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Antiproliferative Mechanisms of a Transcription Factor Decoy Targeting Signal Transducer and Activator of Transcription (STAT) 3: The Role of STAT1

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ABSTRACT

We previously developed a transcription factor decoy targeting signal transducer and activator of transcription 3 (STAT3) and reported antitumor activity in both in vitro and in vivo models of squamous cell carcinoma of the head and neck (SCCHN). Based on the known existence of STAT1-STAT3 heterodimers, the high sequence homology between STAT1 and STAT3, as well as expression of both STAT1 and STAT3 in SCCHN, we examined whether the STAT3 decoy interferes with STAT1 signaling. SCCHN cell lines with different STAT1 expression levels (but similar STAT3 levels) were used. Both cell lines were sensitive to the growth-inhibitory effects of the STAT3 decoy compared with a mutant control decoy. Intact STAT1 signaling was demonstrated by interferon-γ (IFN-γ)-mediated induction of STAT1 phosphorylation (Tyr701) and interferon-regulatory factor-1 (IRF-1) expression. Treatment with the STAT3 decoy (but not a mutant control decoy) resulted in inhibition of IRF-1 protein expression in both cell lines, indicating specific inhibition of STAT1 signaling by the STAT3 decoy. Because STAT1 is a potential tumor suppressor, we also investigated whether STAT1 signaling mitigated the therapeutic efficacy of the STAT3 decoy. In both PCI-15B and UM-22B cells, STAT1 siRNA treatment resulted in decreased STAT1 expression, without altering the antitumor activity of the STAT3 decoy. Likewise, the antitumor effects of the STAT3 decoy were not altered by STAT1 activation upon IFN-γ treatment. These results suggest that the therapeutic mechanisms of STAT3 blockade using a transcription factor decoy are independent of STAT1 activation.

Signal transducer and activator of transcription 3 (STAT3) has emerged as a potential molecular target for cancer therapy. STAT3 is constitutively activated and overexpressed in a variety of human malignancies, including breast, lung, prostate, brain, leukemia, multiple myeloma, and squamous cell carcinoma of the head and neck (SCCHN) (Grandis et al., 1998; Turkson and Jove, 2000). The expression levels of activated or tyrosine-phosphorylated STAT3 have been reported to correlate with decreased survival in several cancers, including oral tongue carcinoma (Masuda et al., 2002). Molecular targeting of STAT3 using a variety of strategies in preclinical models of human cancer has been shown to inhibit tumor growth (Turkson and Jove, 2000). We previously developed a transcription factor decoy based on the STAT3 DNA binding element and demonstrated that this decoy interferes with STAT3 signaling and decreases SCCHN tumor growth in vitro and in vivo (Leong et al., 2003; Xi et al., 2005).

Transcription factor decoys are double-stranded DNA oligonucleotides that resemble the transcription factor-binding site in the promoters of target genes. Transcription factor decoys presumably bind transcription factors and sequester the targeted transcription factor, rendering it unavailable for transcription of downstream target genes. The sequence of the STAT3 decoy was derived from the serum-inducible element of the human c-fos promoter. The therapeutic effects of the STAT3 decoy have also been demonstrated by another group in a chemically induced skin carcinogenesis model

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ABBREVIATIONS: STAT, signal transducer and activator of transcription; SCCHN, squamous cell carcinoma of the head and neck; IFN, interferon; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; hSIE, high-affinity serum inducible element; TBST, Tween 20 in 1× PBS; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; RLU, relative light units; IRF, interferon-regulatory factor; MEF, murine embryonic fibroblast.

(Chan et al., 2004) as well as in psoriasis, where STAT3 hyperactivation plays a major role (Sano et al., 2005). The regulation of STATs and the role of STAT proteins in carcinogenesis remain incompletely understood. In theory, targeting STAT3 using a transcription factor decoy approach might also affect the function of STAT3-associated proteins. Elucidation of the antitumor mechanisms of a STAT3 transcription factor decoy is necessary to optimize the design of clinical studies using this strategy to inhibit STAT3 signaling.

The protein sequence of STAT1 is 72% homologous with STAT3, and STAT1 has been shown to form heterodimers with STAT3. In contrast to the growth stimulatory and antiapoptotic functions of STAT3, STAT1 is generally recognized to have a tumor suppressor function (Bromberg et al., 1996, 1998; Chin et al., 1997; Thomas et al., 2004; Xi et al., 2006). Given the frequent expression of both STAT1 and STAT3 in cancers, including SCCHN, we examined the effects of the STAT3 decoy on STAT1 signaling and the potential role of STAT1 in mediating the antitumor effects of the decoy in SCCHN. Our results demonstrate that although the STAT3 decoy disrupts STAT1 signaling, the therapeutic efficacy of the decoy is independent of STAT1 activation or expression levels.

Materials and Methods

Plasmids and Reagents. The γ-activated sequence-containing luciferase reporter plasmid, pGAS-Luc, was purchased from Stratagene (La Jolla, CA). Human interferon-γ (IFN-γ) was obtained from Roche Applied Science (Indianapolis, IN). Antibodies against STAT1, phospho-STAT1 (Tyr701), STAT3, or phospho-STAT3 (Tyr 705) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against IRF-1 (C-20) and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA) and Oncogene Science (Cambridge, MA), respectively. β-Tubulin antibody was obtained from Abcam Inc. (Cambridge, MA). STAT1 (M-22) and STAT3 (C-20) antibodies used for electrophoretic mobility shift assay were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence kit was purchased from Santa Cruz Biotechnology, Inc. Transfection reagents, Lipofectamine 2000, and Optifect were purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Generation of Stable Clones. All head and neck squamous cell carcinoma cell lines (PCI-37A, 1483, PCI-15B, UM-22A, UM-22B) were of human origin (Lin et al., 2007). 1483 was a kind gift from Dr. Gary Clayman (MD Anderson Cancer Center, Houston, TX) and the UM-22A and UM-22B lines were provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). The PCI-37A and PCI-15B lines were created at the University of Pittsburgh (Heo et al., 1989). Cells were maintained in DMEM with 10% heat-inactivated fetal calf serum (Invitrogen) and 1× penicillin/streptomycin mix (Invitrogen) at 37°C with 5% CO_2. STAT3 knockout and wild-type mouse embryonic fibroblasts were provided by Dr. David Levy (New York University School of Medicine, New York, NY) and were maintained in DMEM with 10% heat-inactivated fetal calf serum (Invitrogen) and 1× penicillin/streptomycin mix (Invitrogen) at 37°C with 5% CO_2 (Lee et al., 2002). STAT5A/B knockout and wild-type mouse embryonic fibroblasts provided by Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN) were grown in DMEM with 10% heat-inactivated fetal calf serum and 1× penicillin/streptomycin mix at 37°C with 5% CO_2 (Teglund et al., 1998). U3A cells that do not express STAT1 were provided by Dr. Jacqueline Bromberg (Memorial Sloan Kettering Cancer Center, New York, NY). U3A cells were cultured in DMEM containing 10% Cosmic calf serum (Hyclone, Logan, UT) and 1× penicillin/streptomycin mix at 37°C with 5% CO_2 (Muller et al., 1993). For the generation of stable clones, UM-22B cells were transfected with pGAS-Luc (Stratagene, La Jolla, CA) or pIRF-1-Luc (Panomics Inc., Redwood City CA) and cotransfected with pcDNA3.1(+) carrying a G418 selection marker. Two days after transfection, cells that stably expressed luciferase were selected by G418 treatment (2 mg/ml) and stable clones were expanded. Expression of luciferase in these clones stably expressing either pGAS-Luc or pIRF-1-Luc was confirmed by luciferase assays.

STAT3 Decoy and siRNA Transfection. The STAT3 decoy and the mutant control decoy sequences (double-stranded deoxyribonucleotides with phosphorothioate modifications in the first three bases and last three bases of the sequences) were generated as described previously (Leong et al., 2003). The mutant control decoy, carrying a single base mutation, was used as a control as in previous studies (Leong et al., 2003; Xi et al., 2005). The DNAs were synthesized and purified using an oligonucleotide purification cartridge (OPC) method by the DNA Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA). STAT1 siRNA SMART pool and STAT3 On-Target Plus SMART pool siRNA was purchased from Dharmacon RNA Technologies (Lafayette, CO). Decoy transfection was performed as described in the manufacturer's manual. In brief, SCCHN cells were plated ($2.5–3 \times 10^5$/well in a six-well tissue culture plate or 0.8×10^5/well in a 24-well tissue culture plate). Eighteen hours after plating, cells were transfected with 102.6 to 1026 pM STAT3 decoy or mutant control decoy as a control. For cytotoxicity studies, the transfection medium was replaced with complete DMEM after 5 h of transfection. For STAT1 signaling studies, IFN-γ was added into the transfection medium 1 h after transfection. For siRNA transfection, 1200 pmol of siRNA was used to transfect a T-75 flask of cells.

Electrophoretic Mobility Shift Assay. UM-22B cell lysate (20 μg) was incubated for 1 h with STAT1 and/or STAT3 antibodies (Santa Cruz Biotechnology). Radiolabeled high-affinity serum-inducible element (hSIE) duplex oligonucleotide or a mutant hSIE duplex oligonucleotide was incubated with the cell extract and antibodies (Wagner et al., 1990). Samples were then run on 4% nondenaturing polyacrylamide gels that were dried at 65°C for 1 h. Supershifted proteins were then visualized by autoradiography.

Western Blotting. Cells were lysed in Western lysis buffer (1% Nonidet-P40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 5 min at 4°C. Lysates were then centrifuged at 4°C, 12,000 rpm for 15 min, and supernatants were collected for protein quantitation. Protein quantitation was performed using the protein assay solution (Bio-Rad Laboratories, Hercules, CA). Proteins (50 μg/lane) were then resolved on 10% SDS-PAGE gels and transferred onto Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) using a semidry transfer machine (Bio-Rad Laboratories). After transfer, membranes were incubated at 4°C overnight in blocking solution containing 5% nonfat dry milk, 0.2% Tween 20 in 1× PBS (TBST). Membranes were then incubated with primary antibody at room temperature for 2 h, then washed three times with TBST (10 min/wash). The membranes were then incubated with secondary antibody for 1 h at room temperature, followed by three washes in TBST. Blots were developed using ECL, according to the manufacturer's instructions (Santa Cruz Biotechnology).

MTT Assay and Cell Counting. To determine survival of SCCHN cells in response to various treatments, MTT assays were performed in 24-well plates. MTT solution was prepared from MTT powder (Sigma, St. Louis, MO) in 1× phosphate-buffered saline (final concentration, 5 mg/ml). Twenty-four hours after the STAT3 decoy treatment, MTT solution was added to each well and incubated at 37°C for 1 h. MTT solution was then removed, and dimethyl sulfoxide (300 μl) was added to each well. The optical density of the contents of each well was determined using a microplate reader set at 570 nm. The percentage cell proliferation was calculated using the following equation: percentage proliferation = (treatment/untreated) × 100%.

Cell counting experiments were performed using trypan blue dye exclusion assay. Cells were trypsinized, and after lifting off of the plate, trypsin was neutralized with DMEM. Cells were centrifuged and pellet was resuspended in fresh media. Cells were then combined with trypan and counted using a hemacytometer. Cell proliferation was then calculated relative to the untreated control using the following equation: percentage proliferation = (treatment/untreated) × 100%.

Luciferase Assay. Stable clones of pGAS-Luc or pIRF-1-Luc were generated in the UM-22B cell line using G418 selection media as described above. Transient transfections of STAT3 decoy and mutant control decoy were performed as described above using 690 pM STAT3 decoy or mutant control decoy. 5 h after transfection, the transfection medium was removed and replaced with DMEM (10% FBS, 1× penicillin/streptomycin) with or without 200 U/ml IFN-γ. After 24 h, cells were lysed in luciferase lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris-HCl at pH 7.8) for 5 min on ice. Lysates were then centrifuged at 14,000 rpm for 5 min at 4°C. Supernatants were collected and assayed for luciferase activity using the luciferase assay kit from Promega (Madison, WI). Luminescence was measured with a luminometer (Wallac Inc., Gaithersburg, MD). Luciferase activity was normalized as relative light units per microgram of total protein in the supernatant. -Fold changes were calculated in reference to the untreated control.

Statistical Analysis. StatXact software with Cytel Studio (Cytel Software Corporation, Cambridge, MA) was used. P values were obtained by the Wilcox-Mann-Whitney test ($P < 0.05$ was considered significant).

Results

STAT3 Decoy Inhibition of SCCHN Growth Does Not Correlate with STAT1 Levels. Given the expression of both STAT1 and STAT3 in SCCHN, we investigated the potential role of STAT1 signaling on the antitumor activity of the STAT3 decoy in SCCHN cell lines. We first examined the expression levels of STAT1 and STAT3 in a panel of SCCHN cell lines, including PCI-37A, 1483, PCI-15B, UM-22A, and UM-22B to compare the effects of the decoy in cells expressing different levels of STAT1 (Fig. 1A). STAT1 was expressed at high levels in two SCCHN cell lines (PCI-15B, and UM-22A), and expressed at relatively lower levels in PCI37A, 1483 and UM-22B. All five SCCHN cell lines expressed high

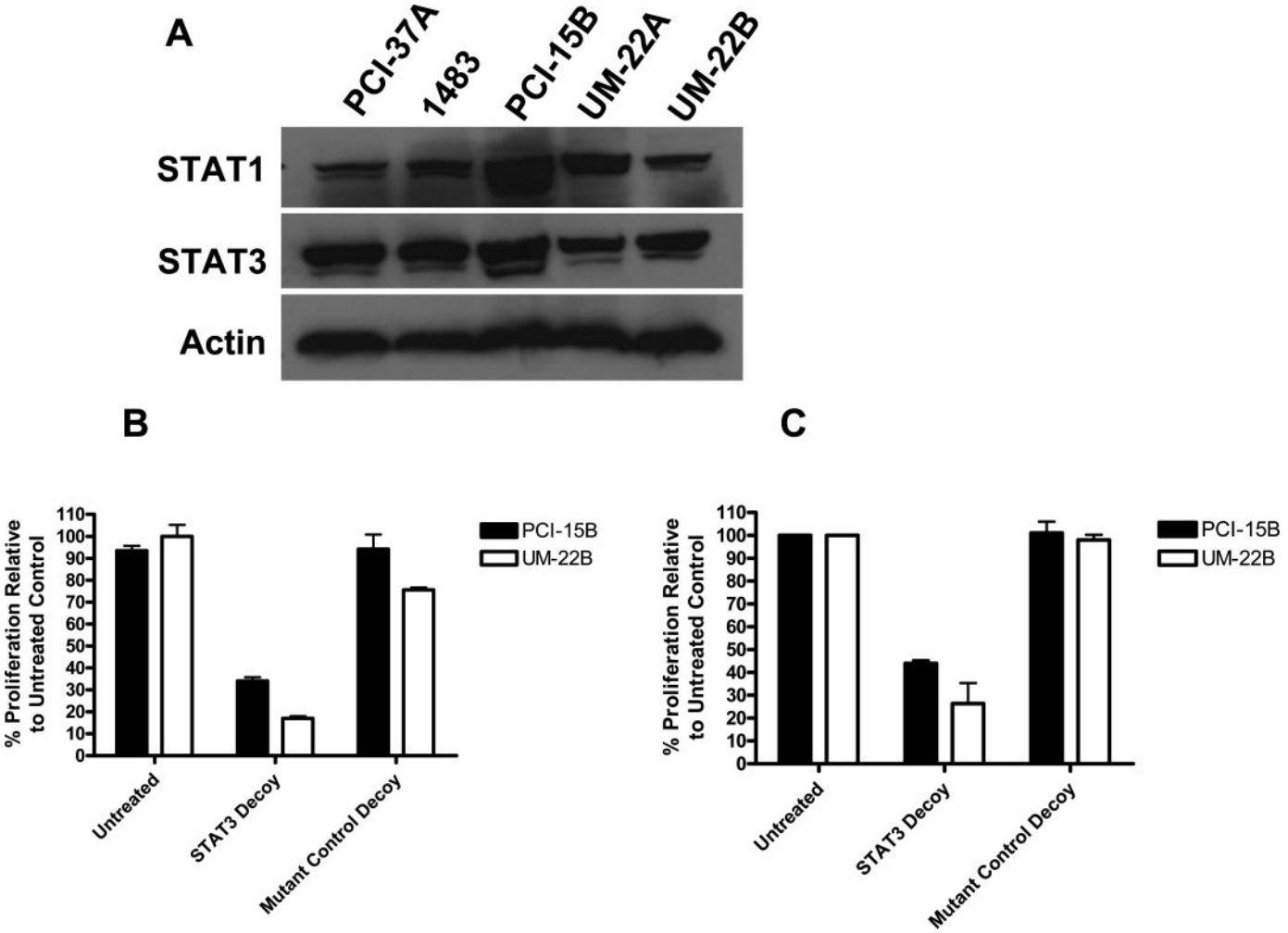

Fig. 1. STAT1 levels do not correlate with SCCHN growth inhibition by the STAT3 decoy. A, expression levels of STAT1 and STAT3 in a panel of SCCHN cell lines (PCI-37A, 1483, PCI-15B, UM-22A, and UM-22B). Fifty micrograms of protein were loaded for immunoblotting with antibodies against STAT1 and STAT3. Immunoblot was probed for β-actin as a loading control. B, STAT3 decoy effects on proliferation in two SCCHN cell lines expressing different levels of STAT1. Both PCI-15B and UM-22B cells (0.6×10^5 cells) were transfected with 690 pM STAT3 decoy or the mutant control decoy and compared with an untransfected control (untreated). Inhibition of cell proliferation was determined by MTT assay 24 h after transfection. C, STAT3 decoy effects on cell proliferation was also examined by trypan blue dye exclusion assays. PCI-15B and UM-22B cells were transfected with 690 pM STAT3 decoy or control decoy and compared with an untransfected control (untreated). Experiments were performed in triplicate wells and performed three times with similar results.

levels of STAT3. Two SCCHN cell lines, PCI-15B and UM-22B, which expressed similar levels of STAT3 but different levels of STAT1 protein, were then chosen for further study. PCI-15B expressed a higher level of STAT1 than UM-22B. As shown in Fig. 1B, treatment of PCI-15B cells with 690 pM STAT3 decoy resulted in only 34% (± 1.8%) proliferation at 24 h, whereas the mutant control decoy treatment resulted in 94.1% (± 6.7%) cell proliferation. In UM-22B, the SCCHN cell line expressing lower STAT1 levels, treatment with the STAT3 decoy resulted in 17.0% (±1%) cell proliferation, whereas the control decoy treatment resulted in 75.5% (± 1.1%) proliferation. Similar results were observed by trypan blue dye exclusion assay (Fig. 1C), in which the STAT3 decoy treatment resulted in 26.4% (± 8.9%) proliferation in UM-22B cells, whereas the control decoy treatment resulted in 98.0% (± 2.3%) proliferation. PCI-15B cells treated with the STAT3 decoy resulted in 43.9% (±1.4%) cell proliferation, and 101% (±5.0%) of the control decoy treated cells proliferated after 24 h. These results demonstrate that SCCHN cell lines (PCI-15B and UM-22B) with high or low expression levels of STAT1, were equally sensitive to the cytotoxic effects of the STAT3 decoy. These results are supported by our previous observations that 1483, a SCCHN cell line with relatively lower levels of STAT1 and high levels of STAT3, was also sensitive to the cytotoxicity of the STAT3 decoy (Leong et al., 2003; Xi et al., 2005).

STAT1 Signaling Is Intact in SCCHN Cells. Because the cytotoxic effect of the STAT3 decoy was not diminished in SCCHN cell lines overexpressing STAT1, we next examined whether the STAT1 activation pathway was intact in SCCHN cells. IFN-γ is known to activate the STAT1 pathway through tyrosine phosphorylation of STAT1 (Tyr 701) and induction of the STAT1 target gene IRF-1. Both PCI-15B and UM-22B cells were serum-starved for 48 h and then treated with IFN-γ for up to 24 h (Fig. 2). In both cell lines, short-term IFN-γ treatment induced a dramatic and persistent phosphorylation of STAT1 (Tyr 701) without significant changes in total STAT1 levels until the 24-h time point. In addition, intact IFN-γ-induced STAT1 signaling was further demonstrated by the rapid induction of IRF-1 expression, a known STAT1 target gene. Maximal IRF-1 induction was observed at 4 and 24 h after IFN-γ treatment in both cell lines. In fact, IRF-1 expression persisted for up to 72 h after IFN-γ treatment, although at much lower levels (data not shown). Therefore, STAT1 signaling is intact in SCCHN cell lines that are susceptible to the cytotoxic effects of the STAT3 decoy.

STAT3 Decoy Disrupts STAT1 Signaling. We and others have shown previously that the STAT3 decoy inhibits STAT3 signaling in several disease models including SCCHN, skin cancer and psoriasis (Xi et al., 2005) (Leong et al., 2003; Chan et al., 2004; Sano et al., 2005). STAT1 shares the highest homology with STAT3 among the STAT family members and it is known to associate with STAT3 through direct heterodimer formation. In theory, targeting STAT3 using a transcription factor decoy approach may also affect the function of STAT3-associated proteins. Among the known STAT3-associated proteins, STAT1 is also of particular interest in this context because, contrary to the growth stimulatory and antiapoptotic functions of STAT3, STAT1 is generally recognized to have tumor suppressor functions (Xi et al., 2006). Therefore, we investigated the effects of the STAT3 decoy on STAT1 signaling by examining the effects of the decoy on STAT1 transcriptional activity and STAT1 target gene expression. SCCHN cells were stably transfected with a STAT1 reporter construct expressing luciferase from a pGAS-Luc containing four γ-activated sequence enhancer elements that are specific for STAT1 (Fig. 3A). In the absence of IFN-γ, the pGAS-Luc stable cell line expressed a low level of luciferase (41.3 ± 1.4 RLU/μg protein), indicating a low level of endogenous STAT1 activation. Treatment with the STAT3 decoy slightly inhibited luciferase activity (13.2 ± 0.3 RLU/μg protein) compared with the mutant control decoy (38.1 ± 1.5 RLU/μg protein). The pGAS-Luc stable cell line was highly responsive to IFN-γ, indicating intact STAT1 signaling. In the presence of IFN-γ, luciferase activity increased markedly by 57-fold (from 41.3 ± 1.4 to 2350 ± 39.6 RLU/μg protein). Treatment with the STAT3 decoy, but not the mutant control decoy, completely abrogated luciferase activity in the pGAS-Luc stable cell line (STAT3 decoy treatment, 25.8 ± 1.8 RLU/μg protein; control decoy treatment, 2260 ± 13.3 RLU/μg protein). We next investigated whether expression of the STAT1 target gene IRF-1 was affected by the STAT3 decoy. When SCCHN cells were treated with the STAT3 decoy for only 5 h (in the presence or absence of IFN-γ), induction of IRF-1 protein was markedly inhibited (Fig. 3B). Consistent with this result, we observed significant inhibition of IRF-1 transcriptional activity using a reporter gene system. A SCCHN cell line stably expressing an IRF-1-Luc reporter gene (carrying six copies of IRF-1-responsive element) was employed (UM-22B). Treatment with the STAT3 decoy, but not the mutant control decoy, completely abrogated the IFN-γ-induced expression of luciferase in the IRF-1-Luc stable cell line (Fig. 3C). We reported previously that the STAT3 decoy abrogates STAT3 DNA binding on gel shift assays (Leong et al., 2003). To determine the effect of the decoy on STAT dimers, we performed supershift experiments using STAT1 and/or STAT3-specific antisera. As shown in Fig. 3D, STAT1 homodimers, STAT3 homodimers, and STAT1/3 heterodimers were all supershifted from the DNA binding complex on gel shift. Taken together, these results demonstrated that STAT3 decoy inhibited STAT1-

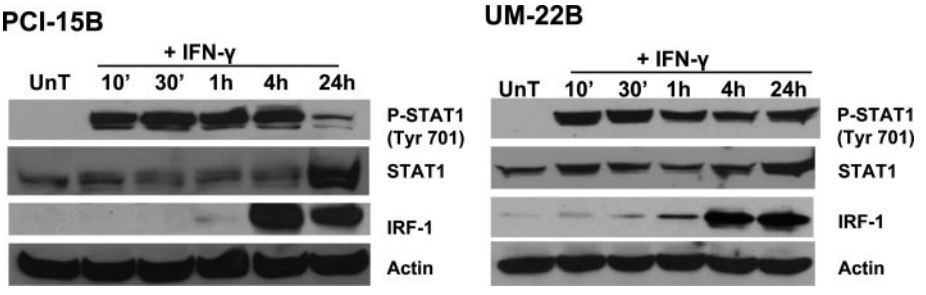

Fig. 2. STAT1 signaling is intact in SCCHN cells. PCI-15B and UM-22B cells were first serum-starved for 48 h and then stimulated with IFN-γ (200 U/ml) for 10 min, 30 min, and 1, 4, and 24 h. The levels of phospho-STAT1 (Tyr 701), total STAT1, and IRF-1 were determined by immunoblotting (50 μg of protein were loaded). Immunoblot was probed for β-actin as a loading control. The experiment was performed three times with similar results.

mediated DNA binding and transcription. These cumulative results suggest that the STAT3 decoy (but not the mutant control decoy) may sequester STAT1 in addition to STAT3 (or sequester STAT1/STAT3 heterodimer) and hence disrupt STAT1 function and transcriptional activity.

STAT1 Does Not Mediate the Cytotoxic Effects of the STAT3 Decoy. STAT1 has been reported to function as a tumor suppressor in human cancer, including SCCHN (Xi et al., 2006). We therefore investigated whether STAT1 contributes to the cytotoxic effects of the STAT3 decoy in SCCHN cells. STAT1 SMART pool siRNA was used to specifically down-regulate the expression of STAT1 in SCCHN cells. As shown in Fig. 4A, STAT1 siRNA transfection of UM-22B for 4 h resulted in a knockdown of STAT1 expression for up to 6 days. Similar results were observed in PCI-15B cells (data not shown). To determine whether STAT1 knockdown using siRNA abrogated STAT1 signaling, we examined the effects of IFN-γ on IRF-1 expression in the presence and absence of STAT1 siRNA. As shown in Fig. 4B, treatment of the SCCHN cells with STAT1 siRNA led to the failure of IFN-γ to induce

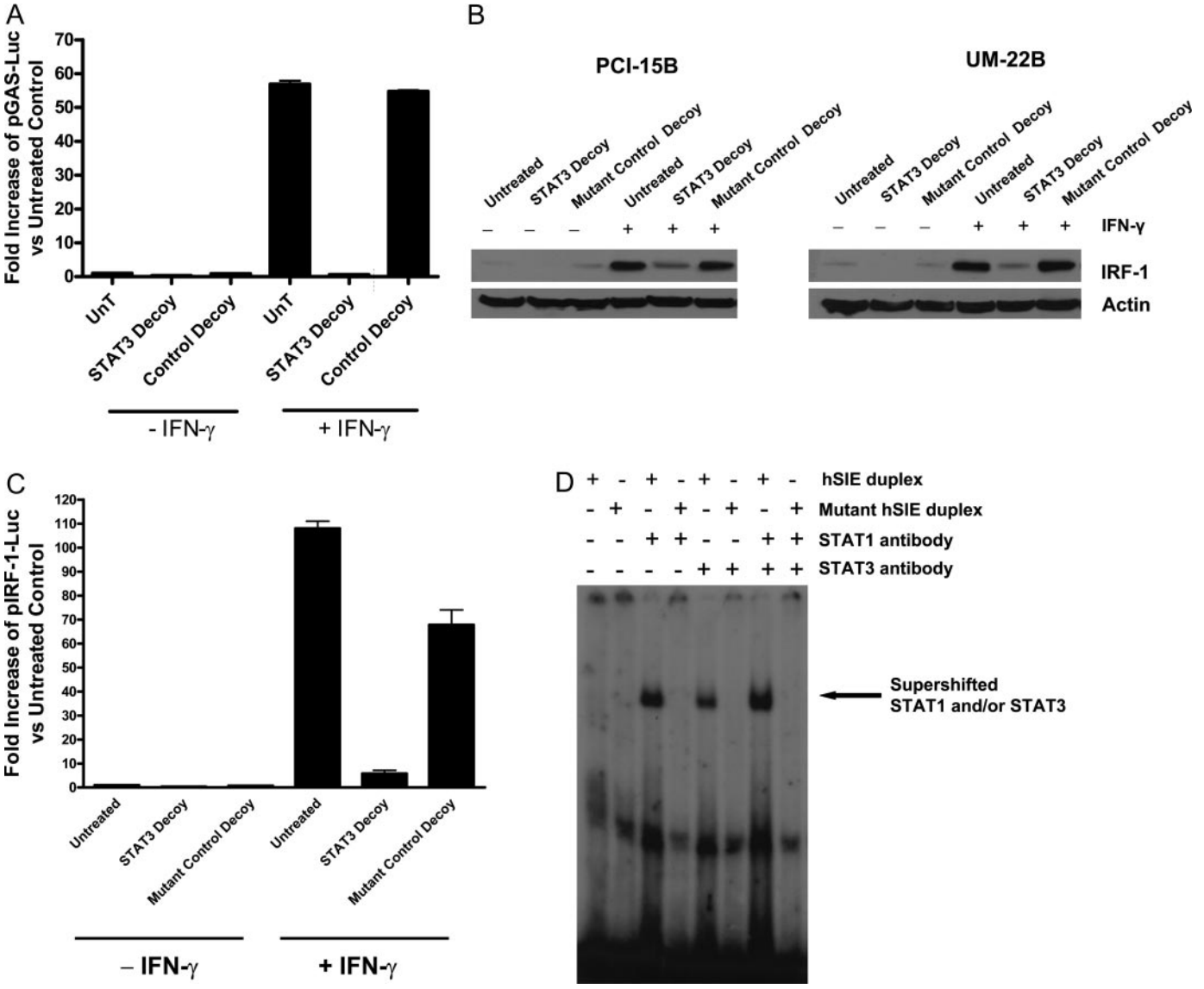

Fig. 3. STAT3 decoy disrupts STAT1 signaling. A, the STAT3 decoy inhibited the expression of STAT1 promoter activity in a cell line (UM-22B) stably expressing pGAS-Luc. The stable cell line was transfected with 690 pM STAT3 decoy or the mutant control decoy. Luciferase assay was performed 24 h after transfection. -Fold change was calculated with reference to the untransfected control (without IFN-γ). Experiments were performed in triplicate wells and performed a total of three times with similar results obtained in each independent experiment. B, expression of an IFN-γ-responsive STAT1 target gene, IRF-1, was specifically down-regulated by the STAT3 decoy upon IFN-γ treatment. SCCHN cells were transfected with the STAT3 decoy or mutant control decoy for a total of 5 h (in a six-well plate). In the IFN-γ-treated group, IFN-γ (200 U/ml) was added 1 h after transfection for an additional 4 h. Cells were then collected for immunoblotting for IRF-1. Immunoblot was probed for β-actin as a loading control. Experiments were performed a total of three times with similar results obtained in each independent experiment. C, specific down-regulation of the transcriptional activity of IRF-1 by the STAT3 decoy. A SCCHN cell line (UM-22B) stably expressing IRF-1-Luc was used to examine the effect of the STAT3 decoy on IRF-1 transcriptional activity. Cells were transfected with 690 pM concentrations of the STAT3 decoy or the mutant control decoy. Luciferase assay was performed 24 h after transfection. -Fold change was calculated with reference to the untreated (no decoy or IFN-γ) IRF-1-Luc control cells. Experiments were performed in triplicate wells and independently performed three times with similar results. D, STAT 1 homodimers, STAT3 homodimers, and STAT1/3 heterodimers can be supershifted from the DNA binding complex after treatment with the STAT3 decoy but not the mutant control decoy. Whole-cell (20 μg) lysate from UM-22B cells was preincubated with STAT1 and/or STAT3 antibodies and then radiolabeled using hSIE or a mutant hSIE probe.

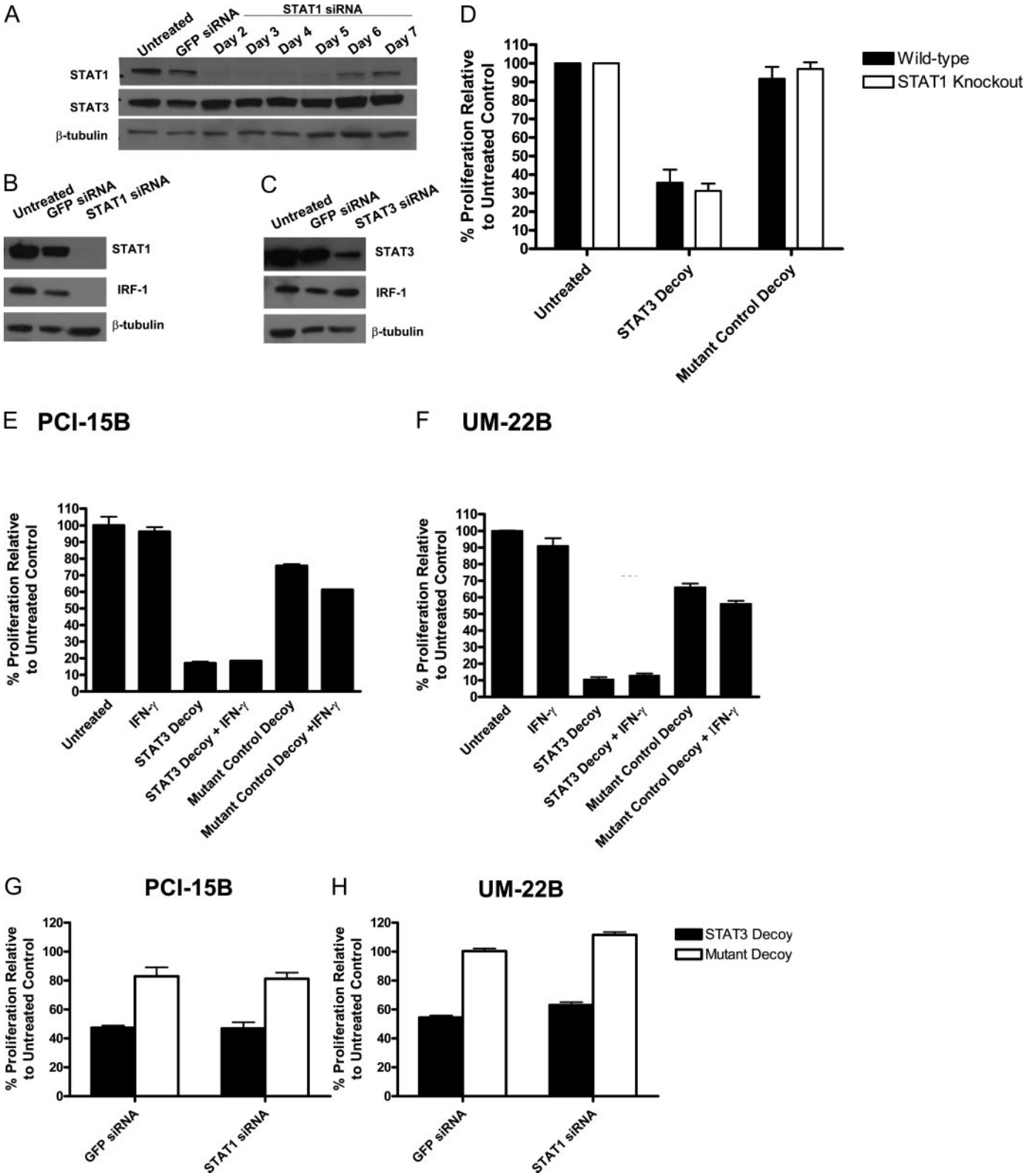

Fig. 4. STAT1 does not contribute to the cytotoxic effects of the STAT3 decoy in SCCHN cells. A, UM-22B cells (a T-75 flask) were transfected with 1200 pmol of GFP siRNA (control) or STAT1 siRNA for 4 h. Cells were collected at days 2, 3, and 4 for the analysis of STAT1 protein levels by immunoblotting. B, untreated, GFP siRNA, or STAT1 siRNA transfected UM-22B cells were stimulated with IFN-γ (200 U/ml) for 4 h. Lysates were collected after 24 h and were immunoblotted for STAT1, IRF-1, and β-tubulin. C, untreated, GFP siRNA, and STAT3 siRNA transfected UM-22B cells were stimulated with IFN–γ (200 U/ml) for 4 h. Lysates were collected after 24 h and were immunoblotted for STAT3, IRF-1, and β-tubulin. D, down-regulation of endogenous STAT1 by STAT1 siRNA did not affect the cytotoxic effects of the STAT3 decoy in SCCHN cell lines. PCI-15B and UM-22B cells were first transfected with the STAT1 siRNA (or GFP siRNA as control) and plated for STAT3 decoy treatment. MTT assay was performed 72 h after decoy treatment. The percentage proliferation after STAT3 decoy treatment (filled bars) and the mutant control decoy (open bars) was calculated using untransfected cells as control. Experiments were performed in triplicate wells and independently repeated three times. E, STAT3

IRF-1 expression in these cells. In contrast, treatment of the same cells with siRNA directed against STAT3 did not mitigate IFN-γ induction of IRF-1 (Fig. 4C). We then evaluated the impact of STAT3 decoy on cells transfected with STAT1 siRNA (or GFP siRNA as control). After transfection with STAT1 siRNA, cells were replated for STAT3 decoy treatment. After an additional 72 h, cell viabilities were determined. As shown in Fig. 4D, STAT1 siRNA transfection did not alter the growth inhibitory effects of the STAT3 decoy in both PCI-15B and UM-22B cells. In PCI-15B cells, STAT3 decoy treatment resulted in 47.4% (± 1.5%) and 46.9% (± 4.3%) cell proliferation in the GFP siRNA transfected and STAT1 siRNA transfected PCI-15B cells, respectively. The mutant control decoy resulted in 83% (± 6.2%) and 81.3% (± 4.1%) cell proliferation in the GFP siRNA- and STAT1 siRNA-transfected PCI-15B cells, respectively. Similar results were observed in UM-22B cells, where STAT3 decoy treatment elicited 54.5% (± 1.3%) cell proliferation in GFP siRNA transfectants and 63.1% (± 2.0%) in STAT1 siRNA transfectants. The mutant control decoy failed to inhibit cell proliferation. To confirm these results in a genetically defined system, the effects of the decoy were examined in STAT1 knockout murine embryonic fibroblasts (MEFs). As shown in Fig. 4E the STAT3 decoy inhibited the growth of STAT1 deficient cells as well as cells derived from wild-type MEFs. These results suggest that expression of STAT1 neither contributes to nor is required for the cytotoxic effects of the STAT3 decoy in SCCHN cells.

STAT1 Activation Does Not Alter the Cytotoxicity of the STAT3 Decoy. We next examined whether activation of STAT1 by IFN-γ would affect the cytotoxicity of the STAT3 decoy. As shown in Fig. 4F, treatment of STAT3 decoy-transfected cells with increasing doses of IFN-γ did not result in any significant changes in the cytotoxicity of the STAT3 decoy ($p = 0.9$ in PCI-15B and $p = 0.6$ in UM-22B). In PCI-15B cells, the percentage cell proliferation with the STAT3 decoy alone, or the STAT3 decoy plus IFN-γ was 17% ± 1.0%, and 18.3% ± 0.17%, respectively. Similar results were observed in UM-22B cells, where the STAT3 decoy alone or the STAT3 decoy plus IFN-γ resulted in 10.7% ± 1.2% and 13.1 ± 1.0% proliferation relative to control, respectively. Thus, activation of STAT1 pathway by IFN-γ does not alter the growth inhibitory effects of the STAT3 decoy in SCCHN cells. This suggests that the efficacy of the STAT3 decoy is independent of STAT1 activation and that the STAT3 decoy can inhibit tumor cell growth even in the presence of STAT1 signaling.

STAT3 Is Required for Growth Inhibition by the STAT3 Decoy. To determine whether STAT3 is necessary for decoy-mediated cell killing, we examined the growth inhibitory effects of the decoy on STAT3 knockout and wild-type MEFs (a kind gift from Dr. David Levy, New York University School of Medicine, New York, NY). We first confirmed that the STAT3 knockout cells did not express STAT3 compared with the wild-type cells, and we also found that both cell lines express comparable levels of STAT1 (data not shown). The MEFs were plated at a density of 5×10^4 cells in 12-well plates and transfected with 1026 pM STAT3 decoy or mutant control decoy. Cell counts, performed after 24 h of transfection, demonstrated that the percentage survival of the STAT3 knockout MEFs treated with the STAT3 decoy was 81.8 ± 9% compared with 29.5 ± 6% survival of the wild-type MEF cells (Fig. 5A). We previously reported a lack of cytotoxic effects of the STAT3 decoy on normal epithelial cells when used at a concentration of 250.3 nM without lipid-mediated transfection (Leong et al., 2003). To verify that the growth inhibition of the wild-type MEFs by the STAT3 decoy was due to the higher concentrations of the decoy used in this assay, we repeated the experiment using a lower concentration of the decoy that we used previously to treat the SCCHN cell lines (102.6 pM); we observed that the survival of wild-type or STAT3 knockout MEFs was not affected when this lower dose of the STAT3 decoy was employed (94% and 102%, respectively) (data not shown). To determine the specific requirement of STAT3 to mediate the growth inhibitory effects of the STAT3 decoy, cells derived from STAT5-deficient mice (and cells from their wild-type counterparts) were also treated with the high concentration of the STAT3 decoy. These cells have been previously reported to express STATs 1 and 3 (Teglund et al., 1998). In contrast to the results obtained in the STAT3-deficient cells, there was no difference in the effects of the STAT3 decoy on the growth of the STAT5 knockout cells or cells derived from their wild-type littermates (Fig. 5B). These results indicate that STAT3 is specifically required for the antiproliferative effects of the STAT3 decoy.

Discussion

In this study, we investigated the potential role of STAT1 on the antiproliferative effects of a STAT3 transcription factor decoy and the reciprocal effects of the STAT3 decoy on STAT1 signaling in SCCHN cells. STAT1 is a potential tumor suppressor that is known to associate with STAT3. Our results demonstrate that the STAT3 decoy inhibits SCCHN growth independent of STAT1 levels and STAT1 activation status. SCCHN cells with either high or low levels of STAT1 were equally sensitive to the growth inhibitory effects of the decoy. Down-regulation of STAT1 levels by siRNA or activation of STAT1 signaling by IFN-γ did not affect the growth inhibitory effects of the STAT3 decoy. In addition, we found that the STAT3 decoy disrupts STAT1 signaling, inhibits STAT1 target gene levels and STAT1 transcriptional activity. These results suggest that STAT1 does not contribute to the antitumor activity of the STAT3 decoy in SCCHN cells. Therefore, a STAT3 decoy has therapeutic potential for treating cancers with active STAT3 and STAT1 signaling.

Transcription factor decoys are double-stranded DNA oligonucleotides that closely resemble the transcription factor-binding site (or DNA binding sequence) in the promoters of

decoy-mediated decrease in cell survival in STAT1 knockout cells is not significantly different from that of wild-type cells ($p = 0.5$). STAT1 knockout cells (U3A) and wild-type MEFs were transfected with 1026 pM STAT3 decoy or mutant control decoy. After 24 h, cell counts using trypan blue dye exclusion assay were performed. Experiment was performed independently three times in triplicate. F, activation of STAT1 signaling by IFN-γ did not affect the cytotoxic effects of the STAT3 decoy in HNSCC. Both PCI-15B (G) and UM-22B (H) cells were transfected with 540 pM concentrations of the STAT3 decoy or mutant control decoy. At 5 h after transfection, the transfection medium was removed and replaced with complete DMEM or DMEM + 200 U/ml IFN-γ. MTT assay was performed at 24 h after transfection. Experiments were performed in triplicate wells and independently performed in triplicate wells and independently performed three times with similar results.

target genes. Decoys presumably bind and sequester the targeted transcription factor, rendering it unavailable for transcription of downstream target genes, thus resulting in specific transcriptional inhibition. A transcription factor decoy approach was originally used for the study of gene expression mediated by transcription factors (Gambarotta et al., 1996; Cho et al., 2002). Because of the sequence-specific characteristics of a transcription factor decoy, it is an attractive approach to target transcription factors. Transcription factor decoys targeting a variety of transcription factors have been developed for E2F, nuclear factor-κB, p53, activator protein-1, ets, Sp1, and estrogen receptor in a variety of disease models (for review, see Gambari, 2004). Many transcription factors have important roles in carcinogenesis and a number of transcription factor decoys have been shown to inhibit human cancer growth in preclinical models (Kuratsukuri et al., 1999; Ishibashi et al., 2000; Alper et al., 2001; Ahn et al., 2003; Leong et al., 2003; Xi et al., 2006). Both STAT1 and STAT3 interact with other proteins and transcription factors. STAT1 binds to the tumor necrosis factor α receptor signaling complex and inhibit nuclear factor-κB (Wang et al., 2000). STAT1 has also been demonstrated to bind to p53 through protein-protein interactions (Townsend et al., 2004). STAT3, like STAT1, interacts with other factors, including PIAS3, GRIM-19, and EZ1 (Chung et al., 1997; Nakayama et al., 2002; Zhang et al., 2003). Although the signal transduction events mediated by STAT1 and STAT3 were initially characterized in the context of DNA binding, it now seems that a coactivator mechanism that does not involve DNA binding can explain some of the consequences of STAT activation. However, the effects of transcription factor decoys, designed to inhibit a specific transcription factor, on other transcriptions factors or interacting proteins is largely unexplored.

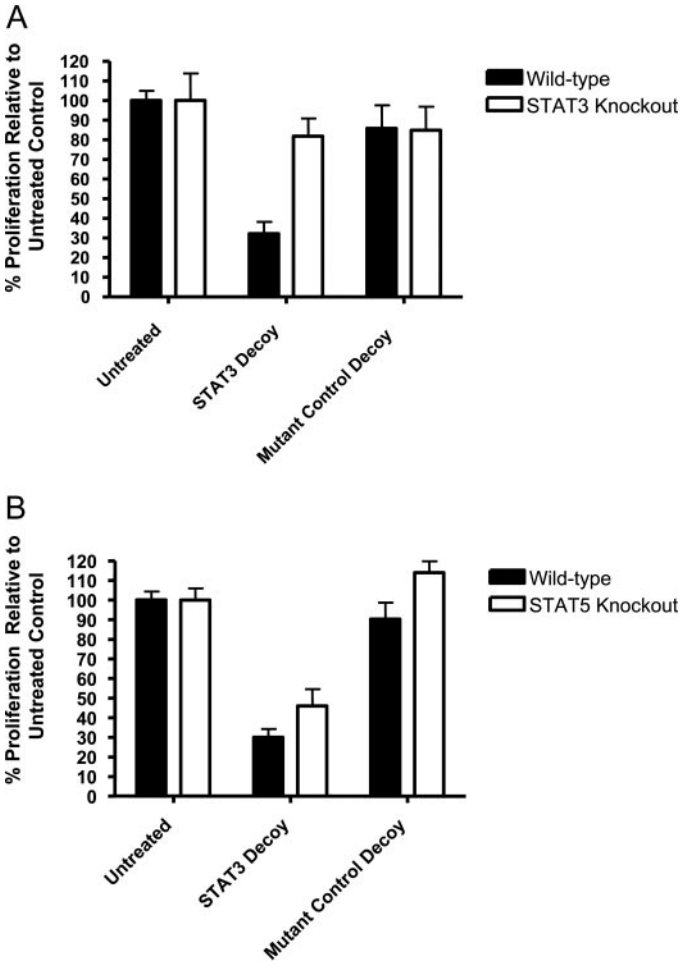

Fig. 5. STAT3 is required for STAT3 decoy-mediated growth inhibition. A, STAT3 knockout or wild-type MEFs (4×10^4 cells) were plated in 12-well plates and transfected with 1025 pM concentrations of the decoy or mutant control decoy. Cell counts were performed 24 h after transfection. EGFP control plasmid was used to measure transfection efficiency (80–90%). This figure represents cumulative results of three independent experiments. B, STAT5 knockout or wild-type MEFs were plated in 12-well plates and transfected with 1025 pM concentrations of the decoy or mutant control decoy. Cell counts were performed 24 h after transfection, and the data represent the cumulative results of three independent experiments.

In theory, inhibition of a tumor suppressor function should lead to enhanced tumor growth. However, this does not seem to be the case when STAT1 signaling is inhibited by the STAT3 decoy. This could be explained by the fact that the function of STAT1 in cancer is still incompletely understood. In addition to a potential tumor suppressor role, STAT1 may also have other unknown functions, such as regulation of apoptosis (Thomas et al., 2004). STAT1 overexpression has been shown to induce chemosensitization in SCCHN (Xi et al., 2006), and STAT1-deficient cells are resistant to tumor necrosis factor-α-induced apoptosis (Kumar et al., 1997). In addition, it is possible that the tumor suppressor activity of STAT1 is restricted to cancer development and not cancer progression. Therefore, abrupt inhibition of STAT1 activity by the decoy may not have an effect on cancer cell proliferation. In a syngeneic model murine squamous cell carcinoma, STAT1 deficiency in the host enhanced interleukin-12-mediated tumor regression (Torrero et al., 2006). These cumulative results suggest that the effects of STAT1 signaling on tumor formation and progression are probably dependent on the specific growth factors, cytokines, and other transcription factors that are present in the tumor microenvironment. The ability of the decoy to inhibit STAT1 as well as STAT3 action raises the possibility that the STAT3 decoy may have actions beyond inhibiting STAT3 in cancer cells, which might limit its potential usefulness as a therapeutic reagent.

Our findings have several clinical implications. Although STAT1 and STAT3 (with relatively opposed functions) are both expressed in a wide variety of cancers, including SCCHN, targeting of STAT3 using a transcription factor decoy approach can still be safely used as an anticancer treatment because inhibition of STAT1 signaling does not mitigate the therapeutic efficacy of the STAT3 decoy. Molecular targeting using a transcription factor decoy approach should be accompanied by a careful examination of the effects on other transcription factors or proteins associated with the transcription factor that is being specifically targeted. In this case, targeting of STAT3 by the STAT3 decoy disrupts STAT1 signaling in SCCHN. Transcription factors are known to function in large multiprotein complexes comprising multiple regulatory proteins, cofactors, and related DNA elements. Therefore, targeting using a transcription factor decoy approach may offer an advantage (compared with an siRNA or antisense approach) of simultaneously inhibiting multiple proteins in the transcription complex.

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