and is associated with worse locoregional control and decreased survival. There is a great need to improve systemic therapy to treat both local recurrence and distant metastatic disease. Thus, defining mechanisms that limit the proapoptotic effects of c-Src inhibitors could result in an ideal combination of therapeutic agents that both inhibit local invasion and lead to significant cytotoxicity.
The STAT family of transcription factors, especially STAT3, is known to be c-Src substrates and can mediate STATs in modulating the biologic effects of c-Src inhibition. The STAT family of transcription factors, especially STAT3 and STAT5, regulates oncogenic signaling in many different tumor types. In HNSCC cells, c-Src’s inhibition results in reduced STAT3 and STAT5 activation and reduced cell proliferation (6). Correspondingly, inhibition of STAT3 in HNSCC leads to increased apoptosis, decreased proliferation, and decreased tumor size (7, 8). However, we found that whereas inhibition of c-Src led to durable inhibition of STAT5, c-Src’s inhibition of STAT3 was only transient, with levels of phospho-STAT3 (pSTAT3, Y705) returning to baseline or above by 7 hours. We confirmed this finding by reducing c-Src specifically with siRNAs (siRNAs) and by measuring STAT3 activity using DNA-binding and transcriptional activity assays (9). We also established the biologic importance of this feedback loop by showing that abrogation of STAT3 reactivation enhanced the cytotoxicity, cell-cycle arrest, and apoptosis caused by c-Src inhibition in vitro. These findings established that the STAT3 compensatory pathway is important for maintaining cancer cell proliferation and survival after sustained c-Src inhibition. Furthermore, the depletion of STAT3 by an siRNA reduced the 50% inhibitory concentration (IC50) of the c-Src inhibitor dasatinib from 23 to 4 nmol/L, increasing sensitivity to STAT3 inhibition, thereby reactivating proliferative signals through Jak2 and STAT3. In addition, the 2 STAT5 isoforms (A and B) are known to have distinct roles in cancer and in embryonic development, but the roles of these isoforms in this feedback loop have never been explored (5, 11). Understanding the basis for STAT3 reactivation is essential to maximizing the proapoptotic effect of c-Src inhibitors.

To test our hypothesis, we measured the levels of all known SOCS family members following c-Src knockdown or inhibition with the ATP-competitive SFK inhibitor, dasatinib, and found that SOCS2 expression was consistently decreased. To further define this novel feedback loop, we manipulated the levels of SOCS2, STAT3, STAT5A, and STAT5B to show that c-Src inhibition leads to STAT5 inactivation, that STAT5A drives SOCS2 protein expression, and that SOCS2 inhibits Jak2–STAT3 binding, Jak activity, and STAT3 activation. We previously showed that c-Src inhibition did not affect total levels of Jak2 protein (9). Moreover, SOCS2 loss caused increased resistance to dasatinib, and SOCS2 overexpression led to increased sensitivity to c-Src inhibitors. We confirmed the biologic importance of this feedback pathway using a heterotransplant model of HNSCC and clinically relevant inhibitors of Jak and c-Src.

Materials and Methods

Cells and reagents

Dasatinib was purchased from Selleck Chemicals and the clinical pharmacy. INCB16562 was provided by Incyte Corporation. Both were prepared as 10 mmol/L stock...
solutions in DMSO. Antibodies used included c-Src, pSFK (Y416), pSTAT3 (Y705), plak2 (Y221), plak2 (Y1007/1008), pSTAT5 (Y694) XP, and SOCS2 (Cell Signaling Technology); total phosphotyrosine and total STAT5B (Upstate Biotechnology); SOCS1 and total Jak2 (BD Biosciences); total STAT5A (Abcam); and β-actin (Sigma Chemical).

Human HNSCC cell lines were obtained from Dr. Jeffrey Myers and maintained as described previously (9). All cell lines were validated by cross-comparing their allelic short tandem repeat profiling (Johns Hopkins Fragment Analysis Core Facility) and patterns generated with the PowerPlex 1.2 platform (Promega) to those from the American Type Culture Collection repository database.

Western blot analysis and immunoprecipitation

Western blot analysis and immunoprecipitation were carried out as previously described (3, 9). Briefly, for immunoprecipitation, cells were lysed and equal amounts of protein cell lysates (800 μg) were precleared with protein A–G–sepharose beads (Invitrogen) for 1 hour. The pre-cleared lysate was incubated with 5 μg agarose-conjugated primary antibody overnight. The immunocomplexes were washed and resolved by SDS-PAGE. Following transfer to nitrocellulose membranes, immunoblots were probed with primary antibody and proteins detected with horseradish peroxidase–conjugated secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescent reagent (Pierce Biotechnology).

Cytotoxicity assay

MTT assay was used to assess cytotoxicity as previously described (3). Eight wells were treated for each experimental condition.

Transfection with siRNA and recombinant plasmids

The siRNAs were predesigned sets of 4 independent sequences (siGENOME SMARTpool, Dharmacon). Controls included cells that were mock transfected (no siRNA) and those transfected with a nontargeting (scrambled) siRNA. The pLUSE STAT5A 1’6, pLUSE STAT5B 1’6 recombinant plasmids, and pMetr7-FLAG-mSOCS2 constructs were used to achieve overexpression of STAT5A and/or STAT5B and mouse full-length SOCS2, respectively, in cells. Mouse SOCS2 shows 94% identity and 95% amino acid sequence similarity with human SOCS2. Cells were harvested, washed, and suspended (10^6/100 μL) in Nucleofector V solution (Lonza Group). siRNA (200 pmol/100 μL), DNA (5 μg/100 μL), or controls were added and electroporated using the U-31 Nucleofector program (Lonza) as described previously (9).

Quantitative PCR

Total RNA was isolated from cells that had been either transfected with siRNAs or incubated with dasatinib (as indicated in the figure legends) by using an RNasy mini kit (Qiagen). Total RNA (2 μg) was converted into cDNA using 1× MMLV buffer, 1 μL RNasin, 10 μmol/L random hexamer, 500 μmol/L deoxyribonucleotide triphosphates, 100 μg/mL BSA, and 1.5 μL Moloney murine leukemia virus (MMLV) reverse transcriptase enzyme. The final reaction volume was 20 μL. The reaction mixture was incubated at 42°C for 2 hours, and the reaction was terminated by heating the mixture at 99°C for 5 minutes and cooling it at 5°C for 5 minutes.

The level of mRNA for the SOCS genes was measured with SYBR green–based real-time PCR in triplicate. The primers were designed by using Primer Express (Applied Biosystems; Supplementary Table S1). Each cDNA sample was amplified by using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s suggested protocol. The PCR products and their dissociation curves were detected using the ABI Prism 7500 fast real-time PCR system. The level of the housekeeping gene L32 ribosomal gene (RpL32) was used as an internal control. Individual data sets were normalized with control vehicle-treated cells; absolute quantities were normalized with L32 as an internal control.

In vitro kinase assay

Purified recombinant Jak2 (Abcam) and SOCS2 (Abnova) proteins were incubated at a 1:1 molar stoichiometric ratio with 15 μCi [γ-32P]ATP (3,000 Ci/mmole), and kinase activity was assayed as described previously (9).

Xenograft nude mouse models

All animal procedures were in accordance with the policies of MD Anderson’s Institutional Animal Care and Use Committee. For the orthotopic models, the tongues of five 6-week-old female Swiss nu/nu mice were injected with 5 × 10^5 Ovx19 cells. For the heterotransplant studies, residual tumor from a patient with untreated oral squamous carcinoma was identified by a head and neck pathologist (A. El-Nagar) at the time of surgical resection and implanted directly into the flank of a nude mouse. The resulting tumor was divided and transplanted into subsequent mice until 40 fifth-generation tumors were produced. The heterotransplant tumors were never cultured in vitro. Dasatinib (20 mg/kg), INCB16562 (60 mg/kg), both, or vehicle was administered by oral gavage daily for 7 days (orthotopic) or 17 days (heterotransplant). Mice were killed 2 hours after the last drug dose, tumors were dissected, and the mice were examined for distant metastases. The tumors were homogenized and subjected to Western blot analysis as described previously (12).

Immunohistochemical analysis

Immunohistochemical staining was carried out as previously described using the following specific conditions: antigen retrieval was carried out using a Dako-Target retrieval at pH 6.0 for proliferating cell nuclear antigen (PCNA), CD31 (Abcam), and pSFK (Y416; Cell Signaling Technology). Peroxide blocking was carried out using 3% water and hydrogen peroxide (pSFK Y416; ref. 13). Primary antibody dilutions were: PCNA (1:100), CD31 (1:50), and pSFK (1:50). Slides were examined by a
blinded observer for the intensity and extent of immunostaining by light microscopy using a ×20 magnification objective. Nuclear PCNA expression was quantified using a 3-value intensity: 0, none; low (1+, weak and 2+, moderate); and high (3+, strong and 4+ very strong). CD31-positive vessels were counted in 5 high-powered fields by a blinded observer.

**TUNEL assay**

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was carried out using the DeadEnd Colorimetric TUNEL system from Promega per the manufacturer’s instructions as previously described (13). TUNEL-positive nuclei were counted for each representative treatment group.

**Statistical methods**

All experiments in which error bars and \( P \) values are provided were done in at least triplicate. The Student test was used to determine if the mean values of these continuous variables were different in the various treatment groups.

**Results**

**c-Src inhibition leads to decreased SOCS2 expression and STAT3 inactivation**

We postulated that the loss of one of the SOCS proteins could contribute to STAT3 reactivation after sustained c-Src inhibition. To test this hypothesis, we determined the expression level of all members of the SOCS family after 7 hours of c-Src inhibition with dasatinib using quantitative PCR analysis in a panel of 6 different HNSCC cell lines (Fig. 1A). Among the 8 family members of SOCS proteins, only SOCS2 showed consistent downregulation in all 6 cell lines. We also measured the expression of the 4 PIAS family members but found no significant alteration in PIAS expression following dasatinib treatment (representative data, Supplementary Fig. S1). STAT3 reactivation was not mediated by an autocrine mechanism such as cytokine release (Supplementary Fig. S2).

To characterize the effect of c-Src inhibition on SOCS2 protein expression, we examined the effect of dasatinib in 2 representative HNSCC cell lines, that grow well both in vitro and in vivo, using Western blot analysis (Fig. 1B). As expected, c-Src phosphorylation was rapidly and durably inhibited at a site associated with its activation (pSFK, Y416). SOCS2 protein expression was significantly downregulated after sustained c-Src inhibition.

To determine whether SOCS2 expression is downstream of c-Src specifically, we transfected HNSCC cells with siRNAs specific to c-Src and examined the effect on SOCS family members’ mRNA (Fig. 1C and D) and protein (Fig. 1E) expression. Upon c-Src depletion, the levels of SOCS2 mRNA and protein decreased significantly. In addition to SOCS2, CIS1 expression was decreased following c-Src knockdown (Fig. 1C and D), but CIS1 was not consistently affected by incubation with dasatinib (Fig. 1A). These experiments show that c-Src activation is upstream of SOCS2 transcription.

Given that STAT5 can regulate SOCS2 expression, we investigated whether c-Src could regulate STAT5 activation in HNSCC cell lines. We incubated cells with dasatinib for 7 hours and measured pSTAT5 (Y694). c-Src inhibition rendered STAT5 durably inactive which is consistent with our previous results showing STAT5 inhibition from 2 to 24 hours following dasatinib treatment (Fig. 1F; ref. 3).

**SOCS2 expression is regulated by STAT5A but not STAT3 or STAT5B**

Previous reports showed that STAT5 can act as a transcriptional regulator for SOCS family proteins in hematopoietic cells (14, 15). We sought to determine whether the modulation of STAT5 activity regulates SOCS2 expression in HNSCC cells. HNSCC cell lines express both isoforms of STAT5 (STAT5A and STAT5B) and their roles may be distinct (11). Likewise, we found that selective STAT5A knockdown using siRNA led to a considerable decrease in SOCS2 expression, whereas STAT5B depletion alone had little effect on SOCS2 expression (Fig. 2A). In contrast, selective STAT3 depletion with siRNA did not affect SOCS2 expression (Fig. 2B).

To further elucidate the function of the STAT5 isoforms in the regulation of SOCS2 expression and STAT3 activation, we selectively overexpressed constitutively active forms of both STAT5 isoforms. STAT5A activation led to increased expression of SOCS2 but not SOCS1 (Fig. 2C). Likewise, STAT5A overexpression resulted in decreased activation of STAT3, thus supporting our hypothesis that STAT5A regulates SOCS2 expression, which subsequently acts as a negative regulator of STAT3 activation. In contrast, STAT5B overexpression alone did not significantly alter basal SOCS2 protein levels or pSTAT3 (Y705) expression.

**Selective knockdown of SOCS2 leads to STAT3 activation**

To determine whether SOCS2 downregulation could lead to STAT3 activation, we selectively decreased SOCS2 expression in HNSCC cell lines using siRNA. Upon SOCS2 knockdown, STAT3 phosphorylation increased markedly by 4.6- and 4.8-fold in TU167 and Osc19 cell lines, respectively, over that in control cells (Fig. 3A). This result supports our hypothesis that SOCS2 has a negative regulatory role in the Jak2–STAT3 signaling pathway. Total Jak2 protein levels were also increased by SOCS2 knockdown, a result consistent with the known role of SOCS in promoting Jak protein degradation. In our previous work, however, we did not observe changes in total Jak2 levels following dasatinib treatment or c-Src knockdown (9).

**SOCS2 depletion results in sustained STAT3 activation despite acute c-Src inhibition**

Our previous experiments have shown that acute c-Src inhibition results in transient STAT3 inactivation (10). We hypothesized that early SOCS2 depletion would allow STAT3 to remain activated despite acute c-Src inhibition.
To test this hypothesis, we examined the effect of dasatinib on STAT3 reactivation in cells with depleted SOCS2. As we showed previously, TU167 cells incubated with dasatinib showed significant downregulation of STAT3 phosphorylation 30 minutes after treatment (SOCS2 levels were not affected at this early time point). In contrast, SOCS2-depleted TU167 cells had incomplete inhibition of STAT3 phosphorylation at 30 minutes after
dasatinib treatment (Fig. 3B). This result shows that SOCS2 expression is required for STAT3 inhibition by c-Src. In contrast, STAT5 was inhibited by dasatinib independently of SOCS2 expression.

**SOCS2 overexpression leads to STAT3 inhibition**

To further explore the role of SOCS2 as a negative regulator of STAT3, we transiently overexpressed SOCS2, which resulted in significant sustained decreases in both STAT3 and Jak2 activation while leaving total STAT3, SOCS1, and pSFK levels unchanged (Fig. 3C). To determine the effect of forced SOCS2 expression following sustained c-Src inhibition, we transfected Osc19 and TU167 cells with either SOCS2 or empty vector and exposed them to dasatinib for 30 minutes to 7 hours (Fig. 3D and E). The overexpression of SOCS2 significantly diminished the basal activation and reactivation of STAT3 compared with controls.

**SOCS2 expression mediates sensitivity and resistance to c-Src inhibition**

To determine the biologic significance of SOCS2 in this feedback loop, we transiently overexpressed or knocked down SOCS2 and estimated cytotoxicity in the presence of the c-Src inhibitor dasatinib (Fig. 4). SOCS2 knockdown led to increased resistance to dasatinib in both HNSCC cell lines compared with results in controls (Fig. 4A and C). In contrast, overexpression of SOCS2 in either line led to increased sensitivity to c-Src inhibition (Fig. 4B and D). The basal differences in dasatinib sensitivity between Osc19 and TU167 cells are likely because of distinct interactions between c-Src and c-Met (16).

Although the manipulation of SOCS2 expression affected sensitivity to c-Src inhibition in a predictable manner, we were concerned that the biologic effects of STAT5 modulation might not parallel what we observed...
with direct SOCS2 manipulation, because STAT5 itself can promote cancer cell survival and proliferation in HNSCC (17). We transfected cells with constitutively active STAT5A or B or both and then measured cytotoxicity in the presence of dasatinib. HNSCC cells that overexpressed STAT5A (and had increased SOCS2 and decreased STAT3 activation, Fig. 2C) were slightly more sensitive to dasatinib. However, those cells overexpressing STAT5B (no change in SOCS2) or both isoforms (increased SOCS2) were more resistant to dasatinib. In TU167 cells, STAT5A and B knockdown led to a modest increase in sensitivity to dasatinib, whereas in Osc19 cells, this observation was reversed (Supplementary Fig. S3A), suggesting that STAT5B promotes cancer survival through an independent mechanism. In TU167 cells, STAT5A and B knockdown led to a modest increase in sensitivity to dasatinib, whereas in Osc19 cells, this observation was reversed (Supplementary Fig. S3A), suggesting that STAT5B promotes cancer survival through an independent mechanism.

**SOCS2 inhibits Jak2–STAT3 binding and Jak2 kinase activity**

Previous reports have shown that SOCS family members bind to Jaks and inhibit their kinase activity, as well as compete with STAT molecules for recruitment to the receptor complex (18). To determine whether SOCS2 affects Jak2–STAT3 binding in HNSCC cells, we overexpressed SOCS2 in TU167 cells and immunoprecipitated total Jak2; complexes were analyzed by immunoblotting (Fig. 5A and Supplementary Fig. S4). In the presence of SOCS2, Jak2–STAT3 binding was significantly decreased. As expected, SOCS2 alone showed no binding to STAT3.

In the presence of SOCS2, Jak2 autophosphorylation and activity toward an exogenous substrate (enolase) were significantly inhibited. As expected, SOCS2 alone showed no binding to STAT3.

**Figure 3.** SOCS2 expression negatively regulates STAT3 activation.

A and B, TU167 and Osc19 cells were mock-transfected with no siRNA, nontargeting (scrambled), or SOCS2-specific siRNA (KD, knockdown). Cells were subjected to no further treatment (A) or incubated with 100 nmol/L dasatinib for 30 minutes before lysis (B) and then harvested and lysed. The indicated molecules were visualized using Western blot analysis. C, TU167 and Osc19 cell lines were transiently transfected with the pMet7-FLAG-mSOCS2 overexpression construct (SOCS2 OE) or vector control. Cells were harvested and lysed 72 hours after transfection, and the indicated molecules were analyzed by Western blot analysis.
kinase activity. These observations confirm that SOCS2 acts as a negative regulator of Jak2–STAT3 signaling by inhibiting Jak2 activity as well as Jak2–STAT3 binding.

Jak inhibition enhances the antitumor effects of c-Src inhibition in vivo

To determine whether the reactivation of STAT3 is biologically significant in vivo, we used a heterotransplant model of HNSCC in which an oral squamous carcinoma tumor was transplanted directly into a mouse. The resulting tumor was divided and serially passaged into mice; the tumors were never cultured in vitro. The resulting tumors maintained the histologic characteristics of the primary tumor from which they were derived (Supplementary Fig. S5). Heterotransplants maintain the gene expression profiles of the original tumors and their pattern of response to chemotherapy resembles those observed in the clinic (19), suggesting that this model may be superior to other
were detected by autoradiography. OE, overexpressed. Radiolabeled proteins were incubated at room temperature in the presence of 15 μCi 32-ATP and the exogenous substrate enolase for 30 minutes. Reactions were terminated with the addition of sample buffer, boiled for 5 minutes, and separated on an 8% SDS-PAGE.

Figure 5. SOCS2 regulates Jak2–STAT3 binding and Jak2 kinase activity. A, lysates from control and SOCS2-overexpressing cells were immunoprecipitated (IP) with Jak2 antibodies, and immunoprecipitated complexes were resolved on SDS-PAGE. The indicated molecules were visualized using Western blot analysis. B, purified recombinant SOCS2 and Jak2 proteins were incubated at room temperature in the presence of 15 μCi 32-ATP and the exogenous substrate enolase for 30 minutes. Reactions were terminated with the addition of sample buffer, boiled for 5 minutes, and separated on an 8% SDS-PAGE. Radiolabeled proteins were detected by autoradiography. OE, overexpressed.

Discussion

Our current findings define the mechanism underlying a novel feedback loop in which sustained c-Src inhibition or knockdown leads to diminished SOCS2 expression via the sustained inhibition of STAT5A. This relieves the negative constitutive inhibition of SOCS2 on the Jak2–STAT3 pathway, specifically allowing the activation of Jak2 kinase activity, Jak2–STAT3 binding, and STAT3 activation. Although SOCS2 can affect Jak2 protein levels by promoting protein degradation, in our previous studies we observed no changes in total Jak2 expression following c-Src inhibition or knockdown. Ultimately, the loss of SOCS2 expression leads to the reactivation of proliferative signals through STAT3 despite sustained c-Src inhibition (Supplementary Fig. S7).

Although it is well established that SOCS proteins can inhibit Jak/STAT function, we are aware of only one other study in which altered signaling led to the loss of SOCS function with subsequent Jak/STAT activation and cancer promotion (20). Jak1 activation is important for v-Abl-induced transformation of pre-B cells. In nontransformed cells, the induction of SOCS1 acts as a negative feedback loop to suppress Jak/STAT function, but v-Abl phosphorylates SOCS1 and inhibits its targeting of Jak1 for degradation. Thus, v-Abl’s inhibition of SOCS1 allows sustained Jak1 and STAT5 activation, contributing to cytokine independence in the transformed cells. Our study showed a distinct role for a SOCS protein in regulating Jak/STAT function; in HNSCC, SOCS2 was regulated at the transcriptional level and not by posttranslational modification and degradation.

SOCS proteins have been most extensively studied in normal immune function and hematologic malignancies, where they function as classic mediators of a negative feedback loop downstream of cytokine receptors (21). The roles of SOCS proteins in epithelial cancers are not as well known, although studies support a tumor-suppressor role for SOCS proteins via Jak/STAT suppression in nonhematologic malignancies. In this context, SOCS1 and SOCS3 are the most extensively studied, although the loss of SOCS2 can promote intestinal growth, polyp formation, and colon cancer progression (22–24). The expression of SOCS1, which is downregulated via methylation in about a third of HNSCC tumors, can inhibit STAT3 activation by Jak in HNSCC cell lines (25). In those cell lines with SOCS1 expression, STAT3 was shown to be activated via epidermal growth factor receptor (EGFR); in those lines lacking SOCS1, STAT3 was activated via interleukin-6 and Jak. The effects of SOCS1 on STAT3 were not examined (25). SOCS3 is commonly hypermethylated and downregulated in HNSCC tumors; its overexpression in HNSCC cell lines leads to apoptosis (26). SOCS3 is also hypermethylated in lung cancer cell lines and tissues (27). In melanoma, the SOCS1 expression was decreased and STAT3 and Jak2 expression increased compared with primary tumor cells. Restoration of SOCS1 expression leads to STAT3 inactivation and inhibition of brain xenograft approaches for therapeutic studies. Both dasatinib and the Jak inhibitor INCB16562 modestly inhibited tumor growth; the combination was significantly more effective than the single agents (Fig. 6A). Likewise, the tumors treated with the combination had significantly more apoptosis (Fig. 6B) and less proliferation (Fig. 6C and Supplementary Table S2). Consistent with our in vitro results, c-Src inhibition did not result in STAT3 inhibition, but Jak inhibition abrogated STAT3 activation (Fig. 6D); c-Src was inhibited in vivo by dasatinib (Fig. 6E).

We also used an orthotopic HNSCC model in which Osc19 cells were implanted into the tongue. Mice were treated with dasatinib or INCB16562 or the combination for 7 days. Tumors consisted primarily of HNSCC cells (>90%) with no distant metastases. As expected, dasatinib treatment inhibited c-Src, and STAT3 remained activated (1.7-fold) over the control level. In the presence of INCB16562, pSTAT3 reactivation upon dasatinib treatment was significantly reduced to 0.2-fold (Supplementary Fig. S6).
metastasis (28). Similarly, exogenous expression of SOCS1, SOCS3, or SOCS5 in thyroid cancer cells reduces STAT3 phosphorylation and sensitizes cells to chemotherapy in vitro and in vivo (29).

In our experiments, SOCS2 had a function distinct from its classically understood role described in hematopoietic cells (21). SOCS2 did inhibit Jak2 kinase activity but does not contain the classic kinase inhibitory region that SOCS1 and SOCS3 proteins possess (21). However, our study was limited in that we used isolated recombinant proteins that may function differently from native proteins in an intact cell. SOCS2 also is classically understood to promote the degradation of Jak2, yet we did not observe changes in total STAT3 or Jak2 levels in HNSCC cells following prolonged c-Src inhibition or knockdown (9). However, we did observe that SOCS2 knockdown led to increased Jak2 expression, showing that SOCS2 is capable of this classical function in HNSCC cells.

SOCS2 expression is dependent upon STAT5 (30). There are at least 5 STAT5A-binding sites in the SOCS2 promoter (intron 1; www.cbrc.jp/research/db/TFSEARCH.html; ref. 31). STAT5A and STAT5B share similar binding sequences (31). Given the high level of homology between STAT5A and STAT5B, it is not clear how the 2 isoforms could be differentially regulating SOCS2 expression based exclusively on sequence data. Another layer of complexity in the regulation of SOCS function is that SOCS2 may compete with or regulate other SOCS proteins. SOCS2 can lead to proteasome-dependent SOCS3 degradation (32). Such a complex system of interregulation may explain why we observed diverse effects on the levels of multiple SOCS proteins in HNSCC cell lines following c-Src inhibition.

Although STAT5A and STAT5B may possess some functional redundancy, their roles in both normal physiology and cancer biology are distinct. Their separate roles in normal physiology are shown by discrete tissue expression patterns, distinct phenotypes of the knockout mice, and different roles in cell signaling [reviewed in (5)]. STAT5 has been studied in multiple cancer types, but the distinction between STAT5A and STAT5B has been examined only infrequently in epithelial tumors (11, 33, 34). STAT5A and STAT5B have differential regulatory roles in HNSCC, breast...
cancer, glioblastoma, and hepatocellular carcinoma (35–39). In HNSCC, STAT5 activation led to increased cell and tumor growth and increased invasion and induced epithelial-to-mesenchymal transition (17). Activated and total STAT5B, but not STAT5A, was found to increase in HNSCC tumors compared with normal-appearing mucosa. Likewise, in a xenograft model of HNSCC, STAT5B antisense was found to inhibit tumor growth in mice, whereas STAT5A antisense did not affect tumor size (40). Cells containing a dominant-negative STAT5B construct fail to proliferate in vitro (11). Erythropoietin mediates invasion in HNSCC through the activation of STAT5A; STAT5A did not promote tumor proliferation (41). These studies support a role for STAT5B, but not STAT5A, in the progression of HNSCC. Although we did not study the differential roles of STAT5A and STAT5B in HNSCC cells with unperturbed c-Src, our model would support a role for STAT5A as a tumor suppressor (by driving SOCS2 expression and subsequent suppression of Jak2–STAT3 activation). Also consistent with the finding that STAT5B promotes HNSCC cancer progression, we found that activation of STAT5B resulted in resistance to c-Src inhibition (Supplementary Fig. S3A). Although STAT5 contributes to the progression of HNSCC, activation of STAT5 correlates with improved survival in breast cancer, where it may promote differentiation rather than progression (36, 42).

Our study has shown that STAT3 and STAT5 are regulated independently. STAT5 activity was predominantly dependent upon c-Src, as the reactivation of Jak activity did not result in STAT5 reactivation. In contrast, STAT3 activation was predominantly Jak dependent, as STAT3 was reactivated in the presence of c-Src inhibition (9). Moreover, acute c-Src inhibition alone did not result in complete STAT3 inhibition unless SOCS2 was present (Fig. 3B). Jak2 are the classic regulators of STAT5 and STAT3, but they are not the only kinases that can do so. ErbB receptor-induced activation of STAT1, STAT3, and STAT5 was found to be mediated by c-Src and independent of Jak (43). Likewise, c-Src can directly phosphorylate STAT5A (Y694) and activate STAT3 (44–46). c-Src can activate STAT5B directly by phosphorylation or indirectly by phosphorylating EGFR (Y845; ref. 47). In HNSCC specifically, c-Src inhibition using both molecular and pharmacologic agents leads to STAT3 and STAT5 inhibition downstream of EGFR (6). EGFR possesses a STAT-binding capacity and can activate STATS in a Jak-independent manner (48, 49). EGFR, though an important mediator of both c-Src and STAT3 activation in HNSCC, does not function in STAT3 reactivation following sustained c-Src inhibition (10). The functions of Jak2, c-Srcs, and growth factor receptors are not independent, as they can cooperate to enhance STAT3 activation during oncogenesis (47, 50).

One unanswered question is what mechanism leads to Jak kinase inhibition. Our previous studies showed that c-Src inhibition led to a rapid and significant inhibition of Jak kinase activity (9). However, Jak is not a known c-Src substrate. Another unresolved issue is the potential role for a cytokine or growth factor receptor as a scaffold for the Jak2/STAT3/SOCS2 complex. Although there is no role for a soluble growth factor or cytokine in this feedback loop (ref. 10 and Supplementary Fig. S2) and our previous work did not support the role for the kinase activity of a growth factor receptor (10), these experiments do not preclude the role of such a receptor as a scaffold for the complex. Future studies will be needed to address these issues.

Our study could have a direct clinical application. We have found STAT3 reactivation in cell lines from lung cancer, mesothelioma, and squamous carcinoma of the skin (10, 51). We have also observed STAT3 reactivation in vivo, after specific c-Src knockdown and using 3 different pharmacologic inhibitors (9, 10, 12); the combination of c-Src and Jak inhibitors leads to significant cancer cell apoptosis in vivo. The reciprocal regulation of c-Src and STAT3 activation in tumors from patients with lung cancer suggests that this pathway functions in human tumors (12). These results show that STAT3 reactivation is likely to occur in patients with a broad range of cancers that are treated with any c-Src inhibitor. Specific and potent kinase inhibitors of c-Src and Jak are well tolerated in humans (1, 5). Specific SOCS mimetics are being developed and may be more specific and presumably less toxic than Jak inhibitors (52). STAT3 inhibitors also are being developed, but none have completed clinical trials (5).

Despite the finding of c-Src expression in epithelial tumors and the availability of agents to sustain its inhibition, the effects of c-Src inhibition on cell survival and proliferation have been moderate and inconsistent. c-Src mediates its effects on cancer cell survival and proliferation via diverse substrates including STATS. We have discovered a heretofore unknown compensatory pathway culminating in STAT3 reactivation and cancer cell survival. Our long-term goal is to use these results to design clinical trials combining these or other more specific c-Src inhibitors with Jak2 or STAT3 inhibitors or SOCS mimetics to improve the survival of patients with HNSCC and other cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

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