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Advances in Brief

Overexpression of a Dominant-Negative Signal Transducer and Activator of Transcription 3 Variant in Tumor Cells Leads to Production of Soluble Factors That Induce Apoptosis and Cell Cycle Arrest[1]

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Abstract

Gene therapy of B16 tumors with a dominant-negative signal transducer and activator of transcription (Stat3) variant, designated Stat3β, results in inhibition of tumor growth and tumor regression. Although only 10–15% of the tumor cells are transfected *in vivo*, the Stat3β-induced antitumor effect is associated with massive apoptosis of B16 tumor cells, indicative of a potent bystander effect. Here, we provide evidence that blocking Stat3 signaling in B16 cells results in release of soluble factors that are capable of inducing apoptosis and cell cycle arrest of nontransfected B16 cells. RNase protection assays using multi-template probes specific for key physiological regulators of apoptosis reveal that overexpression of Stat3β in B16 tumor cells induces the expression of the apoptotic effector, tumor necrosis factor-related apoptosis-inducing ligand. These *in vitro* results suggest that the observed *in vivo* bystander effect leading to tumor cell growth inhibition is mediated, at least in part, by soluble factors produced as a result of overexpression of Stat3β in tumor cells.

Introduction

Effective cancer gene therapies require the killing of genetically untransduced tumor cells ("bystander" cells) concomitant with genetically transduced tumor cells. Because transfection efficiency is one of the rate-limiting steps for gene therapy, the efficacy of any cancer gene therapy depends heavily on bystander effects. Several studies have demonstrated that *p53* gene therapy results in antiangiogenic effects *in vivo*, thereby enhancing its antitumor activity (1–3). Herpes simplex virus-thymidine kinase-based gene therapy relies on the spreading of toxin converted from prodrug by thymidine kinase-expressing tumor cells to adjacent tumor cells via gap junctional intercellular communication (4). The bystander effect exerted by FasL[3]-based gene therapy involves the activation of neutrophils via Fas receptor (5, 6). Activated neutrophils in turn kill both FasL-transfected and parental tumor cells (5, 6). These studies suggest that, in the absence of high efficiency of *in vivo* gene transfection, the antitumor efficacy of a cancer gene therapy is greatly influenced by the strength of its associated bystander effects.

STATs are latent cytoplasmic transcription factors that function as intracellular effectors of cytokine and growth factor signaling pathways (7). STAT proteins were originally defined in the context of normal cell signaling, where STATs have been implicated in control of cell proliferation, differentiation, and apoptosis (8, 9). Recently, a number of studies have demonstrated that aberrant STAT signaling may participate in development and progression of human cancers. In contrast to normal cells in which STAT activation is rapid and transient, in diverse human cancers, including breast cancer, multiple myeloma, lymphomas, leukemias, head and neck carcinoma (10–17) and melanoma,[4] STAT family proteins, especially Stat3, are constitutively activated. These findings raise the possibility that Stat3 may serve as a molecular target for novel cancer therapy.

Recent studies have indicated that inhibition of Stat3 activity in human tumor cells induces apoptosis and/or growth arrest *in vitro*. Stat3β is a dominant-negative Stat3 variant, which is a truncated form of Stat3 that contains the dimerization and DNA-binding domain but lacks the transactivation domain (18). As a consequence, Stat3β can bind DNA but cannot transactivate gene expression, thus blocking Stat3 signaling in a *trans*-dominant negative fashion in most cases. Blocking Stat3 signaling by Stat3β in human myeloma cells down-regulates IL-6-induced expression of the antiapoptotic gene, *Bcl-X_L*, resulting in a dramatic sensitization of cells to Fas-mediated apoptosis *in vitro* (16). In human head and neck squamous carcinoma cells, interrupting Stat3 signaling by antisense oligonucleotides abrogates transforming growth factor-α induced oncogenic growth of these cells (11). Similar to multiple myeloma, disrupting Stat3 signaling in head and neck cancer cells inhibits Bcl- X_L expression and induces apoptosis (17). Constitutive activation of Stat3 in human breast cancer cells correlates with elevated EGF receptor and c-Src activity (19). Blocking Stat3 activity by dominant-negative Stat3β in breast cancer cells leads to apoptosis (19). Our recent results also demonstrate that inhibiting Stat3 activity by Stat3β causes growth inhibition in human melanoma cells.[5] These findings raise the possibility that targeting Stat3 may result in antitumor responses *in vivo* in a wide variety of human cancers.

We have demonstrated recently that *in vivo* transgenic expression of Stat3β in murine B16 tumors results in tumor regression involving massive apoptosis of tumor cells, despite relatively low transfection efficiencies (10 to 15%; Ref. 20). To begin elucidating the cellular and molecular mechanisms underlying the Stat3β-mediated bystander effects observed *in vivo*, we performed *in vitro* studies. Our results show that inhibition of Stat3 signaling in B16 cells leads to production of soluble factors that are capable of inducing both apoptosis and cell cycle arrest. Consistent with the finding that soluble factors are involved in the bystander effects, induction of mRNA encoding the apoptosis effector, TRAIL, is detected in Stat3β-transfected B16 cells.

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[3] The abbreviations used are: FasL, Fas ligand; STAT, signal transducer and activator of transcription; IL, interleukin; EGF, epidermal growth factor; EGFP, enhanced green fluorescence protein; TRAIL, TNF-related apoptosis-inducing ligand; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; PE, phycoerythrin; RPA, RNase protection assay; IRES, internal ribosomal entry site.

[4] G. Niu, T. Bowman, M. Huang, R. Jove, and H. Yu, unpublished results.

[5] G. Niu and H. Yu, unpublished results.

Materials and Methods

Plasmids. Stat3β cDNA was kindly provided by E. Caldenhoven and R. de Groot (University Hospital, Utrecht, The Netherlands; Ref. 18). The bicistronic green fluorescent protein vector, pIRES-EGFP, was obtained from Clontech (Palo Alto, CA). Insertion of Stat3β cDNA into the pIRES-EGFP plasmid to construct pIRES-Stat3β has been described previously (16).

Cell Culture and Transfection. B16 murine melanoma cells were grown in RPMI 1640 containing 10% FBS. Transfections were performed using GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. To determine transfection efficiency, fluorescence intensities of B16 cells transfected with either pIRES-EGFP or pIRES-Stat3β were measured by FACS (Becton Dickinson Immunocytometry, CA) 24 h after transfection.

Nuclear Extracts and EMSA. Nuclear extract preparation and EMSA analysis of STAT DNA-binding activity were performed as described previously (21).

Cell Growth Inhibition Assays. Cells were plated at 1.7×10^5 cells/well in six-well plates, followed by transfection with either pIRES-EGFP or pIRES-Stat3β plasmids 24 h later. Cells were harvested at 24, 48, or 72 h to determine the numbers of live cells. Cell viability was determined by trypan blue exclusion.

Apoptosis Assays. B16 cells transfected with pIRES-EGFP or pIRES-Stat3β were washed with CellScrub buffer (Gene Therapy Systems) 24 h after transfection. Apoptosis of transiently transfected B16 cells was analyzed after staining with Annexin V-PE by two-color flow cytometry. Apoptosis of nontransfected tumor cells in the upper chambers of Transwell units was analyzed after staining with Annexin V-PE and 7-amino+actinomycin D viability probe (VIA-PROBE 7-AAD) (PharMingen, San Diego, CA) by two-color flow cytometry.

Cell Cycle Analysis. Cell cycle analysis based on DNA content was performed. Cells were harvested, washed twice in PBS, and resuspended in 70% ethanol on ice for at least 30 min. After centrifugation, cells were resuspended in 1 ml of propidium iodide staining solution (50 μg of propidium iodide, 1 mg of RNase A, and 1 mg of glucose per ml PBS) and incubated at room temperature for 30 min. The cells were analyzed by FACS using ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). Cells transfected with vectors encoding EGFP were fixed in 1 ml of 0.5% formaldehyde on ice for 10 min before adding 70% ethanol.

Supernatant Studies. The supernatants derived from either empty vector or Stat3β-transfected B16 cells were collected at 12, 24, 36, and 48 h after transfection and filtered through a 0.22 μm filter. Meanwhile, B16 cells were plated 5×10^3/well in 96-well plate in triplicates. After cells were attached to the wells, 100 μl of fresh culture medium and 100 μl of supernatant from each time point were added to each well. Cells in supernatants were cultured for 48 h before analyzing. For direct cell number counting, cells were harvested and counted by trypan blue exclusion. For [^{3}H]thymidine incorporation assays, 0.25 μCi of [^{3}H]thymidine was added to each well during the last 4 h of incubation and transferred to glass fiber filters by an automated cell harvester (Tomtec, Hamden, CT), and [^{3}H]thymidine incorporation was determined with a liquid scintillation beta counter.

Coculturing Studies in Transwell Units. B16 cells in the lower chambers of Transwell units (0.4 μm pore size) were transfected with either pIRES-EGFP or pIRES-Stat3β plasmids. Five h later, 5×10^4 of either B16 or Meth A cells were added to the upper chambers. After 48 h of coculturing, cells in the upper chambers were harvested for both cell cycle analysis and apoptosis assays.

RNA Isolation and RNase Protection Assays. Total RNA was isolated from 5.0×10^6 cells by TRIzol reagent (Life Technologies, Inc., Grand Island, NY). RPAs were carried out using the PharMingen Riboquant mAPO-3 (TRAIL, FasL, CD95, and other death receptor-associated genes) and mAPO-2 (Bcl-2 family members) multi-probe templates according to the manufacturer's protocol (PharMingen). Briefly, the multi-probe template was synthesized by *in vitro* transcription with incorporation of [α-^{32}P]UTP and purified on a G50 Sephadex column (5-Prime to 3-Prime, Boulder, CO). Specific activity was quantitated in a Beckman LS 6500 scintillation counter (Beckman, Schaumburg, IL). Purified probe ($0.8–1.5 \times 10^6$ cpm/μl) was hybridized with 10 μg of total RNA for 16 h, followed by RNase digestion at 37°C for 1 h. Protected RNA fragments were separated on a 5% polyacrylamide denaturing gel and quantified with Image Quant software (Molecular Dynamics, Sunnyvale, CA). RPAs are representative of three individual experiments.

Results

Stat3β Overexpression in B16 Cells Disrupts Stat3 DNA-binding Activity. Fig. 1 shows specific DNA-binding activities of endogenous Stat3 (*Lanes 1* and *2*) and ectopic Stat3β (*Lane 3*). EGF-induced Stat3 binding activity in EGF receptor expressing-NIH3T3 cells (*Lane 4*) was used as a positive control. By supershift analysis with antibody that recognizes Stat3 but not Stat3β or antibody that recognizes Stat3β but not Stat3, we confirm that there are Stat3-Stat3 homodimers in mock-transfected B16 cells and empty vector-transfected B16 cells. Overexpression of Stat3β in Stat3β-transfected B16 cells results in mostly Stat3β-Stat3β homodimer formation. Previous studies suggested that overexpression of Stat3β blocks Stat3-mediated gene expression by displacing endogenous Stat3 from DNA response elements in promoters (16, 18). In B16 cells, overexpression of Stat3β was also shown to block Stat3-mediated gene expression.[5] These data suggest that Stat3β expression disrupts Stat3-specific gene regulation in B16 cells by displacing wild-type Stat3 protein in the DNA-protein complex.

Stat3β-mediated B16 Cell Growth Inhibition Involves Both Cell Cycle Arrest and Apoptosis. To determine whether interrupting Stat3 signaling by enforcing Stat3β expression would inhibit the

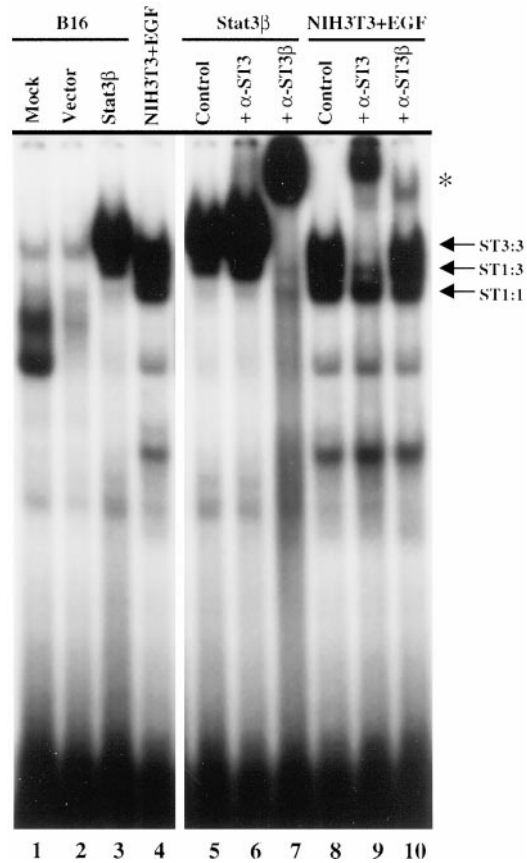

Fig. 1. Disruption of endogenous Stat3 DNA-binding activity in B16 cells by overexpression of Stat3β. EMSA was performed with nuclear extracts prepared from B16 cells transfected with no DNA (*Lane 1*), empty vector (*Lane 2*), or Stat3β expression vector (*Lane 3*). Extracts from EGF receptor-expressing NIH3T3 fibroblasts stimulated with EGF were used as a positive control for Stat1 and Stat3 (*Lane 4*). Supershifts were performed using antibodies recognizing either Stat3 (α-*ST3*) or Stat3β (α-*ST3*β) with extracts derived from B16 cells transfected with Stat3β vector (*Lanes 5–7*). *Lanes 8–10* contained extracts from EGF-stimulated, EGF receptor-expressing NIH3T3 fibroblasts for identification of complexes containing Stat3 or Stat3β homodimers (*ST3:3*), Stat1/Stat3 heterodimers (*ST1:3*), and Stat1/Stat1 homodimers (*ST1:1*), respectively. *, position of supershifted complexes.

growth of B16 cells, pIRES-EGFP control vector and pIRES-Stat3β expression vector were transfected into B16 cells, respectively. Although their transfection efficiencies were similar within each experiment as determined by the percentage of cells that exhibit green fluorescence at 24 h after transfection (by FACS analysis), the number of viable B16 cells decreased dramatically 48 h later in the Stat3β-transfected population (Fig. 2*a*). We next determined whether Stat3β-induced growth inhibition was mediated by cell cycle arrest or apoptosis or both. The cell cycle distributions of empty vector and Stat3β-transfected cells are shown in Fig. 2*b*. The Stat3β-transfected B16 cells showed progressive accumulation in G_0-G_1 phase, with concomitant decrease of the population in S and G_2-M phase. This G_0-G_1 phase arrest could be observed as early as 24 h after transfection. At 48 h after transfection, Annexin V-PE staining followed by FACS analysis to detect apoptotic activity was performed with transfected B16 cells. A high level of apoptosis in Stat3β-transfected cells (75%) relative to empty vector transfected cells (25%) was observed, as shown in Fig. 2*c*. Increased levels of apoptosis as a result of Stat3β transfection were confirmed by confocal laser scanning microscope analysis using rhodamine-labeled terminal deoxynucleotidyltransferase-mediated nick end labeling assay (data not shown). These results demonstrate that Stat3β-mediated growth inhibition of B16 cells involves both cell cycle arrest and apoptosis.

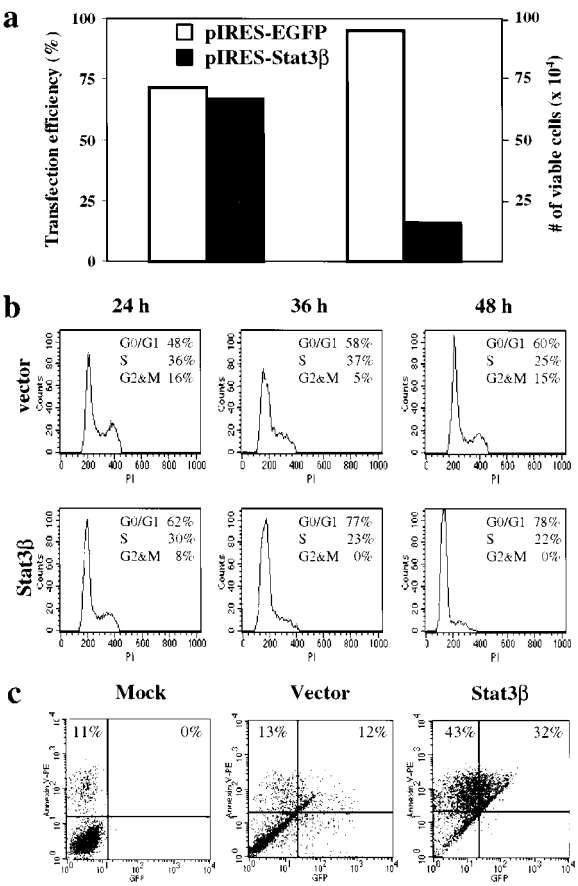

Fig. 2. Overexpression of Stat3β induces cell cycle arrest and apoptosis in B16 cells. *a*, transfection efficiencies of pIRES-EGFP and pIRES-Stat3β vectors in B16 cells, as determined by the percentage of cells positive for GFP expression, and number of viable B16 cells 48 h after transfection, as determined by trypan blue exclusion. *b*, cell cycle analysis was performed by propidium iodide staining at various times after transfection, as indicated. G_0-G_1 phase arrest in Stat3β-transfected B16 cells was detected at 24, 36, and 48 h after transfection. *c*, 48 h after transfection, apoptosis was measured by Annexin V-PE staining, followed by FACS analysis. Data shown represent one of three experiments with similar results.

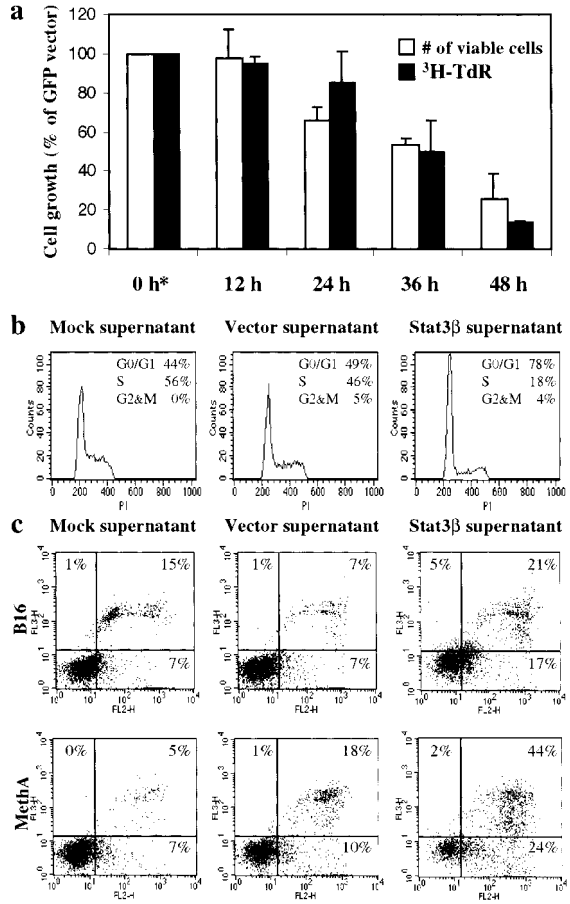

Fig. 3. Soluble factors produced by Stat3β-transfected B16 cells induce growth inhibition of nontransfected B16 cells. *a*, growth inhibition by supernatants derived from Stat3β-transfected B16 cells collected at 0, 12, 24, 36, and 48 h after transfection. B16 cell growth in the presence of supernatants from Stat3β-B16 cells at various times was expressed as a percentage of that of GFP-transfected B16 supernatants at comparable time points, as indicated. Cell growth was determined by both counting viable cells and by [^{3}H]thymidine incorporation assays. Values for cell growth are means of triplicate transfections; *bars*, SD. *b*, cell cycle analysis. *c*, apoptosis assays. B16 cells in the lower chambers of Transwell units were transfected with the indicated vectors, followed by seeding nontransfected B16 cells in the upper chambers 5 h later. Forty-eight hours after that, B16 cells in the upper chambers were harvested for either cell cycle analysis or apoptosis assays. These experiments were repeated at least three times with similar results.

Disrupting Stat3 Signaling Triggers B16 Tumor Cells to Produce Soluble Factors Capable of Inducing Both Cell Cycle Arrest and Apoptosis. A surprising observation was that in the B16 cell culture transiently transfected with Stat3β, nontransfected (GFP-negative) B16 cells also underwent apoptosis (Fig. 2*c*). On the basis of this observation, we tested whether the Stat3β-dependent bystander effect was mediated by cell-cell contact or soluble factors. Supernatants were collected at 12, 24, 36, and 48 h after transfection and used as conditioned medium for nontransfected B16 cells. Fig. 3*a* shows that the conditioned media from Stat3β-transfected B16 cells were able to confer growth inhibition on nontransfected B16 cells, suggesting the involvement of soluble factors in inhibiting the growth of nontransfected tumor cells. To rule out the possibility that the inhibitory soluble factors were induced by apoptosis or stress in general, supernatant derived from UV-irradiated, apoptotic B16 cells was tested for its ability to inhibit B16 cell growth. Results indicate that the supernatant derived from UV-irradiated, apoptotic B16 cells failed to induce any growth inhibition of B16 cells (data not shown).

We next investigated whether soluble factor-induced growth inhibition was mediated through apoptosis or cell cycle arrest or both. As shown in Fig. 3, a significant increase in the percentage of B16 cells arrested in

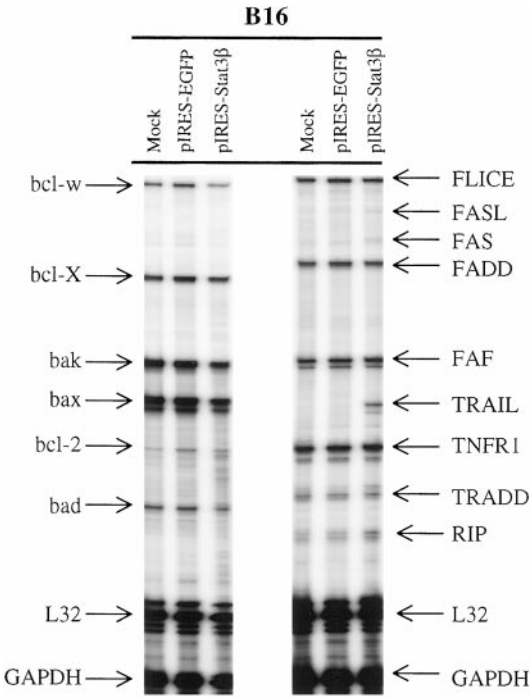

Fig. 4. Stat3β overexpression in B16 cells results in induction of TRAIL mRNA expression. Ten μg of total cellular RNA were isolated from various B16 transfectants, as indicated, and hybridized with each multiple probe before digestion with RNase. Separation of protected fragments was performed by gel electrophoresis. Fragment assignment was determined by migration relative to internal standards. Induction of TRAIL RNA was confirmed by two additional RPA analyses. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *FLICE,* FADD-like interleukin 1β-converting enzyme; *FADD,* Fas-associated death domain containing protein; *FAF,* Fas-associated factor; *TNFR1,* tumor necrosis factor receptor 1; *TRADD,* TNFR-associated death domain containing protein; *RIP,* receptor interacting protein.

G_0-G_1 was observed when cultured in conditioned medium from Stat3β-transfected B16 cells, compared with media from mock or vector-transfected B16 cells. Furthermore, nontransfected B16 cells cultured in upper chambers in which the lower chambers contained Stat3β-transfected B16 cells underwent apoptosis (Fig. 3*c*). These soluble factors produced by Stat3β-transfected B16 cells were also capable of inducing apoptosis of nontransfected Meth A tumor cells (Fig. 3*c*).

Expression of TRAIL, a Tumor-specific Apoptotic Effector, Is Induced in Stat3β-transfected B16 Cells. As a first approach to define what factors may cause apoptosis of untransfected tumor cells as a result of Stat3β expression in B16 cells, RPAs using multi-template probes were performed. Thirty hours after transfection, total RNA was isolated from B16 cell cultures, and RPAs were carried out using probes specific for key physiological regulators of apoptosis. An induction of TRAIL RNA expression in B16 cells as a result of Stat3β overexpression was detected (Fig. 4). This induction of TRAIL was specific, because none of the other genes examined was induced (Fig. 4).

Discussion

Our *in vitro* results demonstrate that overexpression of Stat3β leads to apoptosis and cell cycle arrest of murine melanoma B16 cells. Importantly, disruption of Stat3 signaling in B16 cells also results in the production of soluble factors. The soluble factors are capable of inducing apoptosis and cell cycle arrest of nontransfected tumor cells, suggesting that killing of bystander B16 tumor cells *in vivo* may be mediated, at least partially, by these soluble factors.

It has been reported that bystander effects mediated by other gene therapies also involve soluble factors (22–24). However, it is not known how these soluble factors mediate the bystander effects at the cellular and molecular levels. Our results demonstrate that blocking Stat3 signaling triggers tumor cells to produce soluble factors capable of inducing both cell cycle arrest and apoptosis. At the molecular level, we show that inhibition of Stat3 activity in B16 cells induces expression of the pro-apoptotic effector, TRAIL, which is known to induce tumor cell-specific death (25). TRAIL is a type II membrane protein, but production of a soluble form of TRAIL by various cell types has also been shown. Although we have not been able to directly establish a role of TRAIL in mediating the bystander killing of B16 tumor cells, possibly because of the involvement of multiple factors, it has been demonstrated previously that a high percentage of human melanoma cell lines are sensitive to TRAIL-mediated killing (26). Our recent results also show that blocking Stat3 signaling in human melanoma cell lines triggers them to express TRAIL.[6] These findings suggest that interrupting constitutive Stat3 signaling in cancers, such as melanoma, may lead to TRAIL-mediated tumor killing. Taken together, our findings suggest that interrupting Stat3 signaling induces tumor cells to produce soluble factors capable of mediating bystander tumor cell killing. Further identification and characterization of these soluble factors should provide insight into the molecular mechanism(s) responsible for antitumor bystander effects induced by Stat3-targeted therapy.

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