The Opposing Function of STAT3 as an Oncoprotein and Tumor Suppressor Is Dictated by the Expression Status of STAT3β in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: STAT3 is known to have both oncogenic and tumor suppressive effects, but the regulation of these opposing effects is elusive. We hypothesized that STAT3β, one of the two STAT3 isoforms, is the key determinant in this context.

Experimental Design: The prognostic significance of STAT3β and phospho-STAT3Y705 (pSTAT3αY705) was evaluated in 286 cases of patients with esophageal squamous cell carcinoma (ESCC). STAT3β-induced changes in the chemosensitivity to cisplatin and 5-fluorouracil were assessed both in vitro and in vivo. STAT3β-induced changes in the frequency of cancer stem cells were evaluated using Hoechst and CD44 staining. How STAT3β regulates STAT3α was determined using immunoprecipitation, confocal microscopy, DNA-binding, and chromatin immunoprecipitation-PCR.

Results: STAT3β expression is an independent protective prognostic marker in patients with ESCC, which strongly correlated with longer overall survival (P = 0.0009) and recurrence-free survival (P = 0.0001). STAT3β significantly decreased the cancer stem cell population, and sensitized ESCC cells to cisplatin and 5-fluorouracil in tumor xenografts. Mechanistically, STAT3β markedly attenuated the transcription activity of STAT3α via inducing STAT3α:STAT3β heterodimers. However, the heterodimer formation decreased the binding between STAT3α and PTPN9 (better known as PTP-MEG2), a protein tyrosine phosphatase, thereby promoting the phosphorylation of STAT3αY705 and enhancing its nuclear translocation and DNA binding. Correlating with this, high STAT3β expression converts the prognostic value of pSTAT3αY705 from unfavorable to favorable in patients with ESCC.

Conclusions: STAT3β suppresses chemoresistance and cancer stemness by blocking the transcriptional activity of STAT3α. The paradoxical increase in pSTAT3αY705 induced by STAT3β carries important implications as to how the biologic and prognostic significance of STAT3 in cancers should be interpreted.

Introductions

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family of transcription factors, is responsible for the signal transduction of a host of extracellular stimuli, such as the IL6 family cytokines and various growth factors (1–3). Upon these stimulations, phosphorylation of STAT3Y705 is essential for the subsequent dimerization, nuclear translocation, and activation of STAT3 (1–3). The oncogenic potential of STAT3 has been well documented; specifically, the constitutively activated STAT3 mutant (commonly labeled STAT3C) can effectively induce malignant transformation (4), and many human cancers harbor constitutively active STAT3 (1–3). Nevertheless, accumulating evidence from both experimental and clinical studies has suggested that STAT3 may also carry a tumor suppressor role in specific contexts (5–14). For instance, in a mouse model of intestine adenoma, tissue-specific STAT3 gene knockout was found to enhance tumor invasion and promote tumor progression (5, 6). STAT3 expression was found to be reduced in the majority of skin squamous cell carcinomas compared with adjacent nonmalignant tissues (8). Moreover, nuclear-STAT3 or pSTAT3Y705 was shown to correlate with a better prognosis in...
Translational Relevance

The oncogenic function of STAT3 has been revealed in various cancers. However, recent studies have revealed a tumor suppressor role of STAT3 in multiple cancer types. In esophageal squamous cell carcinoma (ESCC), we revealed a mechanism that may explain the opposing roles of STAT3 in cancer. We found that although STAT3β (a truncated isoform of STAT3) attenuated STAT3 transcription activity, suppressed the cancer stem cell population, and sensitized ESCC cells to chemotherapy in vivo, it substantially increased the Tyrosine705-phosphorylation, nuclear translocation, and DNA binding/promoter occupation of STAT3α (the full-length and oncogenic isoform of STAT3). In support of these findings, high STAT3β expression converts the prognostic value of pSTAT3α705 from unfavorable to favorable in patients with ESCC. These findings suggest that whether STAT3 is oncogenic or tumor suppressive is largely dictated by STAT3β, which carries important implications as to how the biologic and prognostic significance of STAT3 in cancers should be interpreted.

Patient samples

Human ESCC tumors and adjacent nontumorous esophageal epithelial tissues were collected directly after surgical resection between October 2007 and July 2009, at the Department of Tumor Surgery of Shantou Central Hospital (Shantou, China). The cases were selected based on a clear pathologic diagnosis, follow-up data, and had not received local or systemic treatment before surgery. The frozen tissues, including both the case-paired adjacent nontumorous esophageal epithelial tissues and the tumor samples that were used for the Western blot analysis, were rigorously resected by pathologists to ensure that only the epithelium of the normal esophagus was included in the nontumorous tissues, and that no benign tissues were included in tumor samples. The histologic characterization and clinicopathologic staging of the samples were performed in accordance with the 7th edition of American Joint Committee on Cancer Tumor-Node-Metastasis staging system. Detailed clinical information of the patients with ESCC is described in Supplementary Table S1. The study was approved by the ethical committee of the Central Hospital of Shantou City and the ethical committee of Shantou University Medical College, and written informed consent was obtained from all surgical patients to use resected samples for research.

Cell lines and stable cell cone generation

Human ESCC cell line EC109 was obtained from the Chinese Academy of Medical Sciences, Beijing, China. KYSE150 cell line was kindly provided by Dr. Ming-Zhou Guo, Chinese PLA General Hospital, Beijing, China. Both cell lines have been authenticated using short tandem repeat DNA profiling. The generation of the STAT3β and STAT3C Tetacycline-off stable cell clones was performed according to the protocol provided by the manufacturer of the Tetracycline-off system (Clontech). Briefly, EC109 and KYSE150 cells were first transfected with the pTet-Off vector that expresses tetracycline-controlled transactivator, and stable cell clones were selected by exposure to 400 μg/mL hygromycin B (Invitrogen). Then, these stable clones were transfected with the pTRE2hyg-STAT3β or pTRE2hyg-STAT3C vectors, and double stable cell clones were selected using media containing 400 μg/mL hygromycin B (Invitrogen). EC109 and KYSE150 cells were cultured in DMEM and RPMI-1640 medium supplemented with 10% FBS, respectively.

Animal study

Note that $1 \times 10^8$ KYSE150-TetOff-STAT3β cells pretreated with/without 100 ng/mL doxycycline (Dox) were injected subcutaneously into the flank of male nude mice that were 8 weeks old.
old. The Dox+ group mice were treated with Dox by adding Dox in drinking water at a concentration of 100 ng/mL, whereas no Dox was added for the Dox− group mice. Eight days after tumor cell injection when the xenograft tumors were palpable, Cisplatin (2 mg/kg body weight, Calbiochem, Millipore) or 5-fluorouracil (5-FU; 20 mg/kg body weight, Sigma) were injected intraperitoneally every 3 days. Tumor volumes were measured every 3 days. Twenty-three days after tumor cell injection, the mice were sacrificed, and the tumors were surgically resected. Hematoxylin and eosin (H&E) staining and IHC detection of pan Cytokeratin (ZM-0069, ZSGB-BIO) were used to identify the xenografted tumor cells in mice. The expression of Flag and phospho-STAT3αY705

Figure 1.

The expression and prognostic significance of STAT3β in ESCC. A, STAT3β expression was detected by IHC in seven tumor-adjacent normal esophageal epithelial tissues and 286 ESCC samples. An isotype control was used for the negative control staining in a case of tumor-adjacent normal esophageal epithelial tissue. B, the distribution of ESCC cases that show different STAT3β expression. C, Kaplan–Meier survival analysis was used to evaluate the prognostic value of STAT3β in patients with ESCC (log-rank test). D, multivariate Cox regression analysis for overall survival and recurrence-free survival.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th>Recurrence-free survival</th>
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<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
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<td>STAT3β Mod/strong vs. neg/weak</td>
<td>0.711 (0.511–0.960)</td>
<td>0.043*</td>
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<td>Tumor size &gt;5 cm vs. ≤5 cm</td>
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<td>Histological grade G2 + G3 vs. G1</td>
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<td>Primary tumor T3 + T4 vs. T1 + T2</td>
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<tr>
<td>Lymph node N1 + N2 + N3 vs. N0</td>
<td>1.972 (1.403–2.770)</td>
<td>0.001***</td>
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A. EC198-STAT3-RedIF Cells

B. Dox (ng/mL)

C. pSTAT3

D. S-PU

E. NC sSTAT3

F. Vangameli

G. Day 0

H. S-PU treatment

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were detected by IHC using antibodies against Flag (Sigma; F3165) and phospho-STAT3 (9258) (Cell Signaling; #9145), respectively.

Chromatin immunoprecipitation coupled to detection by PCR (ChIP-PCR)

The EZ-ChIP kit (Millipore) was used for this assay, and the experiments were performed according to the manufacturer's instructions. Briefly, 48 hours after the transfection of wild-type STAT3-Flag, mutant STAT3-Y705F-Flag, or empty vector into the EC109 cells, 1 x 10^6 cells were treated with 1% formaldehyde to cross-link proteins to DNA, and the cell lysates were sonicated to shear the DNA into lengths between 200 and 1,000 bp. The sheared DNA fragments were immunoprecipitated with antibodies against STAT3 (Cell Signaling; 1:50), Flag (Sigma; 1:50), or mouse IgG provided by the ChIP kit. The primers used for the PCR amplification of the PLK1 promoter region containing the STAT3 binding sites were: Primer 1, forward: 5'-GAAGTCCTTCGTTGCACTCATGG-3'; reverse: 5'-CITCCGCTCACTTGCCGCTC-3'; Primer 2, forward: 5'-GCCCTCGTGTCAATCAGGTT-3'; reverse: 5'-CCTGACGTCACCTGAGCTC-3'.

Flow cytometry analysis

Cell apoptosis was determined by the propidium iodide and Annexin V double staining assay. Briefly, cells were gently dissociated with trypsin and stained with propidium iodide and Annexin V using the Annexin V-FTC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. For side population cell identification, a previously described method was used (26). For CD44 detection, cells were gently dissociated with trypsin and stained with APC-conjugated CD44 antibody (BD pharmingen, 1:25) or mouse IgG provided by the ChIP kit. The primers used for the PCR amplification of the PLK1 promoter region containing the STAT3 binding sites were: Primer 1, forward: 5'-GAACGGTCCTTCGTTGCACTCATGG-3'; reverse: 5'-CITCCGCTCACTTGCCGCTC-3'; Primer 2, forward: 5'-GCCCTCGTGTCAATCAGGTT-3'; reverse: 5'-CCTGACGTCACCTGAGCTC-3'.

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Statistical analysis

The statistical analyses were performed using either the SPSS V.13.0 statistical software package or the Graphpad Prism 6. To determine the differences between two independent groups of samples, Student t test was used. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The statistical analyses were performed using either the SPSS V.13.0 statistical software package or the Graphpad Prism 6. To determine the differences between two independent groups of samples, Student t test was used. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The statistical analyses were performed using either the SPSS V.13.0 statistical software package or the Graphpad Prism 6. To determine the differences between two independent groups of samples, Student t test was used. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The statistical analyses were performed using either the SPSS V.13.0 statistical software package or the Graphpad Prism 6. To determine the differences between two independent groups of samples, Student t test was used. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test.

Results

High STAT3 expression correlates with a favorable prognosis in patients with ESCC

To our knowledge, the prognostic significance of STAT3 in human cancers has never been assessed. Using IHC and a STAT3-specific monoclonal antibody (29), we surveyed STAT3 expression in a cohort of 286 ESCC tumors. As shown in Fig. 1A, 28 (9.8%) tumors expressed high levels of STAT3, whereas 128 (44.8%) and 130 (45.5%) tumors expressed negative/weak and moderate STAT3 levels, respectively (Fig. 1B). Benign epithelia adjacent to the tumors strongly expressed STAT3 in the suprabasal layer but negative in the basal layer (Fig. 1A). Kaplan–Meier analysis revealed that moderate/strong expression of STAT3 significantly correlated with a longer overall survival (P = 0.0009) and recurrence-free survival (P = 0.0001; Fig. 1C). Moreover, among the 95 patients with ESCC who received radio-chemotherapy, patients with tumors expressing moderate/strong STAT3 had a significantly longer overall survival (P = 0.005) and recurrence-free survival (P = 0.006), as compared with those with tumors expressing negative/weak levels of STAT3 (Fig. 1C). Furthermore, as shown in Supplementary Table S2, tumors with moderate/strong STAT3 expression were significantly less likely to have lymph node metastasis (P < 0.001) and correlated with a low clinical stage (P < 0.001). Multivariate Cox regression analysis revealed that moderate/strong expression of STAT3 is a significant independent protective factor for both overall survival [HR = 0.711, 95% confidence interval (CI) = 0.511–0.990, P = 0.043] and recurrence-free survival [HR = 0.708, 95% CI = 0.515–0.974, P = 0.034; Fig. 1D].

STAT3 decreases the cancer stem cell population and sensitizes ESCC cells to chemotherapy in vitro

The prognostic significance of STAT3 suggests that STAT3 is a tumor suppressor in ESCC. To delineate the mechanism, we employed EC109 and KYSE150 cells that had been stably transfect with STAT3 cloned in a conditional expression vector (i.e., the tetracycline-off system), and these cells were labeled EC109-STAT3 TetOff and KYSE150-STAT3 TetOff, respectively. As shown in Fig. 2A and Supplementary Fig. S1A and S1B, enforced expression of STAT3 (i.e., no doxycycline treatment) in EC109 cells significantly decreased the clonogenic capacity and increased their sensitivity to 5-FU and cisplatin in a STAT3 dose-dependent manner (P < 0.05). Similar observations were made with KYSE150-STAT3 TetOff cells (Supplementary Fig. S1C). To determine whether the tumor suppressor function of STAT3 was mediated via antagonizing the oncogenic function of STAT3, the relative expression of STAT3b and STAT3b were modulated using STAT3 siRNA and doxycycline, respectively, in the same two cell lines. As shown in Fig. 2C and D and Supplementary Fig. S2A, high
expression of STAT3β (i.e., no doxycycline treatment) dramatically decreased the clonogenic ability and markedly enhanced the chemosensitivity of ESCC cells to 5-FU and cisplatin, which was comparable to the effects of STAT3α knockdown.

Because extensive studies have linked cancer stem cells to chemoresistance (30), we therefore determined the impact of STAT3β on the cancer stem cell population of ESCC cells. As shown in Fig. 2E, cells with enforced expression of STAT3β (no doxycycline treatment) or STAT3α knockdown formed approximately 2-fold less tumorspheres compared with the negative controls (P < 0.01). Furthermore, the number of cells per tumorsphere was significantly decreased (~3.5-fold) in response to either treatment (P < 0.001; Fig. 2E). To directly quantify cancer stem cells, we performed Hoechst and CD44 staining, as reported in previous studies (26, 31). As shown in Fig. 2F, enforced expression of STAT3β and STAT3α knockdown significantly decreased the Hoechst-negative cancer stem cells from 1.12% to 0.46% and 0.63%, respectively. Similarly, CD44

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**STAT3β sensitizes ESCC cells to chemotherapy in vivo**

The tumor suppressor function of STAT3β was further tested in nude mice xenografted with KYSE150-STAT3-TetOff cells. As illustrated in Fig. 2G, xenograft formation was detectable consistently on day 8, after which multiple doses of 5-FU or cisplatin was injected into these animals intraperitoneally. We observed significant differences in tumor growth rate between animals that received doxycycline (i.e., low level of STAT3β) and those that did not (i.e., high level of STAT3β) from day 14 for 5-FU and day 17 for cisplatin (Fig. 2G). Under both chemotherapy regimens, xenografts with high STAT3β expression shrank significantly faster than those with low STAT3β expression (P < 0.05; Fig. 2G). The histologic features and the expression status of STAT3β (detectable by FLAG antibody) in these xenografts are shown in Fig. 2H.

**STAT3β enhances the Tyrosine705 phosphorylation and nuclear translocation of STAT3α, which is dependent on the Tyrosine705 residue in STAT3β**

Despite the potent tumor suppressor function of STAT3β as shown above, we observed that enforced expression of STAT3β (controlled by doxycycline) substantially increased pSTAT3αY705 both in vitro (Fig. 2A and C and Supplementary Fig. S1C) and in vivo (Fig. 2H). To further validate these findings, wild-type STAT3β cDNA, mutant STAT3βY705F cDNA, and STAT3 siRNAs were transiently transfected into EC109 and KYSE150 cells to manipulate the relative expression of STAT3α and STAT3β. Consistently, we found that STAT3β transfection again increased pSTAT3αY705 levels (Fig. 3A), and both enforced expression of STAT3β and STAT3α knockdown significantly decreased the chemoresistance and the clonogenic ability in both the cell lines (P < 0.01; Fig. 3A and Supplementary Fig. S3). However, Y705F mutation in STAT3β significantly abrogated its tumor suppressor effect (P < 0.05). These findings suggest that the tumor suppressor effects of STAT3β may be related to its interaction with STAT3α, and Tyrosine705 is an important residue for the function of STAT3β.

Thus, we examined the interplay between these two isoforms closely. As shown in Fig. 3B, enforced expression of STAT3β promoted a more sustained STAT3αY705 phosphorylation upon oncostatin M (OSM) stimulation, as compared with cells transfected with an empty vector. Specifically, although the expression of pSTAT3αY705 diminished to a faint level at 60 minutes after OSM exposure in empty vector-transfected cells, the pSTAT3αY705 signal in STAT3β-transfected cells was maintained at a high level for at least 120 minutes after OSM stimulation. This effect of STAT3β was found to be dependent on its Tyrosine705 residue, as replacement by phenylalanine at this site almost completely abrogated the observed effects (Fig. 3B).

Further studies using the subcellular fractionation experiment showed that the high level of pSTAT3αY705 induced by STAT3β was evenly distributed between the cytoplasm and nucleus (Fig. 3C). Furthermore, even in the absence of OSM stimulation, STAT3β transfection led to a substantial increase in the expression of pSTAT3αY705 in ESCC cells, and the STAT3β-induced pSTAT3αY705 was again evenly distributed between the cytoplasm and nucleus (Fig. 3C). Results from confocal microscopy analysis were in line with these interpretations (Fig. 3D).

Finally, in support that the enhancing effect of STAT3β on pSTAT3αY705 also occurs in patient samples, Western blot studies revealed a significant correlation between STAT3β and pSTAT3αY705 expression in our cohort of ESCC samples (n = 91, P = 0.019; Fig. 3E and Supplementary Fig. S4).

**STAT3β protects pSTAT3αY705 from dephosphorylation by protein tyrosine phosphatases (PTP)**

The molecular mechanism underlying the enhancing effect of STAT3β on the expression of pSTAT3αY705 remains unclear. We hypothesized that STAT3β could interfere with the interaction between PTPs and STAT3α. To this end, we selected PTP-MEG2, a recently identified PTP that specifically interact with STAT3 to dephosphorylate pSTAT3Y705 in breast cancer cells (32). As shown in Fig. 4A, compared with empty vector transfection, STAT3β dramatically decreased the interaction between STAT3α and PTP-MEG2, whereas Y705F mutation of STAT3β diminished the effect exerted by STAT3β. Results obtained from the confocal microscopy studies are in keeping with our model (Fig. 4B). Specifically, co-localization between STAT3α and PTP-MEG2 was observed in empty vector transfected cells, whereas this interaction was abolished in STAT3α transfected cells. In comparison, transfection of the STAT3βY705F mutant was not able to reduce this co-localization (Fig. 4B).

**STAT3β enhances the DNA binding of STAT3α by forming heterodimers, whereas the transcriptional activity of STAT3α was decreased by STAT3β**

The tumor suppressor role of STAT3β and the enhancing effect of STAT3β on the oncogenic pSTAT3αY705 prompted us to determine how STAT3β influences the function of STAT3α in ESCC cells. First, reciprocal co-immunoprecipitation experiment showed that STAT3β was able to form heterodimers with STAT3α, and this interaction was enhanced by OSM stimulation (Fig. 5A). In comparison, the functional defective STAT3βY705F mutant could not effectively form heterodimers with STAT3α (Fig. 5A), suggesting that the heterodimerization is required for the function of STAT3β. We then validated this finding using confocal microscopy (Fig. 5B). Specifically, upon OSM stimulation, transfected STAT3β translocated to the nuclei and overlapped with the nuclear-STAT3α, whereas the STAT3βY705F mutant was largely
localized in the cytoplasm and showed no substantial co-localization with STAT3α.

We then assessed how STAT3β affects the transcriptional activity of STAT3α. A STAT3 reporter was used, and luciferase activity served as the readout. Because STAT3α but not STAT3β carries the domain responsible for transcription regulation, the luciferase activity detectable should reflect the transcription activity of STAT3α alone. As shown in Fig. 5C and Supplementary Fig. S5, Table E shows the statistical analysis of the correlation between STAT3β and pSTAT3α in ESCC samples (n = 91).
although transfection of STAT3b markedly enhanced STAT3αY705 phosphorylation, it dramatically decreased the transcriptional activity of STAT3 as compared with negative control (P < 0.001). Again, the Y705F mutation significantly diminished the effect of STAT3b (P < 0.01). Similar results were observed when these two cell lines were stimulated with OSM (Fig. 5C and Supplementary Fig. S5). To further validate these observations, we repeated the same experiments using EC109-STAT3C-TetOff cells. As shown in Fig. 5C, doxycycline treatment effectively suppressed STAT3C expression and decreased the STAT3 transcriptional activity by approximately 40% (P < 0.01). In comparison, although transfection of STAT3b increased pSTAT3αY705, the STAT3 transcriptional activity was decreased by >80% (P < 0.001).

We then asked whether the decreased STAT3 transcriptional activity induced by STAT3b was caused by a reduction in STAT3 DNA-binding ability. A pull-down experiment using a probe that contains the STAT3 consensus DNA-binding site was performed. Surprisingly, we found that STAT3b markedly promoted the DNA-binding of STAT3α, compared with the negative control and STAT3bY705F (Fig. 5D). Moreover, STAT3b was also able to effectively bind to the DNA probe. To determine whether STAT3b can enhance the occupancy of STAT3α in the promoter region of its downstream target genes, ChIP-PCR was performed. PLK1, a reported oncogenic mediator of STAT3 in ESCC (25), was selected for this experiment. As shown in Fig. 5E, we found that transfection of wild-type STAT3b but not the mutant STAT3bY705F markedly increased the occupancy of both STAT3α and STAT3β (FLAG-tagged) in the PLK1 promoter. However, enforced expression of STAT3b dramatically decreased the expression of PLK1 to an extent that was comparable to STAT3α knockdown in both parental and STAT3β-TetOff cells (Fig. 5F). Again, Y705F mutation in STAT3β abrogated its function in suppressing PLK1 expression (Fig. 5F). Taken together, these data indicate that the transcriptional activity of STAT3α was decreased by STAT3β, although the DNA binding of STAT3α was enhanced by STAT3β via forming heterodimers.

**STAT3β determines the prognostic significance of pSTAT3αY705 in patients with ESCC**

Our collected data indicates that STAT3β inhibits the transcriptional activity and oncogenic function of STAT3α, although it "paradoxically" increases the level of pSTAT3αY705. Thus, a high level of pSTAT3αY705 indicates a tumor suppressive environment when STAT3β is expressed, whereas it indicates an oncogenic environment when STAT3β is negative/weak. Using Western blots, we assessed the relative expressions of STAT3α and STAT3β in 91 frozen tumors, which overlapped with the initial cohort of 286 cases mentioned above (Fig. 6A and Supplementary Fig. S4). In seven pairs of the randomly chosen

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**Figure 4.**

STAT3β protects pSTAT3αY705 from dephosphorylation. A and B, transfected EC109 cells were stimulated with OSM for 45 minutes, and co-immunoprecipitation (co-IP) and confocal microscopy were performed to determine the interaction between PTP-MEG2 and STAT3α. Scale bars, 10 μm.

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samples from our cohort, six tumors showed a dramatic increase in fascin expression compared with the case-matched benign tissues, supporting the validity of our paired ESCC samples in this cohort (Fig. 6A). As shown in Fig. 6A, the expression of STAT3β significantly correlated with the STAT3β immunoreactivity illustrated in Fig. 1 \( (P = 0.0001) \), supporting the validity of our methodology. Results of the expression status of STAT3α and STAT3β, as well as their phosphorylated forms, are summarized in Fig. 6B.

In support of our model, a high level of pSTAT3α\(^{Y705}\) significantly correlated with a longer overall survival in patients with STAT3β-high tumors \( (n = 45, P = 0.039; \) Fig. 6C). In STAT3β-low tumors \( (n = 46) \), although pSTAT3α\(^{Y705}\) did not significantly correlate with the overall survival \( (P = 0.802; \) Fig. 6C), we noted
Figure 6.
STAT3β determines the prognostic significance of pSTAT3α\(^{Y705}\) in patients with ESCC. A, representative Western blots showing the expression of pSTAT3α/β\(^{Y705}\) and STAT3α/β in 7/91 pairs of samples (T, tumor; N, adjacent nontumorous tissue). The complete data and details are described in Supplementary Fig. S4. B, left, STAT3β expression detected by Western blot correlated with STAT3β immunoreactivity illustrated in Fig. 1. Right, the expression status of pSTAT3α/β\(^{Y705}\) and STAT3α/β as detected by Western blot. C, Kaplan-Meier curves showing the prognostic value of pSTAT3α\(^{Y705}\) in STAT3β-High and STAT3β-Low group patients. The correlation between pSTAT3α\(^{Y705}\) and survival time in STAT3β-Low group patients was analyzed. D, Kaplan-Meier curves showing the prognostic value of pSTAT3α\(^{Y705}\) in the entire group of patients (n = 91). E, a schematic model showing how the interplay between STAT3α and STAT3β dictates the dual role of STAT3 in cancer.
that all patients with pSTAT3Y705-high tumors did not survive more than 41 months follow-up, whereas a good number of patients with pSTAT3Y705-low tumors survived past 41 months (P = 0.031, Fisher exact test; Fig. 6C). Finally, in this entire group of 91 patients, pSTAT3Y705 level showed a trend toward a better outcome (P = 0.082; Fig. 6E), which can be attributed to the paradoxical enhancing effect of STAT3β on the oncogenic pSTAT3αY705.

**Discussion**

One of the key findings of this study is that STAT3β is a tumor suppressor in ESCC, as evidenced by the observation that enforced expression of STAT3β in ESCC cells significantly reduced colony formation, increased chemosensitivity, and suppressed cancer stem cell populations. Importantly, we have delineated the key mechanisms underlying the tumor suppressor effects of STAT3β, especially in the aspects of how STAT3β interacts with STAT3α biochemically and functionally. Our data support the hypothetical model illustrated in Fig. 6E. Specifically, upon cytokine (e.g., OSM) stimulation, at least two types of STAT3 dimers are formed, that is, pSTAT3α:pSTAT3α homodimer and pSTAT3α:pSTAT3β heterodimer. Compared with the pSTAT3α:pSTAT3α homodimer, the pSTAT3α:pSTAT3β heterodimer is theoretically more stable due to the absence of the negatively charged C-terminal region in STAT3β, which has been shown to increase the stability of STAT3β homodimers (17, 34). Because only monomerized pSTAT3α can be subjected to dephosphorylation by PTPs (17), the stabilizing effect of pSTAT3α:pSTAT3β heterodimer blocks the translocation and dephosphorylation by PTPs. Thus, a much larger amount of pSTAT3 (as heterodimers) is preserved, which is distributed in both the cytoplasm and the nucleus. Nevertheless, the oncogenic function of the transcriptionally active pSTAT3α is sequestered in this large pool of pSTAT3 heterodimers that are still capable of binding DNA. Therefore, in the presence of sufficient STAT3β, STAT3α signaling is retarded, although the amount of pSTAT3Y705 is markedly increased by STAT3β. In comparison, in tumors in which STAT3β is down-regulated, although pSTAT3Y705 expression is relatively less, the transcriptionally active and oncogenic STAT3α are relatively unchecked. In other words, whether STAT3 is oncogenic or tumor suppressive is largely dictated by the expression status of STAT3β.

The tumor suppressor function of STAT3β has not been extensively studied or published. In addition to its dominant negative effect on STAT3α, it is believed that STAT3β also may regulate the expression of a unique gene set (15, 23, 35, 36). Nonetheless, the mechanism underlying tumor suppressor effects of STAT3β is not completely understood, and the concept is challenging, because at least two studies have shown that STAT3β promotes leukemogenesis and the progression of liver cancer (37, 38). Regarding the evidence supporting the tumor suppressive effects of STAT3β, we have identified only 11 studies in the literature that have discussed about this subject to varying extents. In 7 of these 11 studies, the STAT3β construct was only used as an experimental tool to block STAT3α, believed to act in a dominant negative fashion (21, 22, 23, 43). In the remaining four studies, the main objective was to evaluate how effective STAT3β is as a tumor suppressor (19, 20, 23, 44). Notably, only one of these studies demonstrates that STAT3β directly decreases the transcriptional activity of STAT3α (41). Besides these 11 studies in cancer cells, we are aware of 3 other studies that had evaluated the impact of STAT3β on the transcriptional activity of STAT3α in COS cells: one of these studies revealed that STAT3β significantly decreased the transcriptional activity of STAT3α (16), whereas two other studies described that STAT3β could increase the transcriptional activity of STAT3α (15, 17).

Although our data strongly support that STAT3β is a tumor suppressor in ESCC, we found that this isoform paradoxically increases STAT3αY705 phosphorylation. A similar finding has been reported in one study using murine embryonic fibroblasts (35), but our present study is the first to address this phenomenon in cancer cells and to delineate the underlying mechanisms (Fig. 6E). Importantly, our findings may explain the accumulating controversies regarding the tumor suppressor function of STAT3α in various cancer types (5–14). For instance, there are many publications in which the immune-detection of pSTAT3Y705 or nuclear STAT3 was found to significantly correlate with a better outcome (9–14). The explanations for these seemingly discordant results have not been satisfactory or proven. In our recent review of STAT3 in cancer, we have discussed about the hypothesis that the expression status of STAT3β in cancer cells is a key determinant of the exact biologic effects of STAT3 (3). Thus, in STAT3β-negative/weak tumors, activation of STAT3 increases pSTAT3αY705, which exerts potent oncogenic effects. In contrast, in STAT3β-high tumors, although pSTAT3αY705 is dramatically augmented by STAT3β, the overall oncogenic effects of STAT3α are indeed suppressed by STAT3β. In other words, without the distinction between the two STAT3 isoforms, as in the case of virtually all previously published clinicopathologic studies of STAT3, it is perceivable that one may conclude that a high expression level of pSTAT3αY705 or total pSTAT3αY705 is oncogenic if the vast majority of the tumors in the study cohort are STAT3β-negative/weak. Alternatively, one may conclude that pSTAT3αY705 or total pSTAT3αY705 is tumor suppressor or carries no significance if a substantial proportion of the tumors in the study cohort are STAT3β-high. In support of this model, in our current study, in which 55.2% (158/286) of the tumors carried a relatively high level of STAT3β (i.e., moderate/strong STAT3β staining) as assessed by IHC, pSTAT3αY705 correlated with longer survival (Fig. 6D). Thus, in the presence of high STAT3β expression, the amount of pSTAT3αY705 likely reflects the stabilizing effects of STAT3β on pSTAT3αY705, rather than an authentic indicator of the transcriptionally active and oncogenic pSTAT3αY705, and prognostic value of STAT3 in cancer can be fully assessed only if expression status of both STAT3α and STAT3β is known.

Although our data show that the Y705 residue is important for STAT3β in attenuating the biologic function of STAT3α, the STAT3βY705F mutant could still decrease the transcription activity of STAT3α (Fig. 5C). This observation can be attributed to its dominant negative effect on STAT3α, which is similar to the mechanism reported for the dominant negative STAT3αΔY705 mutant (45). Specifically, like the STAT3αΔY705 mutant, the intact SH2 domain in STAT3βΔY705 can compete with STAT3α for the binding to the tyrosine phospho-peptides on gp130, thereby hampering the phosphorylation and activation of STAT3αΔY705 by JAKs. In support of this hypothesis, our data show that transfection of the STAT3βΔY705 mutant appreciably decreased the expression of pSTAT3αY705 (Fig. 5C), which may explain the decrease of STAT3α transcription activity by this STAT3β mutant.

STAT3 has been shown to regulate cancer cell stemness in several types of cancers, including breast cancer, glioblastoma, and ESCC (3, 26). For instance, in ESCC, the JAK2/STAT3 pathway
was shown to increase the side population cells and CD44High cells (26), two subsets of ESCC cells that have been demonstrated to have cancer stem cell features (26, 31). Our study is the first to show that STAT3β suppresses cancer stemness, as evidenced by the decrease of both side population cells and CD44High cells. This finding is expected, as our data show that STAT3β suppresses the transcription activity and antagonizes the oncogenic function of STAT3α in the ESCC model. Recently, a study reported that morpholinos, a type of artificial small molecules, was able to modulate the STAT3 alternative splicing process to favor the generation of STAT3β at the expense of STAT3α (23). Given that STAT3β opposes the oncogenic role of STAT3α in cancer stemness, these small molecules can be potentially used as a powerful tool to target cancer stem cells.

In conclusion, we have presented data supporting that STAT3β is a tumor suppressor in ESCC, and STAT3β can effectively suppress the oncogenic effects of STAT3α. Our results support the model that STAT3α can be both oncogenic and tumor suppressive, and the expression status of STAT3β is the key regulator of this dual role. Our study has highlighted the importance of interpreting the prognostic value of pSTAT3Tyr705 with the knowledge of the expression status of STAT3β.

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

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