Antitumor Effects of Mucin 1/sec Involves the Modulation of Urokinase-Type Plasminogen Activator and Signal Transducer and Activator of Transcription 1 Expression in Tumor Cells

Dan Ilkovitch,[1] Mary Ellen Handel-Fernandez,[1] Lynn M. Herbert,[1] and Diana M. Lopez[1,2]

[1]Department of Microbiology and Immunology, and [2]Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Florida

Abstract

Expression of the transmembrane isoform of Mucin 1 (MUC1/TM) in an aggressive murine mammary tumor line, DA-3, does not alter tumor development and metastasis, leading to death of the host. However, tumor cells expressing a secreted isoform of MUC1 (MUC1/sec) fail to develop tumors in immunocompetent mice. The rejection of MUC1/sec-expressing tumor cells is immunologically mediated, as, initially, innate cells and, ultimately, T cells are required. After gene array analysis, and confirmation at the protein level, it was discovered that MUC1/sec-expressing tumor cells (DA-3/sec) have a significant reduction in expression of urokinase-type plasminogen activator (uPA) relative to the parental tumor line and tumor cells expressing MUC1/TM. The serine protease uPA has been found to be involved in growth-promoting signaling, angiogenesis, and induction of matrix remodeling leading to metastasis. Although the tumor-promoting Stat3 transcription factor was unaltered in these tumor cells, the tumor-suppressive and IFN-responsive signal transducer and activator of transcription 1 (Stat1) is dramatically up-regulated in DA-3/sec cells. In addition, treatment of various murine and human cell lines with conditioned medium containing MUC1/sec results in up-regulation of Stat1. DA-3/sec tumor cells are also sensitized to the antiproliferative effects of IFN-γ. Furthermore, transfection of the Stat1 gene into DA-3 tumor cells leads to a down-regulation of uPA and delays tumor progression. Thus, Stat1 up-regulation in DA-3/sec cells seems to play a significant role in the mechanism(s) by which rejection of tumor cells expressing MUC1/sec may be occurring. [Cancer Res 2008;68(7):2427–35]

Introduction

Mucin 1 (MUC1) is a glycoprotein that has been described to be overexpressed with altered glycosylation patterns and loss of polarized (apical) surface expression on many epithelial tumor cells (1–3). Several different splice variants of MUC1 exist, including a secreted isoform known as MUC1/sec. MUC1/sec has been detected in both normal (4, 5) and tumor tissues (6, 7), and interestingly, its presence in ovarian lesions has been correlated with benign disease (6). We previously reported the dramatic effect caused by tumor cell expression of the genes for MUC1/sec relative to the full-length transmembrane isoform of MUC1 (MUC1/TM) on tumor development in the DA-3 mammary tumor model (8, 9). Although implantation of the parental DA-3 cell line or cells expressing MUC1/TM (DA-3/TM) lead to metastasis and eventual death of the host, DA-3 cells expressing MUC1/sec (DA-3/sec) fail to develop tumors in immunocompetent BALB/c animals. However, implanting DA-3/sec cells in BALB/c nude mice lacking T lymphocytes leads to tumor growth.

By studying mice lacking a cellular compartment or immune component, or by depletion of a specific cell type, we showed that IFN-γ, natural killer (NK) cells, NKT cells, and macrophages were all important in the early control of DA-3/sec tumor development, as mice lacking any one of these components initially formed tumors, all of which eventually regressed (8). The role of these innate immune cells was further supported by delayed development of progressively growing DA-3/sec tumors in BALB/c nude mice relative to DA-3 or DA-3/TM tumors in these mice. DA-3/sec cells were able to form progressively growing tumors in experiments in which both $CD4^+$ and $CD8^+$ T lymphocytes were depleted from BALB/c animals. Thus, the prevention of DA-3/sec tumor development is, in part, controlled by an early innate immune response but ultimately requires a T-cell–mediated antitumor response (8). Cancer immunoediting involves three stages described as elimination, equilibrium, and escape (10). Although DA-3/sec do not form progressive tumors in BALB/c animals, it is possible that they are either eradicated or contained in equilibrium with an antitumor immune response. In this report, we show by *in vivo* bioluminescence imaging that the majority of DA-3/sec cells are rejected early on in immunocompetent BALB/c mice, and their growth is initially controlled in BALB/c nude mice, although DA-3/TM tumor cells lead to progressive tumor growth in both BALB/c and BALB/c nude mice.

These findings prompted us to explore the possibility that there were tumor-derived factor(s) other than the secreted mucin playing a role in the rejection of these tumors *in vivo*. Gene array analysis showed several differences in expression of RNA transcripts between DA-3/sec and DA-3/TM cells. One of these differences was in the expression of urokinase-type plasminogen activator (uPA), a serine protease implicated in angiogenesis, tumor growth, and correlated with poor prognosis in many human cancers (11). In DA-3/sec cells, the production of uPA is completely down-regulated. This down-regulation of uPA in DA-3/sec cells seems to be tied to a significant up-regulation of the tumor-suppressing and IFN-responsive signal transducer and activator of transcription 1 (Stat1; refs. 12–14). We report here that tumor expression of Stat1, induced by MUC1/sec, down-regulates uPA, an important tumor-promoting molecule. These findings may help explain the immunologic rejection of DA-3/sec cells observed in immunocompetent mice.

Requests for reprints: Diana M. Lopez, Department of Microbiology and Immunology, Miller School of Medicine, University of Miami, P.O. Box 016960 (R-138), Miami, FL 33101. Phone: 305-243-6632; Fax: 305-243-4409; E-mail: dlopez@med.miami.edu.

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Materials and Methods

Animals and cell lines. BALB/c mice (H-2d) between ages 8 and 12 wk were bred at the University of Miami according to guidelines of the NIH. BALB/$c^{nu/nu}$ (nude) mice were purchased from the Charles River Laboratories. The DA-3 mammary tumor cell line developed in our laboratory and the DA-3/sec, DA-3/sec22, and DA-3/TM cells were maintained as previously described (8, 15). B16.F10 cells were purchased from American Type Culture Collection and, human LNCaP, MCF-7, and T47D cells were kindly provided by Dr. Kerry L Burnstein (University of Miami Miller School of Medicine, Miami, FL). Tumor cells were implanted by s.c. injection of 1×10^6 tumor cells in 0.9% saline, resulting in measurable tumors 7 to 10 d after implantation. Tumor volumes were calculated by measuring two diameters of the tumor (x, small diameter; y, large diameter) using digital calipers and entering measurements into the equation tumor volume = $x^2y(0.52)$ as previously published (16). To create the DA-3/sec22-nudes cell line, DA-3/sec22 tumor cells were implanted by s.c. injection of 1×10^6 tumor cells in 0.9% saline in BALB/c nude mice. Four weeks after tumor cells were implanted, the tumor was excised, and a single-cell suspension was stained with anti-CD227(MUC1).FITC (BD Biosciences) followed by magnetic antibody cell separation using anti-FITC microbeads as per manufacturer's protocol (Miltenyi Biotec).

Transfection of DA-3 cells. Stable DA-3/sec and DA-3/TM transfectants expressing firefly luciferase (DA-3/sec-LUC and DA-3/TM-LUC) were generated by isolating the gene-encoding luciferase from pGL4.10[luc2] Vector (Promega) and inserting it into the expression plasmid pcDNA3.1/Hygro (Invitrogen). DA-3/sec and DA-3/TM cells were then plated and transfected using Lipofectamine 2000 as per manufacturer protocol (Invitrogen). Cells were cloned under selection with 400 μg/mL hygromycin and tested for constitutive luciferase expression using a Steady-Glo Luciferase Assay (Promega). To create DA-3/Stat1 cells, the same transfection protocol was followed as above with an expression plasmid pcDNA3.1-Hygro-Stat1 generously provided by Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center, Houston, TX; ref. 12).

Imaging. Mice were given i.p. 2.5 mg of the luciferase substrate D-luciferin (Xenogen) dissolved in 100 μL of 0.9% saline. Five minutes later, they were anesthesized with a xylazine/ketamine solution administered i.p. The mice were then imaged using the bioluminescence optical imager (IVIS 200; Xenogen). Maximal luciferase signals were quantified using Living Image 2.5 (Xenogen) image analysis software. Luciferase signal is reported as photons/sec/cm^2/steradian.

Zymography and ELISAs. Tumor cells were plated at 4×10^5 cells per well in a 6-well plate overnight in the same medium used to maintain the tumor cells. The next day, wells were washed twice with PBS, and culture medium lacking serum was added back to the wells. After 60 h of culturing, conditioned medium/supernatants were collected and final cell numbers were counted using trypan blue exclusion. Gelatin zymography followed a modified procedure of Heussen and Dowdle (17) for detecting enzymatically functional amounts of uPA. Identical amounts of supernatant were electrophoresed under nonreducing conditions using 10% SDS polyacrylamide gels containing 0.33 mg/mL gelatin and 12 ng/mL plasminogen (Roche). The gels were washed twice in 2.5% Triton X-100 for 30 min to remove SDS. After overnight incubation at 37°C in assay/incubation buffer [50 mmol/L Tris-HCl (pH 7.2), 50 mmol/L NaCl, 0.02% NaN_3, 0.005% Brij 35, and 1 mmol/L 1,10-Phenanthroline monohydrate], the gels were stained for 2 h with Coomassie blue R 250 and destained with 7% acetic acid. uPA activation of plasminogen to plasmin produces clear areas of gelatin digestion in the gel. As control, recombinant mouse uPA was loaded at various concentrations (Molecular Innovations). ELISAs for total mouse uPA were carried out according to manufacturer's instructions (Molecular Innovations). Absorbance at 450 nm was read on a Tecan SLT Rainbow Reader (Labinstruments). Absorbance values of samples were converted to nanograms per milliliter against a standard curve produced with the ELISA kit, and then divided by the final cell number obtained when the conditioned medium/supernatants were harvested to provide nanograms per milliliter per million cells.

Western blot analysis. Tumor cells were plated at 1×10^6 cells per 25 cm^2 flasks. Two days later, flasks were rinsed with PBS, and 900 μL of lysis buffer was added [lysis buffer: radioimmunoprecipitation assay buffer (RIPA; Santa Cruz Biotechnologyz), one complete mini protease inhibitor cocktail tablet per 5 mL of RIPA (Roche), and for phosphorylation state detection, Halt Phosphatase Inhibitor Cocktail was added (Pierce)]. Flasks were shaken at 4°C for 15 min, and cell lysates were transferred to Eppendorf tubes, vortexed, and placed on ice for 20 min. Lysates were then centrifuged for 15 min and supernatants containing protein extract were transferred to new tubes and stored at −80°C. Protein concentration was determined using a BCA Protein Assay kit (Pierce). Fifty micrograms of protein were resolved on 10% SDS polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membranes (0.2 μm pore size; Pall Life Sciences). Membranes were blocked for 30 min at room temperature in 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBS-T) and, for phosphorylation state detection, phosphatase inhibitor cocktail A and B (Santa Cruz Biotechnology). Membranes were rinsed in TBS-T and then incubated overnight in primary antibody diluted in the same blocking buffer at 4°C. Primary antibodies used were anti-Stat1 p84/p91 (Santa Cruz Biotechnology), anti-Stat3 (Cell Signaling Technology), antiactin (Sigma-Aldrich), and anti-pStat1(Tyr701; Upstate Biotechnology). Blots were washed for 30 min with two changes of TBS-T solution followed by 1.5 h incubation at room temperature with horseradish peroxidase–conjugated goat anti-rabbit IgG Ab or anti-mouse IgG Ab (Sigma-Aldrich). Blots were washed four times for 10 min with TBS-T and then incubated for 5 min with Supersignal West Pico chemiluminscent substrate (Pierce). Results were visualized by exposing blots to CL-Xposure film (Pierce). Film was then scanned, and data were subjected to densitometric analysis using Scion Image Software (NIH). Protein levels were normalized to the hybridization signals of β-actin, and reported as relative absorbance or as ratios.

Treatment of cells with tumor-conditioned medium. Conditioned medium from 4-d cultures of DA-3/TM and DA-3/sec tumor cells was collected and centrifuged to remove cell debris. Various tumor cell lines were then plated at 2×10^5 cells per mL in 24-well plates and allowed to adhere for 24 h. Medium was then removed, and 75% conditioned medium from either DA-3/sec or DA-3/TM cultures were added back. After 24 h, medium was removed and a second round of conditioned medium was added for 24 h. Cell lysates were then obtained and Western blot analysis was performed as described above.

Flow cytometry. Tumor cells were plated at 1×10^6 cells per 25 cm^2 flasks. Two days later, flasks were rinsed with PBS, and 1 mL of DMEM medium containing 1,000U IFN-γ (Pierce) was added to some flasks. Treated cells were cultured for 15 min at 37°C. Cells were then quickly trypsinized, fixed, permeabilized, and stained for phospho-protein flow cytometry as per protocol provided by Danna and colleagues.[3] Antibody used was Anti–phospho-Stat1 (Y701).PE (BD Biosciences). Cells were analyzed on an LSR I flow cytometer (BD Biosciences).

Results

Expression of MUC1/sec prevents DA-3 tumor development in immunocompetent mice. As explained above, DA-3/sec cells do not grow in immunocompetent BALB/c mice but do develop tumors in immunocompromised BALB/c nude mice. To ascertain whether DA-3/sec cells survive for a period of time or are immediately rejected in immunocompetent BALB/c mice, we chose to monitor tumor development with *in vivo* bioluminescence imaging. We transfected DA-3/sec and DA-3/TM tumor cells to express firefly luciferase (DA-3/sec-LUC and DA-3/TM-LUC), which can be traceable by inoculating mice with D-luciferin. Implanting BALB/c mice s.c. with 1×10^6 DA-3/sec-LUC or DA-3/TM-LUC cells leads to similar initial tumor growth at day 3 (Fig. 1*A* and *C*).

[3] Manuscript submitted.

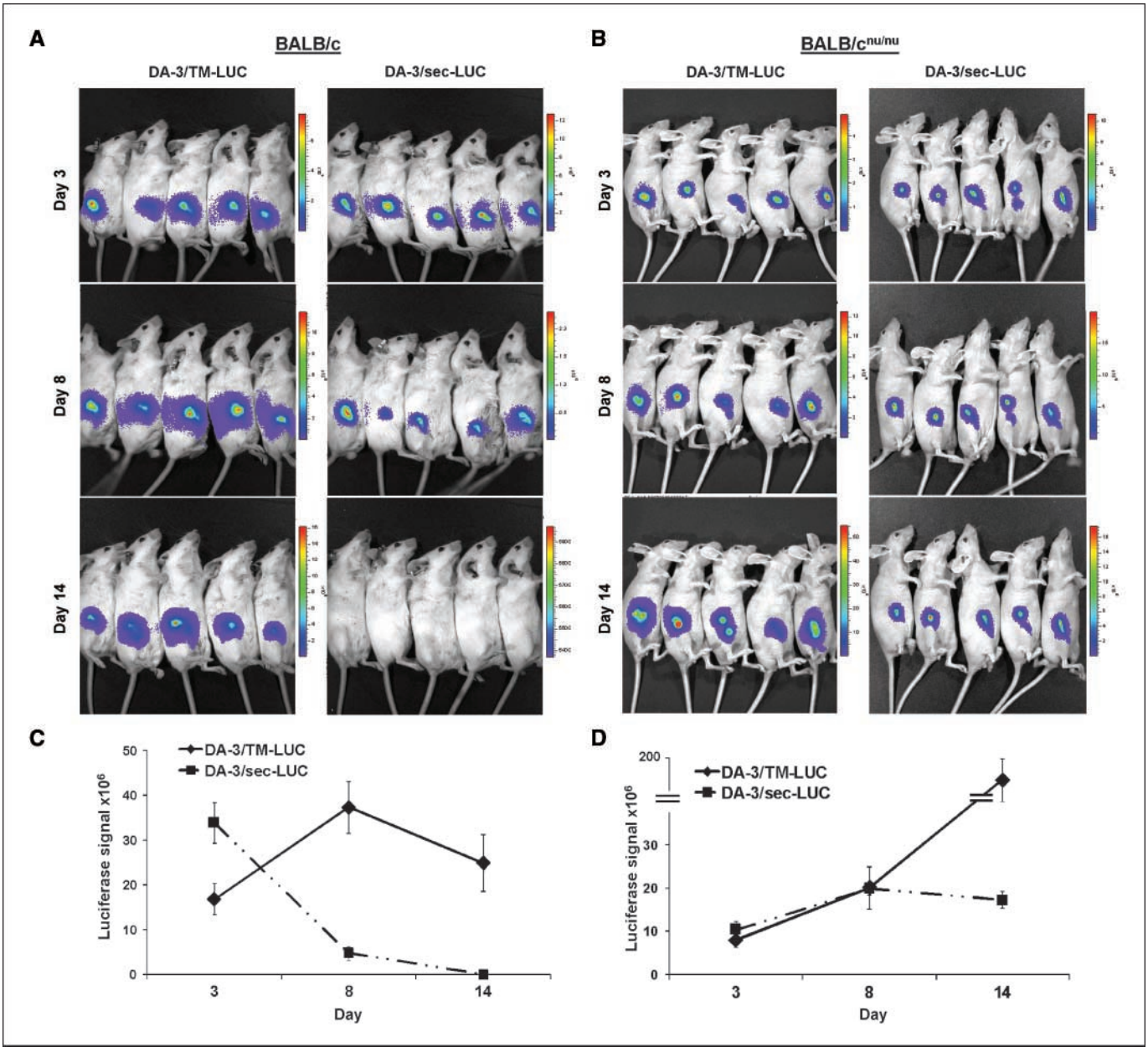


Figure 1. DA-3 cells expressing a secreted isoform of MUC1 (*DA-3/sec*) are rejected in immunocompetent mice, whereas expression of transmembrane MUC1 (*DA-3/TM*) does not alter tumor development. *A,* BALB/c mice were implanted s.c. with 1×10^6 DA-3/TM-LUC or DA-3/sec-LUC cells transfected to express firefly luciferase. Although DA-3/TM-LUC leads to tumor development and luciferase signal is present at days 3, 8, and 14 after inoculation, DA-3/sec-LUC tumor cells begin to regress as seen by decreasing luciferase signal on day 8, falling below the detection limit of the bioimager by day 14. *Color scale,* bioluminescence intensity (p/s/cm^2/sr). *B,* although DA-3/sec-LUC cells are rejected in BALB/c animals, they persist and lead to tumor development in immunocompromised BALB/c $^{nu/nu}$ mice. *C* and *D,* graphs showing the maximal luciferase signal (p/s/cm^2/sr) on days 3, 8, and 14 after tumor cell implantation in BALB/c and BALB/c $^{nu/nu}$ mice, respectively.

DA-3/sec tumor cells, however, begin to regress dramatically by day 8 and are below the detection limit of the bioimager by day 14. In contrast, DA-3/TM tumor cells continue to grow and develop tumors measurable by a caliper as previously described. BALB/c nude mice implanted with the same cells show that DA-3/sec tumor cells, such as DA-3/TM, are capable of persisting and leading to development of tumors in mice lacking T lymphocytes (Fig. 1*B* and *D*). However, whereas DA-3/TM tumors grow rapidly in nude mice, DA-3/sec tumor cells take longer to develop into measurable tumors and, as seen in Fig. 1*D* by the luciferase signal detected, are prevented from progressing during the first 2 weeks. As we have reported previously, this is likely due to the involvement of innate immune cells, NK cells, and macrophages, recruited and stimulated to kill DA-3/sec cells (8).

uPA is dramatically down-regulated in DA-3/sec tumor cells. Various gene products are associated with tumor development, progression, and evasion of antitumor immune responses. To dissect whether MUC1/sec modulates genes in the tumor cells that may lead to successful tumor rejection, several cancer-specific gene arrays were used to determine variation in gene expression between DA-3 and DA-3/sec cells. One of the drastic differences noted in DA-3/sec cells was a 15-fold down-regulation of uPA at the

RNA level relative to DA-3 cells (data not shown). To validate the down-regulated RNA expression of uPA in DA-3/sec cells, we performed a specific zymography assay to test functional uPA enzymatic activity in 3-day culture supernatants from counted DA-3, DA-3/TM, and DA-3/sec cells. Supernatants from DA-3/sec22 cells, a second DA-3 transfectant expressing MUC1/sec, were also tested. After size separation and SDS extraction, active uPA converts plasminogen in the zymography gel to plasmin, which digests the copolymerized gelatin in the gel. Whereas DA-3 and DA-3/TM cells show high levels of expression of enzymatically functional uPA, DA-3/sec and DA-3/sec22 cells show no uPA functional activity (Fig. 2*A*). To confirm that DA-3/sec cells down-regulate total production of uPA and to quantify the levels of uPA that DA-3 and DA-3/TM cells produce, ELISAs for total mouse uPA (active, inactive, and complexed) were performed on supernatants from counted DA-3, DA-3/TM, DA-3/sec, and DA-3/sec22 cells of 3-day cultures (Fig. 2*B*). These results show that DA-3 and DA-3/TM cells expressing transmembrane MUC1 secrete uPA at high levels, 45 ng/mL uPA/10^6 cells and 20 ng/mL uPA/10^6 cells, respectively, whereas DA-3/sec and DA-3/sec22 cells do not secrete detectable amounts. The ELISA for total mouse uPA readily detects recombinant uPA as a positive control at levels as low as 0.3 ng/mL. Thus, expression of MUC1/sec in DA-3 tumor cells leads to complete down-regulation of uPA expression.

Stat1 is up-regulated by MUC1/sec and can be activated in MUC1/sec-expressing tumor cells. Because we have reported previously that expression of the chemokine CCL2 is up-regulated in DA-3/sec cells (8) and are now reporting that uPA is completely down-regulated in these cells, we hypothesized that MUC1/sec may modulate transcription factors that broadly regulate a variety of tumor-promoting or tumor-suppressing gene products. Stat3 is constitutively active in many tumors and has been correlated with tumor-induced immune suppression and oncogenesis. In contrast, Stat1 is down-regulated in many tumors and has opposing gene targets and functions to Stat3 (18). We decided to analyze the relative levels of protein expression of the transcription factors Stat1 and Stat3 in DA-3, DA-3/TM, DA-3/sec, and DA-3/sec22 tumor cells. By Western blot analysis, we see a significant up-regulation of Stat1 in both DA-3/sec and DA-3/sec22, cells that express MUC1/sec (Fig. 3*A*). Densitometric analysis shows however that although Stat3 is relatively the same among the four cell lines, Stat1 is up-regulated 7-fold in DA-3/sec and DA-3/sec22 cells relative to the levels in DA-3 and DA-3/TM cells (Fig. 3*B*). Because Stat1 and Stat3 have opposing functions and gene targets in tumor cells, a higher Stat1/Stat3 ratio may favor a gene expression profile that opposes tumor progression and immune suppression. DA-3/sec and DA-3/sec22 have a 5-fold higher Stat1/Stat3 ratio relative to DA-3 and DA-3/TM cells (Fig. 3*C*). To determine whether MUC1/sec is capable of up-regulating Stat1 in other tumor cells, we treated various tumor cell lines, murine and human, with 75% conditioned medium from either DA-3/TM or DA-3/sec tumor cell cultures. We have previously shown that MUC1/sec can be detected in conditioned medium from DA-3/sec tumor cells but not DA-3/TM cells (8). By Western blot analysis, we see that conditioned medium from DA-3/sec cells can up-regulate Stat1 levels in B16.F10 murine melanoma, LNCaP human prostate carcinoma, MCF-7 human breast epithelial adenocarcinoma, and T47D human breast ductal carcinoma cells(Fig. 3*D*). Treatment of DA-3/sec cells with conditioned medium did not alter the up-regulated levels of Stat1 in these cells.

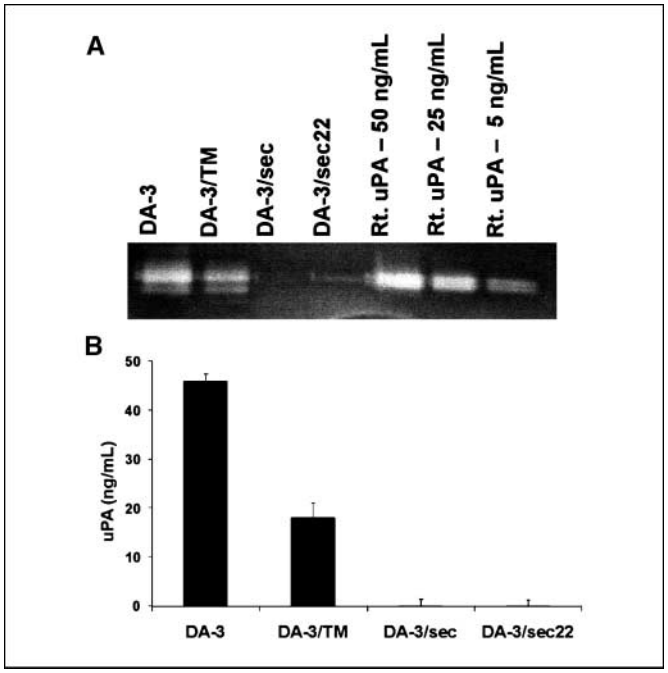


Figure 2. DA-3/sec cells do not produce uPA, whereas DA-3 and DA-3/TM tumor cells express high levels of this serine protease. *A,* a uPA-specific gelatin zymography assay was performed on supernatants from 3-d tumor cell cultures of DA-3, DA-3/TM, and two MUC1/sec transfectants DA-3/sec and DA-3/sec22. As a positive control and to show the sensitivity of the assay, 50, 25, and 5 ng/mL of recombinant mouse uPA (*Rt. uPA*) was loaded in parallel. *B,* ELISA for total mouse uPA performed on 3-d tumor cell cultures of the same cells. Cells were counted on day 3 and used to calculate the amount of uPA produced per million cells in the supernatants. Each assay was performed at least four times with one individual experiment shown. Similar results were obtained from all experiments.

To assume an active Stat conformation that can enter the nucleus and direct transcription of various target genes, Stats are phosphorylated at tyrosine residues to their active form after ligand interaction with various receptors, which allows Stat dimerization, followed by nuclear entry (13). Stats have certain functions in their unphosphorylated cytoplasmic form and have additional functions and targets in their active phosphorylated forms. To test whether up-regulated Stat1 in DA-3/sec cells is capable of assuming an active form, we briefly treated cells for 15 min with IFN-γ, which is known to cause resident Stat1 tyrosine phosphorylation (p-Stat1). Using an antibody that detects the phosphorylation of Tyr^{701} on Stat1 by Western blot analysis, it was found that p-Stat1 is up-regulated in DA-3/sec and DA-3/sec22 cells but not in DA-3 or DA-3/TM cells (Fig. 4*A*). Using a phosflow antibody that similarly detects the phosphorylation of Tyr^{701} on Stat1, we confirmed by flow cytometry that p-Stat1 is clearly up-regulated after brief treatment with IFN-γ in both DA-3/sec and DA-3/sec22 cells (78–91%) but not in DA-3 or DA-3/TM cells (2–11%; Fig. 4*B*). Stat1 is not phosphorylated in DA-3/sec cells in tissue culture without stimulation with IFN-γ; however, *in vivo*, these cells are exposed to IFNs produced by both innate and adaptive immune cells. Thus, high tumor Stat1 expression may be important in the early rejection of DA-3/sec cells. Regarding Stat3, a recent experiment in which the tumor cells were treated *in vitro* with interleukin 6 showed that Stat3 was phosphorylated in DA-3, DA-3/TM, DA-3/sec, and DA-3/sec22 cells to levels correlating with those of Stat3 (data not shown).

Expression of Stat1 in DA-3 tumor cells leads to down-regulation of uPA. Stat1 has been reported to reduce angiogenesis

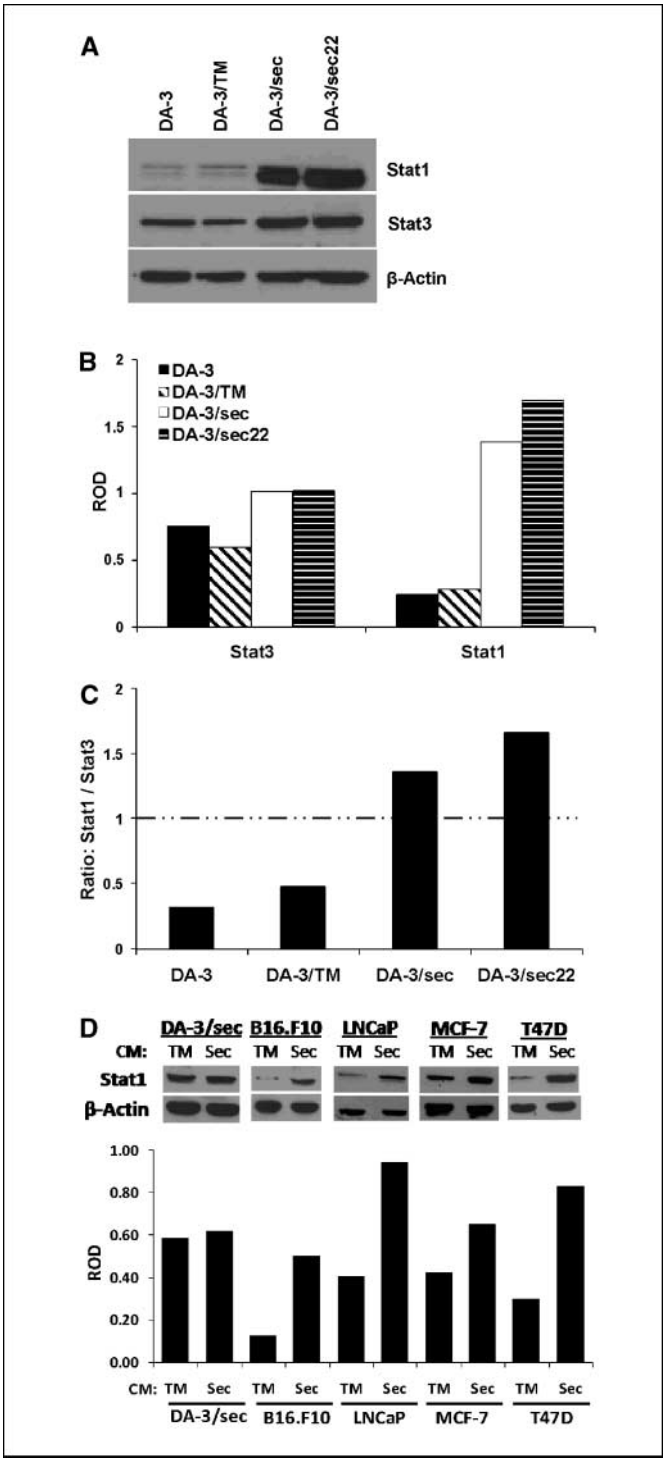


Figure 3. DA-3/sec cells and tumor cells exposed to MUC1/sec have a significant up-regulation of Stat1. *A,* Western blot analysis of cell lysates from DA-3, DA-3/TM, DA-3/sec, and DA-3/sec22 cells shows a significant up-regulation of Stat1, whereas Stat3 is relatively unchanged. *B,* densitometric analysis of Western blot in *A* shows a significant up-regulation of Stat1 after normalization to β-actin in cells expressing MUC1/sec. *C,* densitometric analysis of Western blot reveals a 5-fold higher Stat1/Stat3 ratio in DA-3/sec and DA-3/sec22 cells. *D,* DA-3/sec, B16.F10, LNCaP, MCF-7, and T47D tumor cells were treated for 48 h with 75% conditioned medium (*CM*) from either DA-3/sec or DA-3/TM tumor cell cultures. Cell lysates were then obtained, and Western blot analysis shows up-regulated Stat1 levels in B16.F10, LNCaP, MCF-7, and T47D cells treated with DA-3/sec conditioned medium. Treatment did not alter the elevated levels of Stat1 observed in DA-3/sec cells. The results are representative of one of four experiments.

and metastatic potential of developing tumors by reducing factors such as matrix metalloproteinase (MMP)-2 and MMP-9 and basic fibroblast growth factor (12). uPA has been implicated in driving the metastatic potential of tumor cells with its ability to digest the extracellular matrix and activate MMPs (19). Furthermore, uPA activates plasminogen into plasmin, which can convert suppressive cytokines in the tumor microenvironment, such as transforming growth factor (TGF)-β, into their bioactive forms (20, 21). Because DA-3/sec cells have up-regulated Stat1 and down-regulated uPA, we decided to investigate whether there is a correlation between these two proteins. We transfected the DA-3 tumor cell line with an Stat1 expression plasmid, pcDNA3.1-Hygro-Stat1, to produce the cell line DA-3/Stat1. As a control, DA-3 cells were transfected with the empty expression plasmid, pcDNA3.1-Hygro, to produce the cell line DA-3/Hygro. Although DA-3 and DA-3/Hygro do not show any Stat1 expression by Western blot analysis, DA-3/Stat1 does show high expression as does DA-3/sec and DA-3/sec22 (Fig. 5*A*). However, by densitometric analysis, the Stat1 expression in DA-3/Stat1 is only 60% of the expression levels detected in DA-3/sec and DA-3/sec22 cells (Fig. 5*A*). To test whether Stat1 regulates uPA, an ELISA for total mouse uPA was carried out. Whereas DA-3 and DA-3/Hygro cells produced 50 ng/mL uPA/10^6 cells, DA-3/Stat1 produced significantly lower levels of uPA at 24 ng/mL uPA/10^6 cells (Fig. 5*B*). DA-3/sec and DA-3/sec22 did not produce detectable levels of uPA. The complete down-regulation of uPA seen in MUC1/sec-expressing cells may thus be a result, at least in part, of the significantly high expression of Stat1 observed in these cells.

Stat1 expression in tumor cells delays tumor progression. Given that DA-3/sec tumor cells, which express high levels of Stat1, are rejected in immunocompetent BALB/c animals, we wanted to test whether expression of Stat1 in DA-3 tumor cells alters tumor progression *in vivo*. DA-3/Hygro or DA-3/Stat1 cells (1×10^6) were implanted s.c. in BALB/c mice, and tumor growth was measured during development. Although DA-3/Stat1 cells express lower levels of Stat1 than DA-3/sec cells, their growth is significantly delayed ($P < 0.01$) relative to DA-3/Hygro cells transfected with the vector alone as control (Fig. 6*A*). The untransfected DA-3 cells have the same *in vivo* growth kinetics as DA-3/Hygro cells (data not shown). As we reported previously, and as can be seen in Fig. 1, MUC1/sec-expressing cells have delayed tumor development in BALB/c nude mice (8). We hypothesized that DA-3/sec tumors that eventually develop in nude mice may involve a selection of tumor cells that express lower Stat1. We implanted a BALB/c nude mouse with DA-3/sec22 tumor cells and, after several weeks, excised the tumor. DA-3/sec22 cells were then purified out of a single-cell suspension of the tumor based on MUC1 expression, and the resulting cell line was named DA-3/sec22-nudes. We then analyzed the levels of p-Stat1 using phosflow as described above. Whereas DA-3/sec22 cells up-regulate p-Stat1 to high levels with brief IFN-γ treatment, DA-3/sec22-nudes tumor cells have 30% lower p-Stat1(Fig. 6*B*). These data suggest that the delayed tumor growth of DA-3/sec in nude mice may involve a selection of cells expressing lower activated Stat1.

Host IFN-γ may play an important role in the rejection of tumor cells that express the IFN-responsive transcription factor Stat1. Because DA-3/sec cells have delayed tumor appearance in nude mice and DA-3/Stat1 cells have delayed growth in BALB/c mice, we hypothesized that IFN-γ may play an important role in blocking early growth of tumor cells that express Stat1. We tested whether *in vitro* proliferation of the different tumor cells is

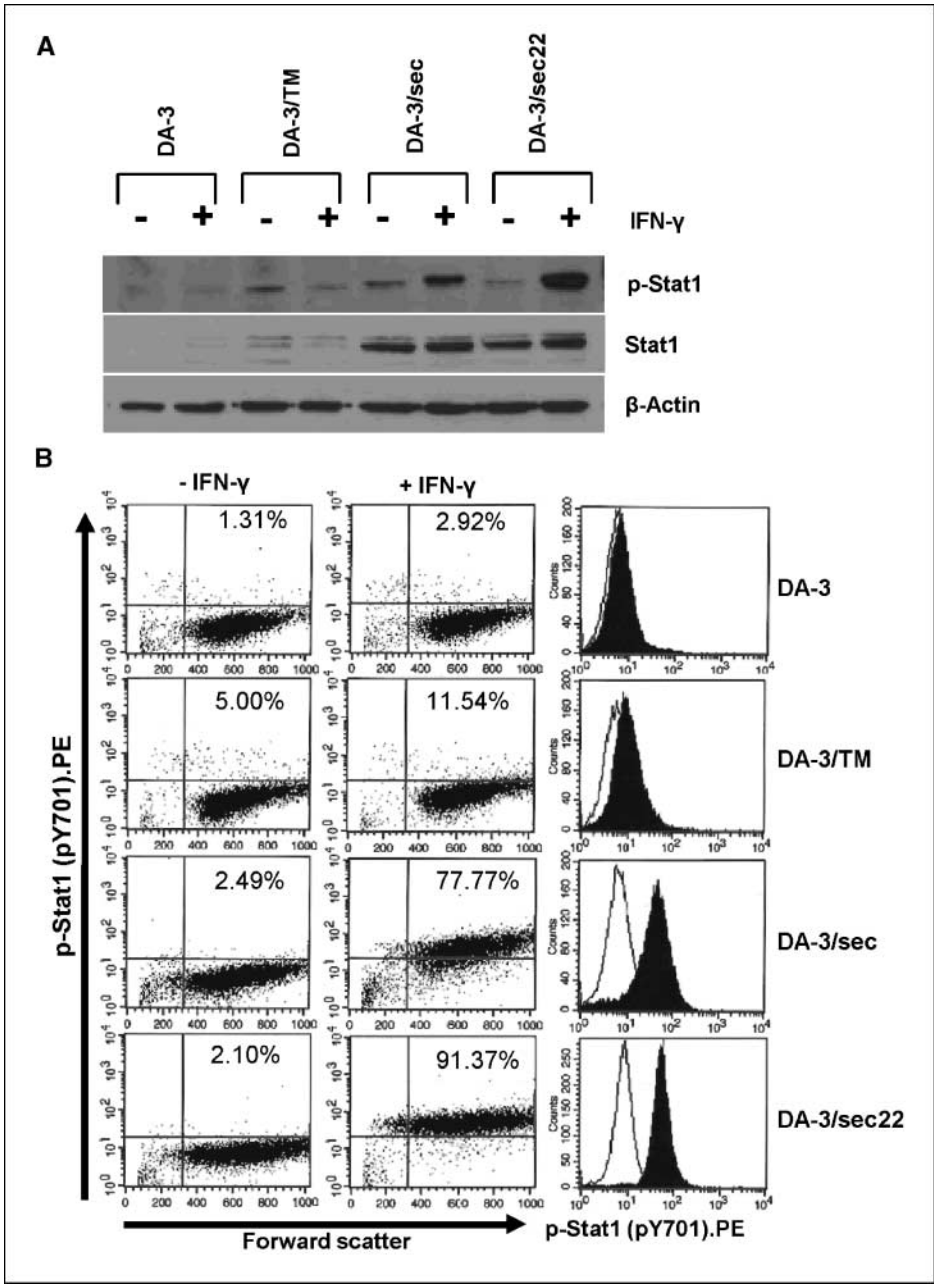


Figure 4. Up-regulated Stat1 in DA-3/sec cells can be activated to the phosphorylated form in response to IFN-γ. *A,* cells were treated briefly with IFN-γ to activate resident levels of Stat1 in the tumor cells. Western blot analysis of cell lysates from DA-3, DA-3/TM, DA-3/sec, and DA-3/sec22 cells either untreated or treated with IFN-γ shows an up-regulation of p-Stat1 in treated DA-3/sec and DA-3/sec22 cells. *B,* using the same brief treatment with IFN-γ, p-Stat1 up-regulation can be detected in treated DA-3/sec and DA-3/sec22 cells by phospho-flow cytometry. DA-3 and DA-3/TM cells do not show significantly high levels of Stat1 or p-Stat1 upon treatment with IFN-γ. The results of one experiment are shown, and each experiment was performed at least thrice.

affected by treatment with IFN-γ. Tumor cells (1×10^5) were plated alone or in the presence of 500 U/mL of IFN-γ, and 3 days later, the cells were counted using trypan blue exclusion. Although DA-3 and DA-3/TM cells, which have very low expression of Stat1, are minimally affected by IFN-γ treatment, DA-3/sec and DA-3/sec22 cells, expressing high levels of Stat1, have a 50% reduction in tumor cell proliferation relative to control cultures (Fig. 6*C*). Furthermore, we have previously reported that DA-3/sec tumor cells initially develop tumors in IFN-$\gamma^{-/-}$ BALB/c animals (8). However, after 3 weeks, those tumors regressed, and it was concluded that T cells were ultimately responsible for the tumor rejection. Thus, IFN-γ may play an important role in the early elimination of cells expressing high levels of Stat1, such as DA-3/sec and DA-3/sec22 tumor cells.

Discussion

Aberrant expression of proteins by tumor cells confers protection from normal cellular growth controls, in part, by stimulating proliferation, induction of angiogenesis, and evasion or suppression of antitumor immune responses. MUC1 is a glycoprotein that has been correlated with an aggressive nature and poor prognosis in many human cancers, including breast (22), ovarian (6), prostate (23), colon (24), pancreas (25), and head and neck cancers (26). Whereas in normal cells, MUC1 protects the cell surface and serves as a sensor of the extracellular environment, in tumor cells, MUC1 overexpression can aid in metastasis by altering adhesion properties, evasion of immune responses by blocking surface marker recognition, and cellular proliferation by activation of key cellular-signaling molecules (27). Furthermore, tumor MUC1 is hypoglycosylated, which can directly affect antigen-presenting

cells and induce their immune-suppressive phenotypes (28, 29). Although splice variants of MUC1 in relation to cancer have not been thoroughly studied, we have previously published that the expression of the secreted isoform, MUC1/sec, in mammary tumor cells confers protection against tumor development, unlike the expression of the transmembrane form, MUC1/TM (8, 9). Obermair et al. (6) have also shown that MUC1/sec expression was correlated with benign disease in ovarian lesions. In this report, we have attempted to dissect changes induced by MUC1/sec in the mammary tumor cells, which may inhibit progressive tumor development and support antitumor immune responses. Focusing on tumor-derived factors that play an integral part in the formation and aggressive development of tumors, we have identified a significant down-regulation of uPA in DA-3/sec cells. uPA is a serine protease used by many cell types including monocytes, dendritic cells, and endothelial cells, mainly for migration and matrix remodeling (19, 30). Tumor cells can thus exploit overexpression of uPA for the same functions, but the protease has also

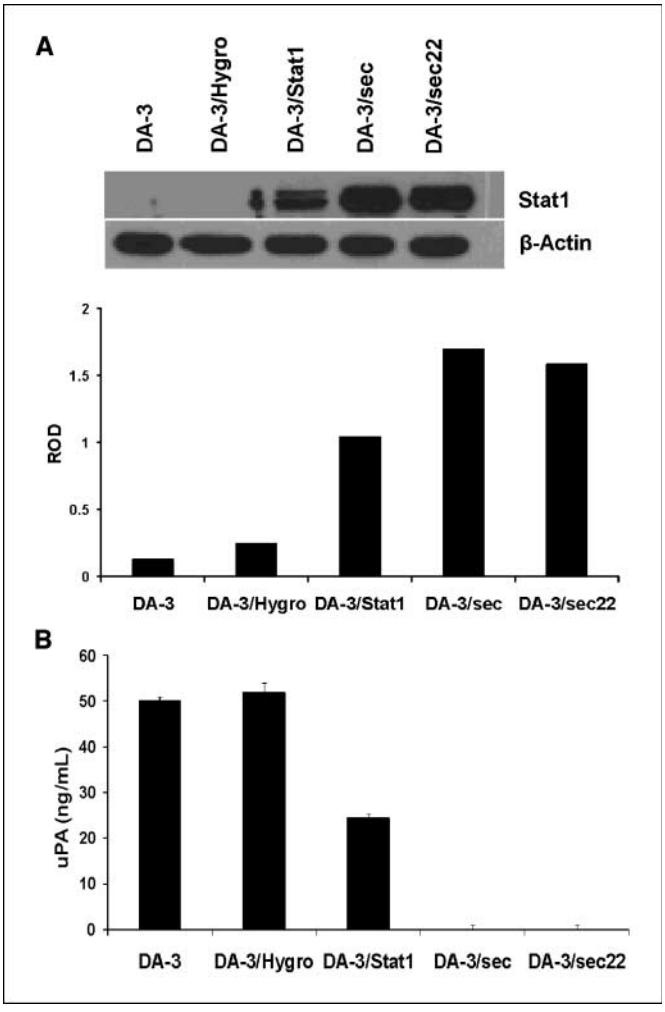
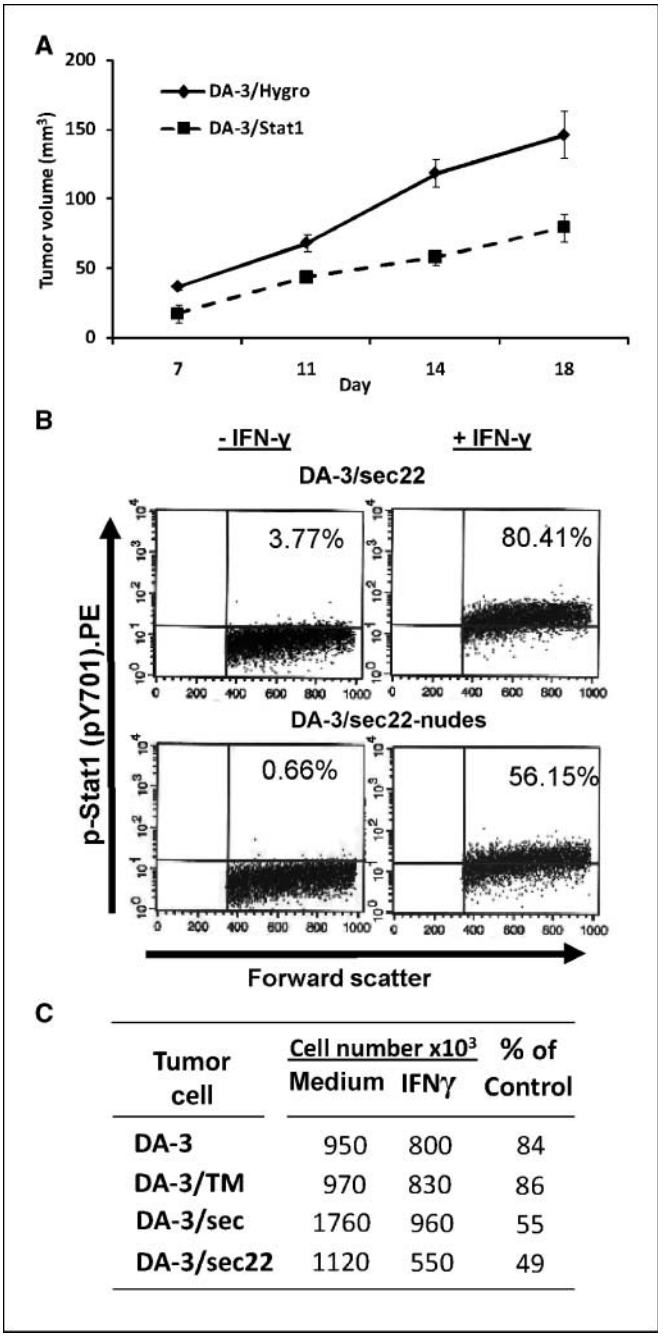


Figure 5. DA-3 cells transfected to express Stat1 down-regulate production of uPA. *A,* DA-3 cells were transfected with either an empty expression vector as control (*DA-3/Hygro*) or with an expression vector containing the Stat1 gene (*DA-3/Stat1*). Western blot analysis shows no Stat1 expression in DA-3 or DA-3/Hygro, with high Stat1 expression in DA-3/sec, DA-3/sec22, and DA-3/Stat1 cells. Densitometric analysis of Western blot with normalization to β-actin shows that DA-3/Stat1 cells express Stat1 at 60% of the level of Stat1 present in cells expressing MUC1/sec. *B,* an ELISA for total mouse uPA was performed on 3-d tumor cell cultures of the same cells. Cells were counted on day 3 and used to calculate the amount of uPA produced per million cells in the supernatants. Although DA-3 and DA-3/Hygro cells produce high levels of uPA, DA-3/Stat1 cells produce approximately half the level of uPA per million cells. Each assay was performed at least four times with one individual experiment shown. Similar results were obtained from all experiments.



Tumor cell	Cell number x10³ Medium	IFNγ	% of Control
DA-3	950	800	84
DA-3/TM	970	830	86
DA-3/sec	1760	960	55
DA-3/sec22	1120	550	49

Figure 6. Stat1 expression in DA-3 tumor cells delays tumor progression. *A,* 1×10^6 DA-3/Stat1 or DA-3/Hygro tumor cells were implanted s.c. in BALB/c animals, and tumor growth was measured at the days indicated. Tumor progression by DA-3/Stat1 cells is delayed relative to DA-3/Hygro cells. *B,* DA-3/sec22 cells were implanted in BALB/c nude mice, and after a tumor developed, it was excised and DA-3/sec22 cells were purified out to produce DA 3/sec22-nudes. Cells were briefly treated with IFN-γ and levels of p-Stat1 were detected by flow cytometry as described in Fig. 4. Levels of p-Stat1 are lower in tumor cells that form a progressively growing tumor in BALB/c nude mice. *C,* 1×10^5 tumor cells were plated *in vitro* alone (*medium*) or in the presence of IFN-γ. After 3 d of culture, IFN-γ treatment greatly decreased DA-3/sec and DA 3/sec22 tumor cell proliferation relative to DA-3 or DA-3/TM tumor cells.

been found to promote cell proliferation through its receptor, uPAR (19). Furthermore, the conversion of plasminogen to plasmin by uPA in the tumor microenvironment leads to the activation of immune-suppressive factors, such as TGF-β, and angiogenic factors, such as MMPs, into their bioactive forms and contributes to tumorigenesis (19, 31, 32). MUC1/sec expression in DA-3 mammary tumor cells leads to the complete down-regulation of uPA, which may play an important role in the rejection of these tumor cells.

The Stat family of transcription factors has been studied in detail, and although Stat3 has been found constitutively active in many tumors (33), Stat1$^{-/-}$ mice are highly susceptible to cancers induced by carcinogens (34). Tumor Stat3 inhibits production of proinflammatory cytokines while inducing factors that hinder adaptive immunity (35). On the other hand, Stat1 reconstitution in tumor cells leads to a down-regulation of proangiogenic factors and renders the cells responsive to IFNs important in immunosurveillance (12). Bowman et al. (36) point out that some transformed cells have both activated Stat1 and Stat3, and they suggest that the growth inhibitory effects of activated Stat1 may be overcome by oncogenic Stat3 expression. In our model, MUC1/sec expression by tumor cells leads to a dramatic up-regulation of tumor suppressive Stat1, and exposure to MUC1/sec conditioned medium up-regulates Stat1 in both mouse and human tumor cells (Fig. 3). However, DA-3 cells, or DA-3 cells expressing either MUC1/sec or MUC1/TM, express relatively the same levels of Stat3. Thus, rather than Stat3 overcoming Stat1, it is possible that a higher ratio of Stat1 to Stat3 within a cell determines whether the scale tips from a protumorigenic profile to one that is antitumorigenic (Fig. 3*C*). Furthermore, in this report, we are showing for the first time that Stat1 expression in tumor cells can lead to the down-regulation of the important protumorigenic factor, uPA (Fig. 5*B*), which has been correlated with tumor aggressiveness in both mouse and human cancers (11).

We have previously reported that DA-3/sec cells do not develop tumors in immunocompetent BALB/c animals but do form tumors that grow for up to 3 weeks in IFN-γ$^{-/-}$ BALB/c animals (8). These tumors eventually regress, and from experiments in T-cell–depleted mice or BALB/c nude mice, we concluded that the permanent removal of DA-3/sec cells requires T cells. However, from these studies, we hypothesized that IFN-γ is important in the early rejection of tumor cells. Although T cells are required for permanent rejection, Fig. 1 shows that in BALB/c nude mice lacking T cells, initial development of DA-3/sec tumors is greatly delayed relative to DA-3/TM tumors that develop in both immunocompetent and nude BALB/c animals. IFN-γ is known to play a great role in the cancer-immunoediting process and can serve as a bridge between innate and adaptive immunity (37). One of the downstream signaling targets of IFN-γ is Stat1, which upon activation, leads to Stat1 phosphorylation and trafficking into the nucleus for activation of various genes. Although DA-3/sec cells do not show activated p-Stat1 *in vitro*, they are capable of activating Stat1 upon brief exposure to IFN-γ (Fig. 4). *In vivo* exposure to IFN-γ would thus have a greater effect on DA-3/sec cells relative to DA-3 or DA-3/TM cells, due to the dramatically up-regulated levels of Stat1. In fact, excising a DA-3/sec22 tumor that eventually develops in BALB/c nude mice showed that DA-3/sec22-nude cells have 30% lower levels of activated Stat1 upon exposure to IFN-γ (Fig. 6*B*). This suggests that IFN-γ immunoedits DA-3/sec tumor cells in BALB/c nude mice and likely contributes to their rejection in immunocompetent BALB/c animals. *In vitro* exposure to IFN-γ was capable of blocking 50% of cellular proliferation of both DA-3/sec and DA-3/sec22 cells, although only reducing proliferation of DA-3 and DA-3/TM cells by 15%. This block in proliferation upon exposure to IFN-γ may also occur *in vivo*, but in addition, there are likely numerous effects of activation of Stat1 in DA-3/sec cells that enhances the antitumor immune response. In addition to the direct effects of IFN-γ on the tumor cells, others have shown that IFN-γ has effects on host cells that enhances production of other antitumor factors by innate cells, such as iNOS and H_2O_2 (38). Li et al. (39) have shown that IFN-γ produced by innate immune cells was sufficient to provide tumor immunity, but that the levels of IFN-γ required were only induced in the presence of T cells. This cross-talk between innate cells and T cells did not require that T cells make IFN-γ themselves. It is thus possible that in BALB/c nude mice, IFN-γ produced by innate cells can control early tumor growth by DA-3/sec, but that ultimately, T cells are required. In immunocompetent BALB/c animals, this mechanism likely plays a crucial role in the early induction of an efficient adaptive immune response that permanently eliminates MUC1/sec-expressing DA-3 cells.

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References

1. Hull SR, Bright A, Carraway KL, et al. Oligosaccharide differences in the DF3 sialomucin antigen from normal human milk and the BT 20 human breast carcinoma cell line. Cancer Commun 1989;1:261–7.
2. Lloyd KO, Burchell J, Kudryashov V, Yin BW, Taylor-Papadimitriou J. Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells. J Biol Chem 1996;271:33325–34.
3. Taylor-Papadimitriou J, Burchell JM, Plunkett T, et al. MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 2002;7:209–21.
4. Imbert Y, Darling DS, Jumblatt MM, et al. MUC1 splice variants in human ocular surface tissues: possible differences between dry eye patients and normal controls. Exp Eye Res 2006;83:493–501.
5. Hey NA, Meseguer M, Simon C, et al. Transmembrane and truncated (SEC) isoforms of MUC1 in the human endometrium and fallopian tube. Reprod Biol Endocrinol 2003;1:2.
6. Obermair A, Schmid BC, Packer LM, et al. Expression of MUC1 splice variants in benign and malignant ovarian tumours. Int J Cancer 2002;100:166–71.
7. Wreschner DH, Hareuveni M, Tsarfaty I, et al. Human epithelial tumor antigen cDNA sequences. Differential splicing may generate multiple protein forms. Eur J Biochem 1990;189:463–73.
8. Grosso JF, Herbert LM, Owen JL, Lopez DM. MUC1/sec-expressing tumors are rejected *in vivo* by a T cell-dependent mechanism and secrete high levels of CCL2. J Immunol 2004;173:1721–30.
9. Herbert LM, Grosso JF, Dorsey M, Jr., et al. A unique mucin immunoenhancing peptide with antitumor properties. Cancer Res 2004;64:8077–84.
10. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329–60.
11. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. Nat Rev Mol Cell Biol 2002;3:932–43.
12. Huang S, Bucana CD, Van Arsdall M, Fidler IJ. Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells. Oncogene 2002;21:2504–12.
13. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3:651–62.
14. Widschwendter A, Tonko-Geymayer S, Welte T, et al. Prognostic significance of signal transducer and

activator of transcription 1 activation in breast cancer. Clin Cancer Res 2002;8:3065–74.
15. Fu YX, Watson G, Jimenez JJ, Wang Y, Lopez DM. Expansion of immunoregulatory macrophages by granulocyte-macrophage colony-stimulating factor derived from a murine mammary tumor. Cancer Res 1990; 50:227–34.
16. Carlsson G, Ekelund L, Stigsson L, Hafstrom L. Vascularization and tumour volume estimations of solitary liver tumours in rats. Ann Chir Gynaecol 1983; 72:187–91.
17. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 1980;102:196–202.
18. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007;7:41–51.
19. Mondino A, Blasi F. uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol 2004;25: 450–5.
20. Bickerstaff AA, Xia D, Pelletier RP, Orosz CG. Mechanisms of graft acceptance: evidence that plasminogen activator controls donor-reactive delayed-type hypersensitivity responses in cardiac allograft acceptor mice. J Immunol 2000;164:5132–9.
21. Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor β 1 by plasmin. J Cell Biol 1990; 110:1361–7.
22. Ceriani RL, Thompson K, Peterson JA, Abraham S. Surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule. Proc Natl Acad Sci U S A 1977;74:582–6.
23. Lapointe J, Li C, Higgins JP, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A 2004;101: 811–6.
24. Beatty PL, Plevy SE, Sepulveda AR, Finn OJ. Cutting edge: transgenic expression of human MUC1 in IL-10−/− mice accelerates inflammatory bowel disease and progression to colon cancer. J Immunol 2007;179:735–9.
25. Hinoda Y, Ikematsu Y, Horinochi M, et al. Increased expression of MUC1 in advanced pancreatic cancer. J Gastroenterol 2003;38:1162–6.
26. Young MR, Neville BW, Chi AC, et al. Oral premalignant lesions induce immune reactivity to both premalignant oral lesions and head and neck squamous cell carcinoma. Cancer Immunol Immunother 2007;56: 1077–86.
27. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 2004;4:45–60.
28. Monti P, Leone BE, Zerbi A, et al. Tumor-derived MUC1 mucins interact with differentiating monocytes and induce IL-10–12low regulatory dendritic cell. J Immunol 2004;172:7341–9.
29. Rughetti A, Pellicciotta I, Biffoni M, et al. Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells. J Immunol 2005;174:7764–72.
30. Ferrero E, Vettoretto K, Bondanza A, et al. uPA/uPAR system is active in immature dendritic cells derived from CD14+CD34+ precursors and is down-regulated upon maturation. J Immunol 2000;164:712–8.
31. Sonoda KH, Nakamura T, Young HA, et al. NKT cell-derived urokinase-type plasminogen activator promotes peripheral tolerance associated with eye. J Immunol 2007;179:2215–22.
32. Shapiro RL, Duquette JG, Roses DF, et al. Induction of primary cutaneous melanocytic neoplasms in urokinase-type plasminogen activator (uPA)-deficient and wild-type mice: cellular blue nevi invade but do not progress to malignant melanoma in uPA-deficient animals. Cancer Res 1996;56:3597–604.
33. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
34. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 1998;95:7556–61.
35. Wang T, Niu G, Kortylewski M, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med 2004;10:48–54.
36. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–88.
37. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48.
38. Pulaski BA, Smyth MJ, Ostrand-Rosenberg S. Interferon-γ-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. Cancer Res 2002;62: 4406–12.
39. Li Z, Pradera F, Kammertoens T, et al. Cross-talk between T cells and innate immune cells is crucial for IFN-γ-dependent tumor rejection. J Immunol 2007;179: 1568–76.