Tumorigenesis and Neoplastic Progression

Tumor Suppressive Protein Gene Associated with Retinoid-Interferon-Induced Mortality (GRIM)-19 Inhibits src-Induced Oncogenic Transformation at Multiple Levels

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Interferons (IFNs) inhibit the growth of infectious pathogens and tumor development. Although IFNs are potent tumor suppressors, they modestly inhibit the growth of some human solid tumors. Their weak activity against such tumors is augmented by co-treatment with differentiation-inducing agents such as retinoids. Previous studies from our laboratory identified a novel gene product, gene associated with retinoid-interferon-induced mortality (GRIM)-19, as an IFN/all-*trans* retinoic acid-induced growth suppressor. However, the mechanisms of its growth suppressive actions are unclear. The *src*-family of tyrosine kinases is important regulators of various cell growth responses. Mutational activation of *src* causes cellular transformation by altering transcription and cytoskeletal properties. In this study, we show that GRIM-19 suppresses *src*-induced cellular transformation *in vitro* and *in vivo* by down-regulating the expression of a number of signal transducer and activator of transcription-3 (STAT3)-dependent cellular genes. In addition, GRIM-19 inhibited the *src*-induced cell motility and metastasis by suppressing the tyrosyl phosphorylation of focal adhesion kinase, paxillin, E-cadherin, and γ -catenin. Effects of GRIM-19 on *src*-induced cellular transformation are reversible in the presence of specific short hairpin RNA, indicating its

direct effect on transformation. GRIM-19-mediated inhibition of the *src*-induced tyrosyl phosphorylation of cellular proteins, such as focal adhesion kinase and paxillin, seems to occur independently of the STAT3 protein. GRIM-19 had no significant effect on the cellular transformation induced by other oncogenes such as *myc* and *Ha-ras*. Thus, GRIM-19 not only blocks *src*-induced gene expression through STAT3 but also the activation of cell adhesion molecules. (Am J Pathol 2007, 171:1352–1368; DOI: 10.2353/ajpath.2007.070241)

The interferon (IFN) family of cytokines regulate development of neoplasia¹ by acting as a tumor surveillance system *in vivo*.² It has been shown in preclinical and clinical studies³ that IFN-induced tumor growth suppression is augmented by differentiation inducers, such as the retinoids,^{4,5} a class of vitamin A metabolites and synthetic homologues. A number of IFN-regulated gene products act as tumor suppressors. For example, mutations in the genes or a loss of their expression of IRF1,⁶ ICSBP,^{7,8} RNaseL,⁹ and DAPK1¹⁰ have been implicated in tumor development in humans and animal models. Each of these proteins targets a different factor(s) involved in cell growth. However, there might be other undefined tumor suppressors controlled by IFNs, given their pleiotropic effects on cells.

We have shown earlier that IFN/all-*trans* retinoic acid (RA) synergistically inhibits tumor growth via induction of apoptosis.⁴ It is not clear what gene products mediate the anti-tumor actions of IFN/RA. Although gene-microarray profiling was used in cataloging the IFN-induced

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genes,¹¹ all genes identified with this method need not necessarily be related to growth suppression. Because IFN/RA induces growth suppression in many cancer cells via an induction of apoptosis, we have applied a genetic method that directly identifies the genes involved in this process.^{3,12,13} In this approach a library of antisense cDNAs, expressed from an episome, is transfected into cells, which are then continuously selected with IFN/RA for identifying surviving cell clones.³ The library-derived antisense RNA-mediated repression of specific endogenous death-associated genes selectively permits the survival of cells in the presence of IFN/RA. The episomes are rescued from the cell clones and sequenced for identification. Based on their original function, we named them as genes associated with retinoid-IFN-induced mortality (GRIM). GRIM-19, one such novel gene product, codes for a 16-kd protein that is present in both nuclear and cytoplasmic compartments. In human breast, prostatic, and renal carcinoma cells, overexpression of GRIM-19 induces apoptosis, which is further augmented by IFN/RA.^{13–15} More recently, we have shown that a loss of GRIM-19 expression occurs in human renal cell carcinomas.¹⁴ The presence of endogenous inhibitors of GRIM-19¹⁶ and mutations in the GRIM-19 gene¹⁷ have been documented in some esophageal and thyroid tumors, respectively. The apoptotic effects of GRIM-19¹³ are also inhibited by certain DNA viral oncoproteins.¹⁸ Together these observations indicate a potential tumor suppressor-like function for this protein.

Oncogenic proteins alter gene expression patterns during cellular transformation. Antioncogenic proteins restrain them for maintaining normal cell growth. However, the role of GRIM-19 in regulating oncogene-induced cell proliferation and tumor formation are unclear. We show here that GRIM-19 overrides src-induced cellular transformation, metastasis, and the expression of genes involved in cell proliferation. One target for GRIM-19 is the transcription factor STAT3 (signal transducer and activator of transcription-3),^{19,20} whose unregulated activity has been suggested to promote tumor development.²¹ It had no effect on myc- and Ha-ras-induced cellular transformation. Although we presumed that GRIM-19 might interfere with the transcriptional activity of STAT3 in src-transformed cells, it also inhibited injury-induced cell migration; phosphorylation of several proteins involved in cell adhesion, such as focal adhesion kinase (FAK), E-cadherin, γ -catenin, and paxillin; and formation of tumors *in vivo*. The inhibitory effect of GRIM-19 on the src-induced phosphorylation of cellular protein seems to occur independently of the STAT3 protein. These data for the first time show that the tumor suppressive effect of GRIM-19 is exerted at multiple levels on activated src.

Materials and Methods

Plasmids and Antibodies

Mammalian expression vectors for Src* (Upstate Biotechnology, Lake Placid, NY) and v-src (provided by Curt Horvath, Northwestern University, Evanston, IL) were

used in these studies. The mammalian expression vector pCXN2-myc and its derivative pCXN2-GRIM-19-myc were described elsewhere.¹³ STAT3-responsive TKS3-Luc, cdc-Luc, and cyclin B1-Luc reporters were described in our previous publication.¹⁹ A *c-myc* expression vector was provided by Robert Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA. c-fos-Luc was described earlier.²² Antibodies specific for STAT3, phospho-STAT3 Y⁷⁰⁵ and phospho-STAT3-S⁷²⁷, Src and phospho-Src-Y⁴¹⁶, and myc-epitope (Cell Signaling Technology, Beverly, MA); actin (Sigma-Aldrich, St. Louis, MO); Ki-67 (Oncogene Science, Cambridge, MA); phosphotyrosine plus (Santa Cruz Biotechnology, Santa Cruz, CA), paxillin, FAK, γ -catenin (BD Biosciences, Franklin Lakes, NJ), histone H1 (Upstate Biotechnology); rabbit anti-*c-myc* polyclonal antibodies (N-262; Santa Cruz Biotechnology); and tubulin (Zymed, South San Francisco, CA) were used in these studies. The monoclonal antibody against myc-epitope, because of its low affinity, does not detect the endogenous *c-myc* protein. Specific antibodies against phospho Y¹¹⁸ and native paxillin (Cell Signaling Technology); p-FAK-Y⁵⁷⁶ and native FAK (Upstate Biotechnology), were used in some of these experiments.

Lentiviral shRNAs

Lentiviral expression vectors carrying shRNAs (short hairpin RNAs) specific for GRIM-19 and STAT3 were purchased from Open Biosystems, Inc., Huntsville, AL, and virus stocks were prepared as recommended by the supplier. The GRIM-19-specific shRNA (pSh-G19) has the following sequence: CCGGCATCGACTACAAACGG-**AACTTCTCGAGAAGTTCCGTTTGTAGTCGATGTTTT**-TG. The STAT3-specific shRNA (psh-S3) has the following sequence: CCGGCCTGAGTTGAATTATCAG-**CTTCTCGAGAAGCTGATAATTCAACTCAGGTTTTT**G. In both cases, the bold nucleotides were scrambled to generate scrambled control shRNA constructs (pSc-G19 and pSc-S3), which could not target the endogenous mRNAs for degradation. All transcript-specific and control shRNAs were expressed using pLKO1-puro (Open Biosystems, Inc.), a lentiviral expression vector, in which shRNAs are generated under the control of human U6 promoter. The shRNA oligonucleotides were inserted into the AgeI and EcoRI sites of pLKO1-puro. This vector also carries a puromycin resistance marker gene under the control of human phosphoglycerate kinase 1 (PGK1) gene promoter, which allows the selection of transfected cells. For generating lentiviral particles, this plasmid was co-transfected along with helper plasmids into human embryonic kidney 293T cells as described earlier.^{23–25} A packaging plasmid pCMV-dR8.2dvpr and a plasmid pCMV-VSVg that codes for the envelope protein were obtained from Addgene Inc., Cambridge, MA. Each shRNA expression plasmid (3 μ g) was mixed with pCMV-dR8.2dvpr (2.7 μ g) and pCMV-VSVg (0.3 μ g) vectors and transfected into human embryonic kidney 293T cells using the Eugene 6 reagent (Roche, Indianapolis, IN). Media from these cultures were collected daily, pooled,

A

Table 1. Primers Used for qRT-PCR

Transcript	Primer sequence	Product size (bp)
Cyclin B1	Fwd: 5'-GGAACATCTGGATGTGCACCTGC-3' Rev: 5'-GGATGGCTCTCATGTTTCCAG-3'	224
Cyclin D1	Fwd: 5'-GAAAATCGTGGCCACCTGG-3' Rev: 5'-GTAGATGCACAACCTCTCGGC-3'	211
c-myc	Fwd: 5'-CGCGCCCACTGAGGATATC-3' Rev: 5'-CCACATACAGTCCTGGATGAT-3'	280
PKD 4	Fwd: 5'-GGACTTCGGTTCAGAAAATGC-3' Rev: 5'-GCCCAGAAGACCAGAAAG-3'	212
rpl 32	Fwd: 5'-TTAAGCCTAACTGGCGGAAACC-3' Rev: 5'-CAGTAAGATTTGTTGCACATCAGC-3'	200

and passed through a 0.45- μ m filter and used as source for lentiviral shRNAs. Knockdown of the target gene was assessed by performing a Western blot analysis with specific antibodies.

Establishment of Stable Cells

3Y1, a nononcogenic rat fibroblastic line (JCRB0734; Japanese Collection of Research Bioresources, Osaka, Japan), was grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.²⁶ This cell has two point mutations in the p53 tumor suppressor gene, resulting in the following amino acid substitutions K¹³⁰T and A¹³⁶T in one of the alleles.²⁷ These mutations do not belong to the category of hotspot mutations observed in human tumors that destroy its DNA-binding capacity.²⁸ The mutational status of Rb in these cells is unknown at this stage. This cell line expresses twofold lower basal level of endogenous GRIM-19 than HeLa cells (data not shown). Cells were electroporated with the indicated plasmids using the Nucleofector technology (Amaya, Inc., Gaithersburg, MD). Initially, the plasmid transfection efficiency was determined by fluorescence-activated cell sorting analysis of cells after transfecting the pEGFP vector, which codes for the green fluorescent protein. After transfection with the genes of interest, cells were selected with G-418 (750 μ g/ml) for 10 to 12 days. Drug-resistant clones were isolated and expanded. All gene expression studies were conducted using pools of colonies ($n \sim 80$) to avoid a clonal bias. For experiments shown in Figure 1, A–C, cells were seeded directly onto 10-cm culture dishes in an agar-media mixture containing G-418. G-418 was added the top agar layer (in less than 1 ml of culture medium,

which absorbs into the top agar layer) on every 3rd day until the experiment was completed. In experiments that used cells pre-expressing v-src (which are already resistant to G-418), a Zeocin resistance marker plasmid (pcDNA3.1-Zeo; Invitrogen, Carlsbad, CA) was mixed with a GRIM-19 expression vector at a ratio of 1:10 and transfected into cells. Cells were selected with G-418 (750 μ g/ml) and Zeocin (250 μ g/ml) simultaneously to eliminate untransfected cells and enrich the double transfectants.

Gene Expression Analyses

Total RNA was extracted using the RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) as recommended by the manufacturer. Approximately 5 μ g of total RNA was reverse-transcribed using a commercially available Superscript III enzyme (Invitrogen). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using the Mx3005P real-time PCR machine (Stratagene, La Jolla, CA). PCR was performed with gene-specific primers (Table 1) in a 30- μ l final reaction containing ~ 20 ng of cDNA, 200 nmol/L of primers, 0.6 U of TaqDNA polymerase, and SYBR Green dye. Expression of different genes was normalized to *rpl32*. Triplicate reactions were performed for each sample, and each experiment was repeated three times with independent batches of RNA.

Cells were transfected with different Luciferase reporters, along with a β -actin- β -galactosidase reporter for normalization, using Lipofectamine reagent (Invitrogen); and reporter activities were monitored as described earlier.¹⁹ All experiments were repeated at least three times. Western blotting and immunoprecipitation analyses were per-

Figure 1. Effect of GRIM-19 on src-induced cellular transformation. 3Y1 cells were electroporated using the Nucleofector technology (Amaya, Inc.) with the indicated expression vectors, as recommended by the manufacturer, and used for soft-agar growth assays in the presence of continuous selection with G-418 (750 μ g/ml). **A:** Photomicrographs of representative fields from various transfections are shown. **B:** Quantification of colony formation. Bars: mean $\pm$ SE of triplicates. The Western blots below this graph show the expression levels of Src, GRIM-19, and actin proteins in these cells. A portion of the cells used for colony formation assays (**A**) was plated in parallel, without soft agar, and selected with G-418 for 3 weeks. An equal quantity of protein (45 μ g) from each transfectant was used for the Western blot analyses. **C:** Effect of GRIM-19 on myc-induced cellular transformation. The blots below it show the expression of the transfected gene products in the transfectants. Total protein (60 μ g) was used for Western blot analysis with the indicated antibodies. Different antibodies were used for detecting c-myc (N-262, rabbit polyclonal antibody; Santa Cruz Biotechnology) and myc-tagged GRIM-19 (mouse monoclonal antibody, which has a very low affinity for endogenous rodent myc protein; Cell Signaling Technology). **D:** GRIM-19 suppresses soft agar colony formation by cells pretransformed v-src. A v-src-expressing cell line was transfected with various plasmids along with a pcDNA3.1 zeo. Stable cell lines were isolated after selecting for 3 weeks with G-418 (750 μ g/ml) and Zeocin (250 μ g/ml). An equal number of cells (2000 per well in a 12-well plate) was used for soft agar assays. Six replicates were used for each transfectant, and experiments were repeated at least three times. Mean $\pm$ SE colony numbers were plotted. **E:** Western blot (WB) analysis of the expression of transfected genes in various cells. Total protein (60 μ g) was used. Western blotting with actin-specific antibodies was used as a loading control for these blots. Original magnifications, $\times 100$.

formed as reported in our previous publication.¹⁹ Nuclear extracts were prepared as described earlier.²⁹

Soft Agar Colony Formation Assay

For these assays, a bottom layer containing 0.7% agarose with growth medium was poured first. Cells were mixed with growth medium containing 0.35% agarose and seeded on the solidified bottom agar layer. Plates were incubated for ~3 weeks in a humidified incubator at 37°C. Colonies were counted. Triplicate samples were used for each cell line.

In Vitro Wound-Healing Assay

Cells lines expressing various genes were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and penicillin-streptomycin in a six-well plate. Confluent monolayer was scratched with a 200- μ l pipette tip to make a wound. The monolayer was washed twice with phosphate-buffered saline to remove the detached cells, supplemented with full medium, and incubated for 4 hours at 37°C. Images were captured to monitor the cell movement into the wounded area. Each experiment used triplicate samples and was repeated three times.

Cell Migration Assays

These assays were performed using Transwell chambers (6.5-mm diameter, 8- μ m pores; Costar Corp., Cambridge, MA), in which the upper and lower chambers were separated by a polycarbonate membrane. The lower chamber was filled with 0.6 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The upper chamber was seeded with 25,000 cells in 0.1 ml of Dulbecco's modified Eagle's medium without serum and incubated at 37°C for 24 hours. Nonmigrated cells in the upper membrane surface of chamber were removed after swiping gently with a cotton swab, and the cells on the bottom surface of the membrane (migrated population) were fixed with ice-cold methanol and stained with 0.01% crystal violet prepared in 20% ethanol. The number of migrated cells was determined after eluting the bound dye with a mixture of acetic acid-methanol. Absorbance of the eluate was measured at 595 nm. In each case, a parallel control without removing the cells was used. Absorbance obtained with this control was considered as total input. Absorbance obtained with the migrated cells was expressed as a percentage of total input. Migration data obtained with naïve 3Y1 cells were considered as 100%. Triplicate measurements were performed. All experiments were repeated three times.

Cell Growth Assays

Cells (3000 per well) were plated in 96-well plates. Each group had eight replicates. Cells were fixed with trichloroacetic acid (final concentration, 10%) at 4°C for 1 hour at the end of the experiment and stained with 0.4% sul-

forhodamine B (Sigma-Aldrich, Inc.). The bound dye was eluted with 100 μ l of Tris-HCl (pH 10.5), and the absorbance was measured at 570 nm.³⁰ Eight hours after plating the cells, one plate was fixed with TCA for determining the input. Absorbance obtained with this plate provided the input cell numbers.

Tumorigenic Assays

Three- to 4-week-old athymic nude (*nu/nu*) NCr mice (Taconic Farms, Germantown, NY) were used for these studies as described earlier.⁴ Procedures involving animals and their care were conducted in conformity with an institutionally approved protocol that is in compliance with United States national and international laws and policies. Each experimental group contained 10 mice, with one tumor per mouse. Cells (1×10^6) were inoculated into flanks. Tumor volume was calculated using caliper measurements and the formula for a prolate spheroid: $V = (4/3) \times \pi a^2 b$, where $2a$ = minor axis, $2b$ = major axis of prolate spheroid. Student's two-tailed *t*-test was used to assess the statistical significance of difference between pairs of means of tumor volume. Tumor specimens were fixed, sectioned, and stained with Ki-67-specific antibodies using the commercially available Vectastain ABC kit (Vector Laboratories, Burlingame, CA). For metastasis experiments ~50,000 cells per mouse were injected via the tail vein. Animals ($n = 10$) were monitored for metastatic tumor development 6 weeks later by necropsy. Tumors were found only in the lungs.

Results

Effect of GRIM-19 on Src-Induced Cellular Transformation

Previous studies from our laboratory have shown that the overexpression of GRIM-19 in several human cancer cell lines suppresses cell growth and induces apoptosis.^{13,15,18} Because these tumor cell lines may have acquired multiple genetic alterations and consequently possessed several different activated-oncogenic gene products, it was difficult to pinpoint if GRIM-19 inhibited a specific activated oncogenic event in those studies. Therefore, we transfected a nononcogenic rat fibroblastic cell line 3Y1 with expression vectors coding v-src and Src*. In the case of Src* the negatively regulated phospho-acceptor Y⁵²⁷ was mutated to a phenylalanine to generate an active form.³¹ We also coexpressed GRIM-19 to determine its impact on Src-induced cellular transformation. Transfection of these Src variants into cells caused a robust cellular transformation, as revealed by the formation of numerous soft agar colonies (Figure 1, A and B). Src*-transformed colonies were consistently smaller in size than those transformed by v-src. In the presence of GRIM-19, significantly fewer src-transformed soft agar colonies appeared ($P > 0.005$). These colonies were very small in size when compared with those formed by v-src and src*. Although GRIM-19 induces apoptosis in several carcinoma cells,³ it did not cause a significant apoptosis

in these fibroblasts. Under our conditions, less than 6% of 3Y1 cells underwent apoptosis (data not shown). The plasmid transfection efficiency is ~45% in these experiments. Thus, the reduction in the numbers of src-induced soft agar colonies in the presence of GRIM-19 cannot be solely attributed to GRIM-19-induced loss of transfected cells because of rapid apoptosis. In fact, we were able to recover cells expressing both gene products (see below). Even though GRIM-19 alone causes some apoptosis in certain carcinoma cell lines, a moderate expression of it is tolerated, but such cells grew slower than the controls.¹³ As expected, the empty vector (pCXN2) and GRIM-19 alone did not cause any cellular transformation. Thus, GRIM-19 suppresses Src-induced cellular transformation. We have also verified the overexpression of GRIM-19 and Src proteins in a parallel experiment. A baseline expression of endogenous src protein was seen in vector- and GRIM-19-transfected cells. Approximately 2.5-fold more Src protein was present in the transfectants. The presence of GRIM-19 did not significantly alter Src expression in these cells. Last, GRIM-19 did not block myc (Figure 1C)- and activated Ha-ras (data not shown)-induced cellular transformation in this model, indicating its specificity toward src. There was a basal level of expression of c-myc in 3Y1 cells, which was increased by approximately threefold after transfection with the myc-expression vector (see the Western blots below, Figure 1C). The presence of GRIM-19 did not down-regulate the expression of myc protein.

Because the above experiment showed the effect of a co-transfected GRIM-19 on src-induced cellular transformation, we next examined if GRIM-19 could similarly suppress the colony formation by a cell line expressing an activated src. Therefore, we stably transfected GRIM-19 into a 3Y1 cell line that was already transformed by v-src. The resultant cell colonies were used for the soft-agar and cell growth assays. To avoid a clonal bias, we used pooled populations of stable colonies for these experiments. As expected, several soft agar colonies were observed with the v-src transformants. Transfection of GRIM-19, but not the empty vector, caused a marked reduction in the number of colonies (Figure 1D). The GRIM-19-transfected v-src-expressing cells formed significantly fewer colonies ($P > 0.01$). We have also ascertained the expression of Src and GRIM-19 in these cells (Figure 1E). A twofold higher amount of Src protein was found in the v-src-transfected cells compared with the control. As expected, the myc-tagged GRIM-19 protein was detected only in cells transfected with pCXN2-GRIM-19-myc. A comparable loading of protein was ensured by probing these blots with actin-specific antibody.

GRIM-19 Inhibits Cell Injury and Mitogen-Driven Migration of Src-Transformed Cells

One characteristic of cancer cells is higher motility than normal cells, which permits them to be metastatic. Therefore, in the next experiment we examined whether GRIM-19 affected src-induced cell motility. We used in-

jury- and mitogen-induced migration assays for these studies. In the injury-induced migration assays, a scratch was introduced into a confluent monolayer of cells. Cell migration into the denuded area was monitored 4 hours later (Figure 2A). Fewer cells migrated into the injured area when this experiment was performed with 3Y1 cells expressing GRIM-19 or pCXN2. However, a number of cells expressing the v-src and src* proteins migrated into the injured area under the same conditions. Such rapid migration was virtually absent in src*/GRIM-19 and v-src/GRIM-19 cell lines. These data show an inhibitory effect of GRIM-19 on src-induced cell motility.

Cell motility was also assessed using another approach. In these experiments, migration of cells across a polycarbonate membrane toward growth medium was monitored using a colorimetric assay (Figure 2B). A significantly higher ($P > 0.01$) percentage of cells expressing src* or v-src migrated across the membrane toward growth medium in these assays, compared with the vector-alone-transfected cells. GRIM-19 completely suppressed src*- or v-src-induced migration. GRIM-19 alone did not have any statistically different effect on cell motility compared with the control.

Next, cells expressing the Src and GRIM-19 combinations were used for growth assays using sulforhodamine B staining as described previously.³⁰ Equal numbers of cells from different cell lines were plated; and cell growth was monitored after 2 and 4 days (Figure 2C). The v-src- or src*-transfected cells grew robustly compared with the controls expressing pCXN2 or GRIM-19 alone. However, in the presence of GRIM-19 there was a significant decline ($P > 0.005$) in src-promoted growth. The starting cell numbers were comparable among various cell lines, indicating no differences in plating efficiency. Thus, the differences in cell growth patterns are not attributable to variations in the input numbers of cells.

Last, we examined if these differential effects of src on cell growth were related to phosphorylation of Src at the critical Y⁴¹⁶ using Western blot analyses with pY⁴¹⁶-src-specific antibody (Figure 2D). A normal tyrosine phosphorylation of Y⁴¹⁶ was observed in the src-transfected cells, consistent with transforming characteristics described above. In the presence of GRIM-19, a significant reduction of src phosphorylation at Y⁴¹⁶ occurred, although it was not directly proportional to the amount of GRIM-19 expression. The difference in the levels of pY⁴¹⁶-src cannot be attributed to a reduction of total src levels in the cells, because all src transfectants had a comparable expression.

In Vivo Effects of GRIM-19 on Src-Induced Tumor Formation

To determine whether src-transformed cells have differential abilities to form tumors in the presence of GRIM-19, 3Y1 cell lines carrying various genes were transplanted into athymic nude mice, and tumor formation was monitored for 25 days. As shown in Figure 3, A and B, the pcDNA 3.1-transfected cells grew slowly and formed small size nodules, especially at the end of the 3rd week.

Figure 2. Effect of GRIM-19 on src-dependent cell motility. **A:** Injury-induced migration of cells into the wounded area was monitored. White line indicates the edge of injured site. Note the rapid migration of src* and v-src-expressing cells, but not the controls, into the wounded area. **B:** Invasion assay was performed using Transwell chambers. Serum (10%) was used as an attractant in the lower chamber. Cells ($n = 25,000$) were seeded in the upper chamber of insert and incubated for 24 hours, migrated cells were fixed and stained with crystal violet, and the bound dye was eluted and quantified at 595 nm. **C:** Growth of various cell lines as measured by a colorimetric assay. **D:** Western blot analysis of src expression and its activating phosphorylation (pY⁴¹⁶) in the transfectants. Approximately 75 μ g of total protein was used for Western blotting. Original magnifications, $\times 100$.

The *in vivo* growth of these cells was not significantly different from naïve 3Y1 cells (data not shown). The src-expressing cells formed large tumors, which grew robustly compared with those expressing Src/GRIM-19. GRIM-19 significantly knocked down the ability of src to form tumors ($P > 0.001$). Remarkably, GRIM-19 even depressed the baseline growth of 3Y1 cells ($P > 0.05$) in this model, indicating its growth-suppressive effect. This observation is consistent with the growth inhibitory effect of GRIM-19 *in vitro* in other cell types.^{13–15} Although not apparent in Figure 2C, because of the scale, there was some retardation of growth of 3Y1 cells *in vitro*, especially on the 2nd day, after the expression of GRIM-19. Last,

mitogen-induced motility of GRIM-19-expressing cells is relatively slower compared with the vector-transfected cells ($P > 0.07$). These probably are responsible for the slower growth of GRIM-19-alone-transfected 3Y1 cells *in vivo*. We also examined a cellular basis for the loss of tumor formation by examining the intratumoral expression of a proliferation-associated antigen, Ki-67 (Figure 3, insets). As expected, the src tumors stained highly positive for Ki-67. The tumors carrying GRIM-19 and pCDNA3.1 were negative for Ki-67. More importantly, the src/GRIM-19 and v-src/GRIM-19 tumors had an extremely low expression of Ki-67. Thus, GRIM-19 overrides the src-dependent tumor proliferation *in vivo*.

Figure 3. Effect of GRIM-19 on the growth of Src-expressing cells *in vivo*. Cell lines (in 3Y1 background) expressing various genes were subcutaneously transplanted into athymic nude mice ($n = 10$), and tumor growth was monitored. Untransfected 3Y1 cells were used as an additional control in these experiments. The growth of these cells was similar to the pCDNA3.1-transfected cells (data not shown). **Insets** show the immunohistochemical analyses of the tumors grown in nude mice. Tumors were excised and fixed, and an immunohistochemical analysis was performed with Ki-67-specific antibodies. Original magnifications, $\times 40$.

In a separate experiment cells were injected into mice via tail vein for determining their metastatic potential. Metastases were monitored after 6 weeks by performing necropsy. Metastases were found only in the lungs. The number of metastases found in each case was counted and is shown in Table 2. Neither the vector- nor the GRIM-19-expressing cells formed any metastases. The v-src-transfected cells formed the highest number of metastases with an average of 2.3 metastases per mouse. The v-src/GRIM-19 cells formed significantly ($P > 0.005$) fewer numbers of metastases compared with the control (13% of that observed v-src-transformed cells).

GRIM-19 Suppresses the src-Induced Transcription of a Number of STAT3-Regulated Genes

We next investigated a molecular basis for the loss of src-induced oncogenic function. One of the targets of

Table 2. Effect of GRIM-19 on Metastases Induced by v-src

Cells expressing	Number of metastases	Average per mouse	Percent
pCXN2	0	0	0.00
GRIM-19	0	0	0.00
v-src	23*	2.3	100.00
v-src/GRIM-19	3	0.3	13.04

Athymic nude mice were injected 0.5×10^5 in a volume of 0.1 ml via the tail vein. $n = 10$ mice/group. Six weeks later, mice were euthanized, and necropsy was performed. Metastases in the lungs of each group were counted.

* $P > 0.05$.

activated Src is transcription factor STAT3.^{32,33} Recent studies in human cell lines have shown that STAT3 induces a number of genes involved in growth promotion.^{34,35} We have selected several growth-associated genes, with known rodent homologues, for these analyses, and their relative expression was quantified using qRT-PCR. Three of these, *c-myc*, cyclin B1, and cyclin D1, are involved in cell proliferation.^{36–38} One other gene, *PKD4*, a protein kinase involved in the regulation of carbohydrate metabolism,³⁹ is down-regulated in cells expressing high STAT3.³⁴ As shown in Figure 4A, the expression of cyclin B1, cyclin-D1, and *c-myc* transcripts was induced severalfold more than the controls in the presence of the src* and v-src proteins, and GRIM-19 inhibited it. The levels of these transcripts in v-src/GRIM-19 and src*/GRIM-19 cell lines were comparable with or lower than those observed in cells expressing GRIM-19 or vector alone. In the case of *c-myc*, GRIM-19 caused a stronger repression of the src-induced expression of the gene, even below the baseline level. A converse picture was seen with *PKD4*; it was significantly ($P > 0.01$) repressed in the presence of activated src proteins. GRIM-19 derepressed the src-induced inhibition of *PKD4* expression. Thus, GRIM-19 antagonizes the effects of src on specific cellular genes.

To determine whether these effects are exerted at the transcriptional level, we transfected luciferase reporter plasmids driven by the native promoters of three different STAT3-regulated genes, *cdc2*, cyclin B1, and *c-fos*, into the cells and measured gene expression (Figure 4B). The expression of all reporters was induced severalfold by the src proteins. In every case, GRIM-19 suppressed the src-induced transcription of the reporters. Another reporter, TKS3-Luc, was driven by minimal STAT3-binding elements. These data show that src-induced STAT3-driven gene expression occurring through different native promoter elements, and the minimal STAT3-responsive promoter element(s) is inhibited by GRIM-19. GRIM-19 did not exert a negative effect on the CMV-enhancer-driven transcription (Figure 5), indicating its specificity.

Because the activity of STAT3 is regulated by phosphorylation at the Y⁷⁰⁵ (dimerization) and S⁷²⁷ (transactivation) residues,⁴⁰ we next examined the influence of GRIM-19 on the phosphorylation status of STAT3 (Figure 4C). As expected, the src proteins, in particular v-src caused a strong tyrosine phosphorylation of the Y⁷⁰⁵ residue. Src* also mildly, but significantly, increased it. In

Figure 4. GRIM-19 represses src-dependent expression of STAT3-inducible genes. **A:** Endogenous gene expression was quantified using qRT-PCR, and transcript abundance was measured after normalizing to an internal control, rpl32. Baseline level of each transcript (pCXN2 vector-transfected cells) was subtracted from the experimental samples. **B:** Effect of GRIM-19 on src-induced expression of the Luciferase reporters driven by the STAT3-responsive gene promoters. The indicated luciferase reporters and a β -galactosidase reporter (0.2 μ g each) were transfected into the indicated stable cell lines. Luciferase activity was normalized to that of β -galactosidase and plotted. Bars, relative luciferase activity (RLA) $\pm$ SE RLA was calculated and plotted after comparing to the data obtained with pCXN2-3Y1 cells. **C:** Effect of GRIM-19 on the phosphorylation of STAT3. Western blot analyses were performed with the indicated antibodies. **D:** Lack of an inhibitory effect of GRIM-19 on IL-6-induced phosphorylation of STAT3. The indicated 3Y1 cell lines were treated with IL-6 (100 ng/ml) along with soluble IL-6 receptor (100 ng/ml) for 30 minutes (R&D Systems, Minneapolis, MN). Rodent fibroblasts express an extremely low level of the ligand binding chain of the IL-6 receptor. Hence, we treated these cells with the soluble receptor, during stimulation with IL-6.

Figure 5. Effects of GRIM-19- and STAT3-specific shRNAs on Src-dependent gene expression. **A** and **D**: shRNA-mediated knockdown of GRIM-19 and STAT3 expression in cells. Approximately 150 μ g of total protein was used for Western blot analysis. **B**: Effect of STAT3-specific shRNA on v-src-induced expression of the indicated reporter genes. Various plasmids were co-transfected into cells along with the luciferase reporters, and the promoter activity was measured as RLA. **C**: Down-regulation of GRIM-19 by specific shRNA. V-src/GRIM-19 cells were infected with specific lentiviral vectors, and the loss of GRIM-19 expression was monitored using Western blot analyses of the cell extracts (120 μ g) with myc-tag-specific antibodies. **D**: v-src/GRIM-19 cells were infected with lentiviral vectors carrying the indicated shRNAs (overnight) and then transfected with the indicated reporters as indicated in Materials and Methods. **E** and **F**: Effects of STAT3 (**E**) and GRIM-19 (**F**)-specific shRNA on the expression of endogenous STAT3-regulated mRNAs. A qRT-PCR analysis was performed as in Figure 4. Vector- and v-src-alone-transfected 3Y1 cell lines were used as negative and positive controls. For **E** and **F**, v-src and vsrc/GRIM-19-expressing cell lines were infected with the indicated lentiviral vectors, carrying specific shRNA, for determining the effects on gene expression. **G** and **H**: GRIM-19-specific shRNA reverses the inhibitory effect of GRIM-19 on soft agar colony formation in v-src/GRIM-19 cells. Cells were infected with lentiviral vectors coding for the indicated shRNAs and then used for soft agar colony formation assays. **I**: Interaction between GRIM-19 and STAT3. Immunoprecipitation and Western blot analyses were performed with the indicated nuclear extracts (250 μ g). Immunoprecipitation and Western blot were performed with myc-tag- and STAT3-specific antibodies, respectively. Histone H1 antibodies were used as an internal control.

the presence of GRIM-19 such phosphorylation was strongly reduced, consistent with the src pY⁴¹⁶ data (Figure 2). The constitutive phosphorylation of STAT3 at S⁷²⁷ was not affected significantly by src and/or GRIM-19 proteins. The differences in STAT3 phosphorylation were not attributable to a difference in STAT3 levels in these cell lines.

In a previous report we have shown that GRIM-19 does not interfere with cytokine-induced activation of STAT3 via tyrosyl phosphorylation in human cells.¹⁹ To test whether this is also true for rodent cells, we treated the 3Y1 cells expressing pCXN2 and GRIM-19 with interleu-

kin (IL)-6, a known activator of STAT3. In both these cells, no tyrosyl phosphorylation of STAT3 was observed in the absence of IL-6 treatment. However, IL-6 treatment caused an equivalent activation of STAT3 tyrosyl phosphorylation at Y⁷⁰⁵ (Figure 4D). There was no change in the serine phosphorylation at the S⁷²⁷ residue. Total STAT3 levels were comparable under both conditions. Thus, GRIM-19 does not interfere with the cytokine-induced tyrosyl phosphorylation of STAT3. Based on these observations there is a differential inhibition of STAT3 phosphorylation in response to cytokines and activated src oncogene.

To demonstrate the STAT3 dependence of these promoters, we performed a control experiment in which lentiviral shRNA vectors that can target rodent STAT3 were used. First, we confirmed the specificity of the shRNAs by performing a Western blot analysis of STAT3 protein in these cells (Figure 5A). Only the STAT3-specific shRNAs, but not the controls, promoted a loss of STAT3 expression in these cells. We chose a representative STAT3-driven reporter cyclin B1-Luc and a STAT3-independent CMV-Luc for examining the effects of STAT3-specific shRNAs in coexpression studies (Figure 5B). As expected, the expression of cyclin B1-Luc, but not of CMV-Luc, was induced by v-src. In the presence of the STAT3-specific shRNA, the src-induced expression of cyclin B1-Luc was down-regulated. The control shRNA had no such effect on cyclin-B1-Luc. Neither v-src nor the STAT3-shRNA affected the expression of CMV-Luc. Based on these observations, we conclude that GRIM-19 targets STAT3 for suppressing the gene expression driven by v-src.

To determine whether this is a direct effect of GRIM-19, cells were infected with lentiviral shRNAs capable of targeting GRIM-19 into v-src/GRIM-19 cells. In these experiments, we expected a specific reversal of inhibitory effects of GRIM-19 by GRIM-19-specific shRNA on src-induced expression of STAT3-regulated genes. We first confirmed the knockdown of expression of myc-GRIM-19, but not that of STAT3, in these cells using a Western blot analysis of cell extracts (Figure 5C). Only the GRIM-19-specific shRNA ablated GRIM-19 expression in the cells. In the reporter experiments, the control shRNA expression vectors, which lacked any GRIM-19 sequences (pLKO1 or contained a scrambled GRIM-19 sequence (psc-G19), did not promote the v-src-driven expression of cyclin B1-Luc (Figure 5D). However, the lentiviral vectors carrying GRIM-19-specific shRNA reversed the inhibitory effect of GRIM-19 on v-src-driven expression of this reporter. Neither v-src nor GRIM-19 had any effect on CMV-Luc, indicating their promoter-specific effect.

We next examined whether the endogenous STAT3-regulated genes were similarly affected by the STAT3 (Figure 5E)- and GRIM-19 (Figure 5F)-specific shRNAs. We chose cyclin B1 and cyclin D1 as representatives for these experiments. For studying the effects of STAT3 and GRIM-19 shRNAs on the expression of endogenous mRNAs, we used v-src and v-src/GRIM-19 cell lines, respectively. As expected, the scrambled and empty vector controls showed no effect on cyclin B1 and cyclin D1 gene expression in v-src-transfected cells. Consistent with the reporter data, only the STAT3-specific shRNA down-regulated the Src-induced expression of cyclin B1 and cyclin D1 transcripts (Figure 5E). In the complementary experiment, only the GRIM-19-specific shRNA, but not the controls, relieved the inhibitory effects of GRIM-19 on the src-induced expression of these transcripts in v-src/GRIM-19 cells (Figure 5F). In the presence of GRIM-19-specific shRNA, almost all of the v-src-induced expression was recovered (compare v-src control with the psh-G19). These data indicate that no other secondary genetic changes, only GRIM-19, is responsible for the

repression of src-induced gene transcription in the v-src/GRIM-19 cells. To test the biological relevance of this observation, we infected the v-src/GRIM-19 cells with lentiviral particles carrying GRIM-19-specific shRNA and the control scramble shRNA. Only the GRIM-19-specific shRNA, but not the controls, reversed the inhibitory effects of GRIM-19 on colony formation (Figure 5, G and H).

To determine the mechanisms of tumor suppression by GRIM-19, GRIM-19-myc was immunoprecipitated, and the resultant proteins were subjected to Western blot analysis with STAT3-specific antibody (Figure 5I). We have used nuclear extracts for this experiment because both STAT3 and GRIM-19 are present in the cytoplasmic and nuclear compartments of the cells, and it is the nuclear fraction that contributes to transcriptional effects. Furthermore, we wanted to exclude the possibility that GRIM-19 might interfere with the nuclear translocation of STAT3.²⁰ STAT3 was seen in the nuclei only when cells expressed an activated src. Importantly, GRIM-19 did not significantly block the nuclear translocation of STAT3 as reported in another study.²⁰ The myc-tag-specific antibody clearly detected GRIM-19 in cells. Its nuclear localization was not affected by src. The myc-tag-specific antibodies co-immunoprecipitated STAT3 protein only when cells expressed an activated Src. No detectable level of STAT3 was present in the immunoprecipitation products of cells expressing GRIM-19 alone. Thus, GRIM-19 binds only to activated STAT3. There was no change in the levels of STAT3 in the src-expressing cell nuclei. However, there was a reduction in the level of phosphorylation at Y⁷⁰⁵ of STAT3 in the presence of GRIM-19, even though the same amount of nuclear STAT3 was present. Thus, under these experimental conditions, src-induced nuclear migration of STAT3 seems to occur independently of tyrosyl phosphorylation, and GRIM-19 did not prevent it. Last, an equal loading of the proteins was confirmed by probing the blots with histone H1-specific antibodies.

GRIM-19 Blocks Src-Induced Tyrosyl Phosphorylation of Other Cellular Proteins

Because src also phosphorylates multiple cellular proteins for exerting its other effects, such as cell adherence and motility,^{31,41–44} we next examined if GRIM-19 had a similar inhibitory effect on other cellular proteins. Western blot analysis of cell extracts with phosphotyrosine-specific antibody revealed a src-dependent tyrosyl phosphorylation of a number of proteins (Figure 6A, arrows). Such tyrosyl phosphorylation was strongly diminished in the presence of GRIM-19. Notably, the profiles of tyrosyl-phosphorylated proteins between src* and v-src were different, suggesting a probable substrate preference of these proteins. The differences in tyrosyl phosphorylation of proteins do not seem to be attributable to a differential loading of proteins into these lanes, as revealed by a reprobing of these blots with tubulin-specific antibody. Based on the molecular weight data shown in Figure 6A, we have used antibodies against paxillin, FAK, E-cadherin, and γ -catenin for immunoprecipitation analysis.

Figure 6. Effect of GRIM-19 on src-induced phosphorylation of cellular proteins. **A:** Effect of GRIM-19 on src-induced tyrosine phosphorylation of cellular proteins. Total protein (60 µg) was used for a Western blot analysis with phosphotyrosine-specific antibodies. Specific phosphorylated bands are indicated. Common bands between Src* and v-src-expressing cells are indicated with **arrows**. **Arrowheads** indicate the bands differentially phosphorylated between Src* and v-src. A reprobing of the blot with tubulin-specific antibodies showed a comparable loading. **B:** Effect of GRIM-19 on src-induced tyrosyl phosphorylation of various cellular proteins. Immunoprecipitation and Western blot analyses performed with the indicated antibodies. Approximately 500 µg of total cellular protein from each transfectant was used. Blot regions corresponding to the appropriate bands are shown in each case. **C:** Loss of STAT3 does not affect the src-induced cellular protein tyrosyl phosphorylation profiles and the inhibitory effects of GRIM-19 on it. Cells were infected with lentiviral vectors coding for STAT3-specific shRNA (psh-S3) or a control vector (pLKO-1) as in Figure 5. Total cell lysates (70 µg) were used for Western blot analysis with the indicated antibodies. **D:** Effect of STAT3 knockdown on src-induced tyrosyl phosphorylation of paxillin and FAK and its inhibition by GRIM-19. Specific antibodies that detect the pY¹¹⁸ paxillin and pY⁵⁷⁶-FAK proteins were used for the Western analyses. Total cellular lysates (80 µg) were used in each case. Total paxillin and FAK levels were determined using native paxillin- and FAK-specific antibodies. Actin levels were determined for a comparable protein loading.

The products were then subjected to Western blot analyses with phosphotyrosine-specific antibody. Indeed, GRIM-19 inhibited Src-induced tyrosyl phosphorylation of all proteins. Importantly, GRIM-19 alone did not affect the baseline tyrosine phosphorylation of these proteins. The presence of GRIM-19 did not diminish the levels of these proteins in cells. Interestingly, only v-src, but not Src*, activated the phosphorylation of E-cadherin and γ -catenin in these cells. GRIM-19 suppressed such phosphorylation effectively.

Although the above experiments show an inhibitory effect of GRIM-19 on the src-induced tyrosyl phosphorylation of certain cellular proteins, they cannot exclude the possibility that these effects are also exerted through STAT3. To investigate this aspect, we have used a shRNA that could knock down the expression of STAT3 (shown in Figure 5) and determined their influence on the src-induced tyrosyl phosphorylation of cellular proteins. 3Y1 cell lines expressing pCXN2, GRIM-19, v-src, and v-src/GRIM-19 were infected with lentiviral particles carrying shRNA specific for rodent STAT3. After this, cells were selected with puromycin to eliminate uninfected cells. Because no significant differences were noted between the empty vector and scrambled STAT3-shRNAs in the preliminary experiments (data not shown and Figure 5) in terms of their ability to knock down STAT3, we have chosen to show pLKO-1 as a control for these experiments. The effects of STAT3-shRNA (psh-S3) were compared with those of empty vector (pLKO1). First, we examined the global tyrosyl phosphorylation in response to v-src. Several tyrosyl phosphorylated proteins were detected in the v-src cells, compared with the GRIM-19 and pCXN2-transfected cells, on probing of the Western blots with a phosphotyrosine-specific antibody (Figure 6C; compare lanes 1, 3, and 5). The src-induced phosphorylation of such proteins was strongly reduced in the v-src/GRIM-19 cell line (Figure 6C, compare lanes 5 and 7). Most importantly, the STAT3-specific shRNA did not significantly diminish src-induced tyrosyl phosphorylation profile in the v-src cells (Figure 6C, compare lanes 5 and 6). The GRIM-19-mediated inhibition of src-induced phosphorylation of these cellular proteins continued to occur efficiently even after a knockdown of STAT3 (Figure 6C, compare lanes 7 and 8). Last, the shRNA did not affect baseline phosphorylation observed in pCXN2- or GRIM-19-transfected cells. We have confirmed the knockdown of STAT3 in these transfectants. As expected, the STAT3-specific shRNA knocked down (~85% of the control) the expression of endogenous STAT3 in all cell lines to a comparable extent. An equivalent loading of protein in these lanes was ensured by probing these blots with tubulin-specific antibody. We next determined src-induced phosphorylation of specific sites on representative proteins, paxillin and FAK, using phospho-isoform-specific antibodies (Figure 6D). Cell lysates were prepared, and phosphorylation of paxillin at Y¹¹⁸ and FAK at Y⁵⁷⁶ was monitored using specific antibodies. Total levels of paxillin and FAK were also compared in these experiments. The v-src-induced phosphorylation of paxillin at Y¹¹⁸ and FAK at Y⁵⁷⁶ occurred only in the src-expressing cells but not in the controls,

and it was robustly inhibited in the presence of GRIM-19 (Figure 6D; compare lanes 1, 3, 5, and 7). More importantly, there was no discernible difference in paxillin at Y¹¹⁸ and FAK at Y⁵⁷⁶ phosphorylation between the pLKO1 and psh-S3-transfected v-src cells (Figure 6D, compare lanes 5 and 6), indicating its independence from STAT3. Conversely, the GRIM-19-mediated inhibition of v-src-induced phosphorylation of paxillin and FAK was unaffected by the loss of STAT3 expression (Figure 6D, compare lanes 7 and 8). The baseline phosphorylation of these proteins was not affected by the shRNA. Total paxillin and FAK protein levels were comparable among these samples. A comparable loading of proteins was ensured with a probing of these blots with actin-specific antibodies. Thus, the loss of STAT3 expression does not seem to significantly affect either the src-induced phosphorylation of cellular proteins or the inhibitory effects of GRIM-19 on src.

Discussion

In this study we have shown that GRIM-19 suppresses src-oncogene-induced cell growth, cell motility, and tumor formation *in vitro* and *in vivo*. Interestingly, GRIM-19 suppressed cellular transformation by an activated tyrosine kinase src, but not myc, and Ha-ras, indicating its specificity (Figure 1). Although one previous study reported an inhibitory effect of GRIM-19 on v-src-induced colony formation in a very preliminary experiment,²⁰ it did not fully investigate the underlying mechanisms, including *in vivo* effects. Our study provides a detailed insight into the anti-oncogenic effect of GRIM-19. The previous study presumed that all GRIM-19 effects are targeted at STAT3 alone.²⁰ Further, that study assumed that GRIM-19 prevents nuclear translocation of STAT3, which is in contrast to our observations that GRIM-19 and STAT3 are in the nuclei of src-transformed cells (Figure 5I). Expression of a number of STAT3-regulated genes is inhibited at the transcriptional level (Figure 4). More importantly, we find that GRIM-19 not only inhibits the transcriptional activity guided by the activated src but also suppresses the oncogene-induced modifications of proteins involved in cell adhesion and motility (Figure 6). We also showed a suppression of the metastatic activity of v-src-transformed cells in the presence of GRIM-19 (Table 2). Our results are consistent with a previous report that indicated an inhibitory effect of IFNs on the src-induced cellular transformation⁴⁵ via inhibition of pp60src activity. Those studies, however, did not show a molecular basis of the inhibitory action of IFNs on src. IFNs have been reported to exert direct anti-oncogenic activities on a number of oncogenes.^{46–51} For example, the H-ras-induced transformation of NIH-3T3 cells is ablated by lysyl oxidase (also known as rrg, the ras regressor gene)⁴⁸; the myc and fos-induced transformation is inhibited by IRF1,⁵¹ and the BCR-Abl-induced leukemogenesis is suppressed by IRF8.⁸ Our study, thus, identifies another novel anti-oncogenic effect of IFNs, via GRIM-19. It is important to note that although IFN/RA is the most potent inducer of GRIM-19, IFN alone can induce it significantly.¹³

One of the many targets of activated src is transcription factor STAT3.^{32,33,52} STAT3 is transiently activated by the JAKs via tyrosyl phosphorylation in normal cells after cytokine treatment, which is turned off by feedback inhibitors.⁴⁰ In contrast to this, a chronic tyrosyl phosphorylation of STAT3 has been noted in a number of human tumors and tumor cell lines.²¹ Although the tyrosyl phosphorylation of STAT3 is important for some biological processes, especially the cytokine-driven responses,⁴⁰ STAT3 Y⁷⁰⁵F, a mutant that cannot be tyrosyl phosphorylated, is still capable of inducing the expression of a number of cellular genes involved in growth promotion.^{35,53,54} For example, the *SOCS-3*, *c-myc*, *dp1*, *c-fos*, *c-jun*, and *Bcl-x*, *cyclin B1*, *cdc2*, and *m-ras* genes are induced by Y⁷⁰⁵F mutant of STAT3. Some of these genes were examined in this report. We have already shown that inactivation of the critical S⁷²⁷ residue in the transactivation domain of STAT3 strongly diminishes the binding of GRIM-19.¹⁹ Based on the above observations, we believe that GRIM-19 specifically inhibits the transcriptionally active STAT3 and these effects can be Y⁷⁰⁵-independent. Indeed, src induced an equivalent nuclear migration of STAT3, despite a diminution of Y⁷⁰⁵ phosphorylation (Figure 5I). It is likely that such nuclear migration of STAT3 may occur through other mechanisms. For example, a recent study showed that the STAT3-Y⁷⁰⁵F mutant could migrate to nucleus and induce gene expression, in association with the NF- κ B-p65 subunit.⁵⁵ Whether these or other undefined mechanisms participate in promoting the nuclear migration of STAT3 independently of its tyrosyl phosphorylation in the v-src-transformed cells remains unknown at this stage. These will be investigated in our future studies. Irrespective of the mechanisms used for the src-induced nuclear migration of STAT3, GRIM-19 seems to ablate the transcriptional function of STAT3. In addition, many studies have shown an important role for src and src family kinases in stimulating the NF- κ B activity in other cell types.^{56–60} Taken together, p65 and STAT3 may collaborate with each other in a v-src dependent pathway. In addition, GRIM-19 seems to discriminate IL-6-induced tyrosyl phosphorylation of STAT3 from activated-Src-induced tyrosyl phosphorylation. Although the former is not inhibited by GRIM-19, the latter is (Figure 4, C and D). In the case of IL-6, JAKs play a central role in regulating STAT3 activation.^{61–64} Just as GRIM-19 inhibits src-induced STAT3 tyrosyl phosphorylation, IL-6-dependent JAK, but not the v-src-activity, is inhibited by SOCS proteins,⁶⁵ indicating their specificities. It should be noted that IL-6 transiently induces phosphorylation of STAT3 in the target cells, whereas v-src persistently activates STAT3. Thus, it is natural that these processes are controlled by distinct inhibitors. The GRIM-19-specific shRNA alleviated the negative regulatory effects of GRIM-19 on src and allowed the STAT3-dependent gene expression and anchorage-independent growth (Figure 5). Thus, GRIM-19-induced secondary changes may not be responsible for the inhibitory effects on gene expression and transformation. Because the current model uses a rat cell line and the gene promoters of STAT3-regulated genes in rat cells are not fully defined, we were not able to show direct recruitment

of STAT3 and GRIM-19 to the corresponding promoters. However, in a separate study we have demonstrated the recruitment of these proteins to the endogenous STAT3-regulated genes in human tumor cell lines.⁶⁶

GRIM-19 not only blocked src-induced cellular transformation and tumor formation, but also suppressed cell motility in response to injury and mitogenic signals. Cell motility is controlled by a number of cellular factors, including proteins of the extracellular matrix, cytoskeleton, and those involved in the formation of lamellipodia, adherens junctions.⁶⁷ Some important targets of src include FAK, cadherins, catenins, and R-ras.^{41,68–72} Indeed, GRIM-19 blocked the src-induced tyrosyl phosphorylation of these proteins (Figure 6B). These results are consistent with the loss of v-src-induced metastatic activity in the presence of GRIM-19 (Table 2). Surprisingly, we observed a differential profile of tyrosyl phosphorylation of some proteins between Src* and v-src-transfected cells (Figure 6). For example, E-cadherin and γ -catenin belong to this type. Although, the basis for this difference is not clear at this stage, the src* and v-src proteins differ from each other quite significantly at their C termini.⁷³ The v-src protein completely lacks C-terminal eight amino acids, unlike Src*. Because of these differences Src* may limit the access of substrates to the kinase.^{74,75} Last, v-src induced tyrosyl phosphorylation of certain cellular proteins and its inhibition by GRIM-19 were not affected after the knockdown of STAT3 in cells (Figure 6C), excluding the possibility that these effects are STAT3-dependent. Therefore, some effects of GRIM-19 on v-src seem to occur beyond STAT3. In summary, STAT3 alone does not seem to be the sole target for GRIM-19. The observation that GRIM-19 inhibits src activity but not its levels, suggests that Cbl, a negative regulator src activities, which induces its degradation,⁷⁶ may not participate in this process. At this stage, it is unclear how this effect is mediated. Because both proteins (src* and v-src) lack the Y527 residue, a residue phosphorylated and negatively regulated by the C-terminal src kinase (CSK),^{77–79} CSK may not participate in the GRIM-19-dependent negative regulation of src activity. It is reasonable to assume that some GRIM-19-induced unknown cellular inhibitor may mediate the negative regulatory effects of GRIM-19 on activated src. Our future studies are directed at identifying such inhibitory factor(s) and determining how it interferes with src activities. In light of these observations, it is conceivable that inactivation of GRIM-19 may be a critical step involved in pushing the cell into a transformed state by activated src, and a mere tyrosine phosphorylation of STAT3 may be insufficient for promoting growth. These observations may also be important in light of the fact that some advanced human colon carcinomas possess an activated mutant version of src.⁸⁰

Interestingly, a metabolic regulatory enzyme PDK4 is also repressed by Src, and GRIM-19 restored its expression. PDK4 inactivates mitochondrial pyruvate dehydrogenase complex (PDHc) via a direct phosphorylation of its subunits.³⁹ At this stage it is not clear why Src suppresses this gene. It may have an undefined relationship to altered glucose metabolism in aggressively growing

tumors, where glycolytic generation of ATP supersedes that of tricarboxylic acid cycle-dependent oxidative phosphorylation.⁸¹ Indeed, *c-myc*, a target of STAT3, drives up the expression of glycolytic enzymes.⁸² Src-transformed cells have high levels of hypoxia-inducible factor-1,^{83,84} which correlates with a shutdown of tricarboxylic acid cycle. Because GRIM-19 is also present in the mitochondrion as a part of the ATP generation complex I⁸⁵ and its presence may also reverse these metabolic abnormalities in tumor cells, these observations suggest that not all src effects are STAT3-mediated. In the same vein, GRIM-19 can block src activities above and beyond STAT3 as evidenced by the loss of src activity and tyrosine phosphorylation of multiple cellular proteins in src/GRIM-19-expressing cells (Figure 6), even in the absence of STAT3.

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