Reciprocal Regulation of SOCS1 and SOCS3 Enhances Resistance to Ionizing Radiation in Glioblastoma Multiforme

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Abstract

Purpose: The expression of *suppressors of cytokine signaling 1* (*SOCS1*) and *SOCS3* genes is dysregulated in several solid tumors, causing aberrant activation of cell growth and survival signaling pathways. In this study, we analyzed SOCS1 and SOCS3 gene expression in glioblastoma multiforme (GBM) and studied the role of each protein in GBM cell signaling and radiation resistance.

Experimental Design: SOCS1 and SOCS3 gene expression was analyzed in 10 GBM cell lines by reverse transcription-PCR and Western blotting. SOCS3 expression was also studied in 12 primary GBM tissues by immunohistochemistry. The methylation status of the SOCS1 and SOCS3 loci was determined by methylation-specific PCR. Extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) activation in GBM cell lines overexpressing SOCS1 or lacking SOCS3 was determined by phosphorylated-specific Western blotting. Radiation responses in SOCS1-positive and SOCS3-deficient GBM cell lines and fibroblasts from wild-type and SOCS1 or SOCS3 knockout mice were studied in a clonogenic survival assay.

Results: All GBM cell lines tested lacked SOCS1 expression, whereas GBM cell lines and primary GBM tumor samples constitutively expressed SOCS3. SOCS1 gene repression was linked to hypermethylation of the SOCS1 genetic locus in GBM cells. Reintroduction of SOCS1 or blocking SOCS3 expression sensitized cells to radiation and decreased the levels of activated ERK MAPKs in GBM cells.

Conclusions: SOCS1 and SOCS3 are aberrantly expressed in GBM cell lines and primary tissues. Altered SOCS gene expression leads to increased cell signaling through the ERK-MAPK pathway and may play a role in disease pathogenesis by enhancing GBM radioresistance.

Glioblastoma multiforme (GBM) is an aggressive cancer of glial cells. It is the most common type of brain cancer, with a mean survival time of only 12 months following diagnosis, and only 2% of GBM patients survive to 3 years (1, 2). A major reason for the poor outcome of GBM is the difficulty of treating this disease. The standard treatment regimen for GBM is surgery followed by radiation therapy. However, GBM cells often develop resistance to ionizing radiation (IR), rendering radiotherapy ineffective. The molecular basis of radioresistance in GBM is not well understood, and thus, the potential for development of novel targeted therapies for GBM is limited.

Attempts to sensitize GBM cells to radiation have focused on the use of broadly acting inhibitors of kinases known to be mutated or amplified in this disease. In particular, targeting the epidermal growth factor receptor (EGFR) has been shown to sensitize GBM cells to IR (3–6) and might prove to be an effective radiosensitizing approach in the subset of GBM tumors (36-50% of *de novo* GBM and 8% of secondary GBM), which display constitutive EGFR activation (2, 7–9).

Molecular targeting of other signaling pathways in GBM is also a major focus of current research. In particular, platelet-derived growth factor A, platelet-derived growth factor receptor α chain, and the PTEN-Akt/mammalian target of rapamycin pathways are constitutively active in GBM and are currently being explored as potential targets for novel therapeutics (10–16).

Recently, several studies have shown that increased activation of the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway is correlated with radiation resistance in solid tumors (17–21). The finding that activation of the Ras-ERK pathway is critical in GBM pathogenesis (22, 23) suggests that modulation of the ERK pathway may be an effective adjunct to radiation therapy of GBM.

Recently, a novel family of signal transduction regulators, the suppressors of cytokine signaling (SOCS), has been identified and plays an important role in the regulation of oncogenic

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signaling pathways and cancer pathogenesis. These proteins were initially identified as immediate-early genes induced following cytokine stimulation that act as feedback inhibitors of cytokine-induced and growth factor–induced signal transduction. In particular, SOCS gene expression is induced by cytokine-activated signal transducers and activators of transcription (STAT) and blocks Janus-activated kinase-STAT signaling in a classic negative feedback loop (24, 25). The SOCS family encompasses a large superfamily of proteins, all characterized by sequence homology within a COOH-terminal 40-amino acid protein-protein interaction domain known as the SOCS box, which regulates the ubiquitination and turnover of SOCS and SOCS-associated proteins (26). The NH_2 termini of SOCS proteins, in contrast, are highly divergent and are characterized by the presence of distinct protein-protein interaction motifs. The SOCS superfamily is divided into subfamilies based on these NH_2-terminal domains, which include WD40 repeats, SPRY domains, ankyrin repeats, and SH2 domains (26). The large number of proteins (>50) within this superfamily suggests that SOCS represents an important class of molecules that regulate growth and survival pathways through multiple extracellular stimuli.

Although well characterized as inhibitors of cytokine-driven STAT activation, recent studies suggest that SOCS has a broader spectrum of action and may also regulate MAPK, phosphatidylinositol 3-kinase, and nuclear factor-κB signaling (27–31). In agreement with this idea, our laboratory has identified a novel function for SOCS3 as a positive regulator of the ERK-MAPK pathway (32). SOCS3 is tyrosine phosphorylated by activated Janus-activated kinase and receptor tyrosine kinases. Phosphorylated SOCS3 binds to the Ras inhibitor p120 RasGAP, inhibiting its function and sustaining MAPK signaling. Thus, SOCS proteins can also be considered multifunctional proteins that act as molecular "switches," blocking a subset of signaling pathways while allowing others to remain active.

The involvement of SOCS in the pathogenesis of multiple cancers has recently been established. Repression of SOCS1 gene expression has been described for solid tumors of the liver, breast, colon, and pancreas (33–40). Interestingly, loss of SOCS1 expression seems to occur through the same mechanism in all of these diverse cancers: epigenetic silencing of the SOCS1 locus by CpG island methylation of the SOCS1 promoter and coding sequence. Likewise, the *SOCS3* gene is methylated and silenced in cancers of the lung, liver, colon, and head and neck (41–44). These data argue that, in many cancers, SOCS seems to function as tumor suppressors, and cells that inactivate SOCS expression acquire a selective growth advantage. However, recent data have shown that SOCS genes may also be overexpressed in tumors as well. Both SOCS1 and SOCS3 mRNA are constitutively expressed in some breast cancer cells, and SOCS3 overexpression has been described in lymphoid and myeloid cancers, such as T-cell cutaneous leukemia and chronic myelogenous leukemia (45–48). In the latter case, SOCS3 overexpression was shown to contribute to disease pathogenesis, as cells with high SOCS3 expression were resistant to the growth-inhibitory effects of IFNs (45–48). Thus, there seems to be a complex relationship between SOCS and cell transformation, and depending on cellular context, SOCS proteins may have tumor suppressor function or may also impart a selective advantage to cancer cells when overexpressed. Despite the description of SOCS involvement in multiple solid tumors and leukemias, the role of SOCS in the pathogenesis of GBM and potential regulation of biological responses to anticancer therapies, such as IR, has not been determined.

In the present study, we have analyzed SOCS gene expression in a panel of cell lines representing primary, *de novo* human GBM. We have identified a reciprocal expression pattern of two SOCS family members: SOCS1 and SOCS3. SOCS1 expression is repressed epigenetically by DNA hypermethylation. In contrast, SOCS3 is constitutively expressed through activated STAT3 in GBM cells. Importantly, we show that altered SOCS expression may be involved in disease pathogenesis and contributes to the radioresistant phenotype of GBM. Thus, targeting SOCS expression or function in GBM cells may be a useful strategy to sensitize tumor cells to IR and thus improve the outcome of radiotherapy for GBM patients.

Materials and Methods

Cell lines and tissue culture. Human GBM cell lines U87, T98, A172, CD, HG, KW, SF188, SF767, U251, and U343 were obtained from Drs. Paul Mischel and William McBride (University of California at Los Angeles, Los Angeles, CA). Retroviral packaging cell lines PlatE and PA317 were described previously (32). Wild-type (WT) and SOCS1- or SOCS3-deficient murine embryonic fibroblasts (MEF) were prepared as follows: embryonic day 14.5 embryos from SOCS3-flox/flox mice were isolated by cesarean section, and their heads and blood were removed. The remainder was cut into small pieces and trypsinized. Cells were separated by centrifugation and plated on culture dishes. MEFs were cultured in DMEM containing 10% FCS (49). MEFs were immortalized by transfection of large T as described (50, 51). SOCS3-flox/flox MEFs were then infected with adenovirus carrying Cre recombinase gene (multiplicities of infection of 100; ref. 51) and cultured further. Genotyping was carried out as described (51). SOCS3 deletion status was maintained more than 3 months of culture.

All other cell lines were cultured in DMEM (Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 10 units/mL penicillin G, and 10 mg/mL streptomycin (Mediatech, Herndon, VA). All cells were grown in 100-mm dishes at 37°C in a humidified 5% CO_2 incubator.

Retroviral infection. PA317 packaging cells carrying the control green fluorescent protein (GFP) retroviral vector (pMX-IRES-GFP) or pMX-IRES-GFP encoding SOCS1 or dominant-negative STAT3 (DN-STAT3) were made by transient transfection of the ecotropic packaging cell line PlatE. PA317 amphotropic packaging cells were infected with supernatants from PlatE cells and sorted for GFP expression by flow cytometry according to standard procedures (29, 49). Viral supernatants were collected from packaging stable PA317 cell lines, filtered with a 0.45-μm filter (Fisher Scientific, Pittsburgh, PA), and added to GBM cells with Polybrene (Sigma, St. Louis, MO) at a final concentration of 8 μg/mL. Fresh medium was replenished after a 4-h incubation. After 48 h of infection, the cells were washed and resuspended in fresh culture medium. Cells stably expressing the GFP fusion constructs were sorted by flow cytometry for GFP expression. The DN STAT3 cDNA (STAT3 Y705F) was a generous gift from the laboratory of Dr. James Darnell (The Rockefeller University, New York, NY).

Irradiation. Cells were trypsinized, counted, and diluted. The cell suspensions were immediately irradiated at room temperature using a MARK-1-30 irradiator (cesium-137 source; J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 5 Gy/min.

Clonogenic assay. Irradiated and untreated cells were plated in triplicate into 100-mm culture dishes. Before plating, the viability of the cells was determined by trypan blue dye exclusion test. After 14 days, colonies were fixed and stained with 1% crystal violet in methanol.

Colonies consisting of more than 50 cells were scored. The fraction of cells surviving irradiation was normalized to the surviving fraction of the corresponding untreated control.

RNA preparation and reverse transcriptase PCR. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). Total RNA (1 μg) was used for reverse transcription with the SuperScript II RNase Reverse Transcriptase kit (Invitrogen Life Technologies, Carlsbad, CA). PCR primers were designed using the software Primer3.[6] Primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL). The thermal cycling conditions were as follows: 3 min at 94°C followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR products were analyzed on 2% agarose gels. Sequences of the PCR primers (human genes) used are as follows: *SOCS1*, 5′-tggtagcacacaaccaggtg-3′ (forward) and 5′-gaacggaatgtgcggaagt-3′ (reverse); *SOCS3*, 5′-gccacctactgaaccctcct-3′ (forward) and 5′-acggtcttccgacagagatg-3′ (reverse); *interleukin-6* (*IL-6*), 5′-aaagaggcactggcagaaaa-3′ (forward) and 5′-caggggtggttattgcatct-3′ (reverse); *CIS*, 5′-actatgtggcctcctgcact-3′ (forward) and 5′-gcactgcttctgcgtacaaa-3′ (reverse); *PIM1*, 5′-cgagcatgacgaagagatca-3′ (forward) and 5′-ggtggatctcagcagtttcc-3′ (reverse); *NG4*, 5′-gtgcgcacaagtcctgagta-3′ (forward) and 5′-tggaaccactcaatggaaca-3′ (reverse); and *GAPDH*, 5′-gagtcaacggatttggtcgt-3′ (forward) and 5′-gacaagcttcccgttctcag-3′ (reverse). Primer sequences for murine genes are as follows: *SOCS1*, 5′-tgcttcttcgcgctcagcgtg-3′ (forward) and 5′-gcggcgctggcgcagcggggc-3′ (reverse) and *GAPDH*, 5′-gtggatctgacgtgccgcctggag-3′ (forward) and 5′-ggaaatgagcttgacaaagttgtc-3′ (reverse).

DNA methylation. Genomic DNA was extracted from GBM cell lines using DNeasy Tissue kit (Qiagen). Methylation-specific PCR for the detection of methylation of the *SOCS1* and *SOCS3* genes was carried out following sodium bisulfite treatment of genomic DNA using the EZ DNA Methylation kit (Zymo Research, Orange, CA). PCR products were analyzed on 2% agarose gels. Primers for SOCS1 and SOCS3 methylation-specific PCR have been described previously (33).

Immunohistochemistry. Formalin-fixed, paraffin-embedded human tissues, including glioblastoma and morphologically normal brain derived from surgical procedures and autopsy, respectively, were obtained from the Department of Pathology at the University of California at Los Angeles Medical Center under approval by the University of California at Los Angeles Institutional Review Board. Whole tissue sections (5 μm thick) were mounted on conventional charged glass slides. All sections were heated at 56°C for 25 min, deparaffinized in three changes of xylene, and rehydrated through a descending series of ethanol. Heat antigen retrieval was done by immersing the section in 0.01 mol/L sodium citrate (pH 6.0) in a pressure cooker (Nordic Ware, Minneapolis, MN) placed in a 1,100-W microwave set to "high" for 15 min. Following endogenous peroxidase blocking with a 0.03% hydrogen peroxide solution for 5 min, 5% normal goat serum was applied for 30 min to block nonspecific protein binding sites. The sections were then incubated for 1 h at room temperature with an anti-SOCS3 rabbit polyclonal antibody (2.5 μg/mL final concentration; ref. 32). A biotin-free immunohistochemical staining detection technique was used for antibody visualization (EnVision+ System, HRP Rabbit kit, DAKO, Carpinteria, CA). Subsequently, the sections were incubated for 30 min at room temperature with a goat anti-rabbit secondary antibody conjugated with peroxidase-labeled polymers (EnVision+). The sections were visualized with application of 3,3′-diaminobenzidine substrate chromagen solution and hematoxylin counterstain. Pooled nonimmune irrelevant rabbit IgG (Vector Laboratories, Burlingame, CA) applied at the same final concentration was used as a negative control for all samples. Sections of morphologically normal human hepatic tissue were used as positive tissue controls for SOCS3 expression. Rabbit anti-vimentin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive assay control.

[6] http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Immunoprecipitation. Cells were lysed in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 2 mmol/L EDTA, 0.875% Brij 97 (Sigma), 0.125% NP40 (British Drug Houses, Poole, United Kingdom), 10 μg/mL aprotinin (ICN, Aurora, OH), 10 μg/mL leupeptin (ICN), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), and 1 mmol/L Na_3VO_4 (Sigma). Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Total protein (300 μg) was immunoprecipitated with antibodies as described in the figure legends. Rabbit polyclonal anti-FLAG antibodies and Sepharose-conjugated M2 monoclonal antibody were purchased from Sigma. The precipitates were washed thrice in lysis buffer, boiled in sample buffer, and resolved by SDS-PAGE. The proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bradford, MA). The membranes were probed with antibodies as described in the figure legends followed by incubation with horseradish peroxidase–labeled anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ). The proteins were detected with chemiluminescent substrate (Pierce, Rockford, IL).

Western blot analysis. Protein (50 μg) was denatured in sample buffer [10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mmol/L Tris-HCl (pH 6.8)] by boiling for 5 min and then subjected to SDS-PAGE followed by electrotransfer to polyvinylidene difluoride membrane. The membranes were probed with antibodies as described in the figure legends followed by incubation with horseradish peroxidase–labeled anti-mouse or anti-rabbit secondary antibodies. Rabbit anti-SOCS3 (32) and anti-Akt was purchased from New England Biolabs (Ipswich, MA), and anti-ERK and anti-STAT3 were purchased from Santa Cruz Biotechnology. The proteins were detected with chemiluminescent substrate. For the production of nuclear and cytoplasmic lysates, cells were first lysed in buffer A containing 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.4% Igepal, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. The supernatant (cytosolic fraction) was collected. The pellet was lysed in buffer B containing 20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, with shaking vigorously at 4°C for 2 h. The supernatant (nuclear fraction) was saved.

Results

The* SOCS1 *gene is repressed and* SOCS3 *is constitutively expressed in GBM cells. GBM is characterized by constitutive cytokine (IL-6)-mediated Janus-activated kinase-STAT signaling or EGFR activation. Therefore, we postulated that SOCS1 and SOCS3 expression may be altered in GBM. We analyzed SOCS1 and SOCS3 mRNA levels by reverse transcription-PCR (RT-PCR) and SOCS3 protein expression by Western blot in 10 GBM cell lines. As shown in Fig. 1, expression of SOCS1 and SOCS3 is differentially regulated in GBM cells. In Fig. 1A, SOCS1 mRNA could not be detected in any of the cell lines. In contrast, SOCS1 mRNA was readily detected in a human GBM cell line (U87) engineered to express SOCS1 ectopically (*top*, *last lane*). Surprisingly, SOCS3 mRNA was constitutively expressed in all GBM cell lines that we tested (Fig. 1B). Likewise, constitutive SOCS3 protein expression was detected in GBM cells by Western blot (Fig. 1C).

Constitutive SOCS3 expression in GBM cells is STAT3 dependent. The constitutive SOCS3 expression in all GBM cell lines was unusual, given that *SOCS3* is a Janus-activated kinase-STAT–inducible gene and is generally not highly expressed in the absence of cytokine or growth factor stimulation (32, 52–55). GBM cells often display constitutive IL-6 receptor or EGFR signaling, which enhances tumor

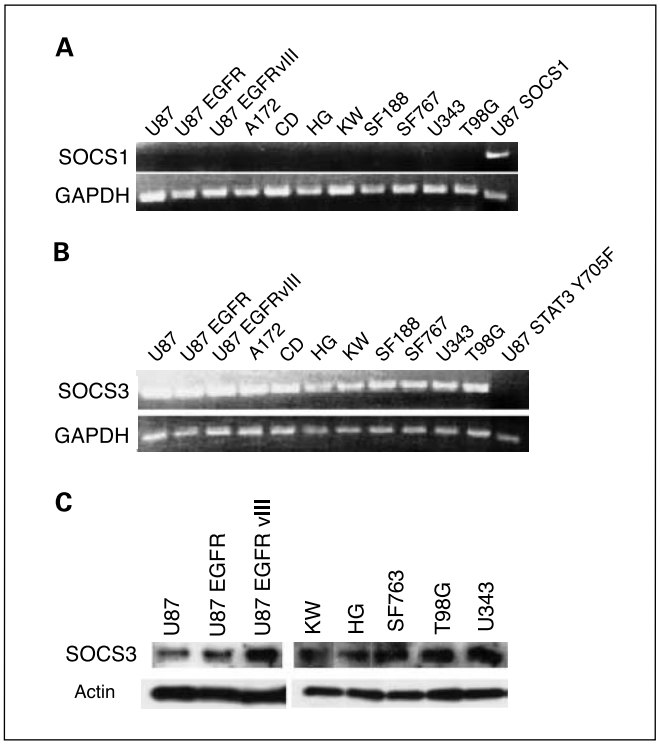

Fig. 1. SOCS1 and SOCS3 are aberrantly expressed in GBM cell lines. *A,* mRNA from 11 GBM cell lines was isolated and subjected to RT-PCR with primers specific for SOCS1. SOCS1 mRNA was not detectable in these cell lines. Control cell line, U87-SOCS1 expressed SOCS1 mRNA. *B,* the same RNA samples were used for RT-PCR for SOCS3 detection. SOCS3 expression was constitutive in GBM cell lines. *C,* protein extracts from several GBM cell lines were analyzed by SDS-PAGE followed by Western blotting with an anti-SOCS3 antiserum. *Top,* all GBM cell lines constitutively expressed SOCS3 protein; *bottom,* the same extracts were blotted with anti-actin antibodies as a protein loading control.

proliferation, survival, and radioresistance (56). Both of these receptors activate STAT3, which transcriptionally up-regulates SOCS3 expression. Furthermore, previous reports have shown that U87 cells display constitutive nuclear STAT3 DNA binding activity in electrophoretic mobility shift assays (57–59). Therefore, we determined whether constitutive SOCS3 expression in GBM cells was dependent on STAT activation in GBM. We stably expressed a phosphorylation-deficient DN STAT3 allele (STAT3 Y705F) in U87 cells via retroviral-mediated gene transfer (Fig. 2A) and analyzed SOCS3 mRNA and protein expression. As shown in Fig. 2B, we readily detected SOCS3 mRNA in U87 as well as U87 expressing the control GFP vector by RT-PCR. In contrast, U87 expressing DN STAT3 expressed barely detectable levels of SOCS3 mRNA. As expected, we also observed the repression of several other STAT-dependent genes in U87 STAT3 Y705F cells, such as *PIM1*, and another SOCS family member, *CIS* (Fig. 2C). We also confirmed by Western blotting that SOCS3 protein levels were greatly reduced in U87 cells expressing DN STAT3 (Fig. 2D), suggesting that STAT activation is required for the constitutive SOCS3 expression we observe in GBM cell lines.

The* SOCS3 *locus is unmethylated but the* SOCS1 *locus is methylated and silenced in GBM. We next investigated whether the apparent repression of *SOCS1* gene expression in GBM cells was due to epigenetic silencing of the *SOCS1* locus as has been reported for other human tumors (33–40). Sodium bisulfite–treated genomic DNA from several GBM cell lines was subjected to PCR using primers specific for methylated or unmethylated sequences in exon 2 of the *SOCS1* locus (33). As shown in Fig. 3A, the *SOCS1* locus was methylated in all GBM cell lines tested. In 7 of 10 cell lines, we observed high levels of SOCS1 gene methylation compared with unmethylated SOCS1 genomic DNA. In three other cell lines, we detected similar levels of methylated and unmethylated sequences. The bisulfite-treated DNA was also subjected to methylation-specific PCR using SOCS3-specific primers (33). Consistent with our finding that SOCS3 was constitutively expressed in GBM cells, we found that the *SOCS3* locus was unmethylated and thus presumably accessible to transcription factors (Fig. 3B) in all of the cell lines we analyzed.

SOCS1 and SOCS3 regulate radioresistance of GBM cell lines. We next determined whether changes in SOCS expression can

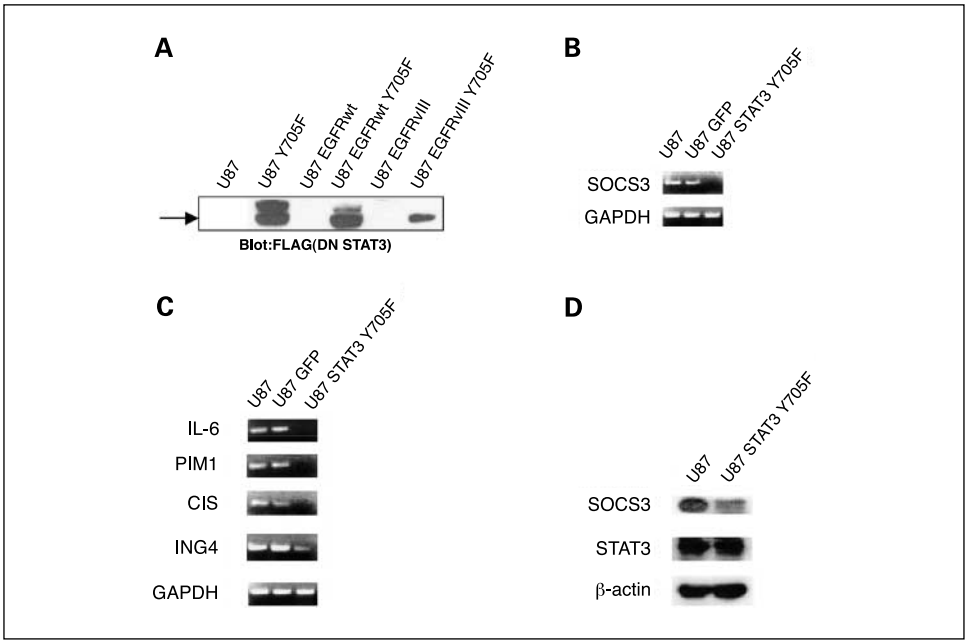

Fig. 2. Constitutive SOCS3 expression in GBM cells is STAT3 dependent. *A,* U87 cells were engineered to stably express a FLAG-tagged DN STAT3 (STAT3 Y705F) allele by retroviral-mediated gene transfer (see Materials and Methods). Western blot analysis with anti-FLAG antibody shows expression of DN STAT3 in the indicated cell lines. *B* and *C,* DN STAT3 repressed the expression of SOCS3 and other STAT-dependent genes. mRNA from U87, U87-GFP vector controls, and U87 STAT3 Y705F was isolated and subjected to RT-PCR with primers specific for the indicated genes. DN STAT3 repressed the expression of SOCS3 as well as IL-6, PIM1, and CIS. *D,* U87 STAT3 Y705F expresses greatly reduced levels of SOCS3 protein compared with the U87 parental cell line but had no effect on STAT3 expression.

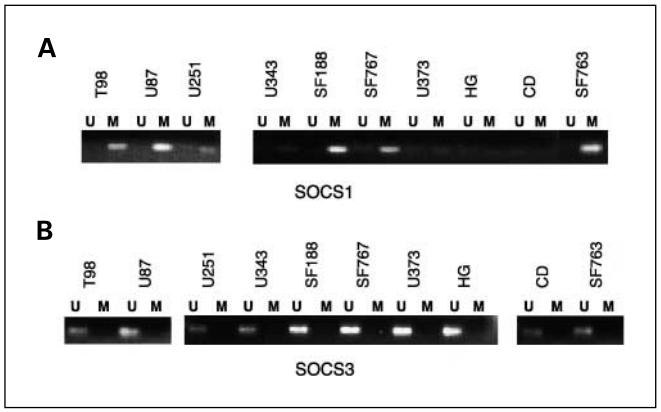

Fig. 3. The *SOCS1* locus is hypermethylated and the *SOCS3* gene is unmethylated in GBM cell lines. *A,* sodium bisulfite – treated DNA from GBM cells was subjected to PCR with primers specific for methylated (*M*) or unmethylated (*U*) DNA. SOCS1 exon 2 was highly methylated in all GBM cell lines we analyzed. *B,* the same DNA was subjected to PCR with methylation- or unmethylation-specific SOCS3 primers. The *SOCS3* locus was unmethylated in all cell lines tested.

contribute to GBM radiation resistance. SOCS1 and SOCS3 are known to modulate several signaling pathways, most notably STATs, but also MAPK and phosphatidylinositol 3-kinase as well (27–29, 32). All of these pathways may affect the radioresistant phenotype of GBM cells. Thus, we determined whether altering SOCS expression in GBM would affect cellular radiation responses. Using retroviral-mediated gene transduction, we ectopically expressed SOCS1 or DN STAT3 in U87 cells and compared clonogenic survival of these cell lines to the U87 parental cells and U87 cells expressing the GFP control empty vector. As shown in Fig. 4A, expression of DN STAT3 sensitized U87 GBM cells to the cytotoxic effects of IR. Survival fraction was reduced from 43% in U87 parental cells to 22% in U87-DN STAT3 at 4 Gy ($P < 0.01$). Likewise, U87 cells expressing Myc-tagged SOCS1 were more sensitive to radiation than U87 or U87-GFP. Clonogenic survival was reduced from 70% in U87-GFP to 19% in U87-SOCS1 cells at 2 Gy irradiation ($P < 0.05$), suggesting that repression of SOCS1 and constitutive expression of SOCS3 contribute to radioresistance of GBM. To show specifically that endogenous SOCS3 and SOCS1 are involved in radiation responses, we compared clonogenic survival of MEFs from WT mice and gene-targeted mice lacking SOCS1 or SOCS3 expression. In agreement with experiments using DN STAT3 and SOCS1 in U87, MEFs lacking SOCS3 were markedly more sensitive to the cytotoxic effects of IR than WT MEFs, whereas SOCS1-deficient MEFs were protected from radiation (Fig. 4C and D).

Modulation of SOCS1 and SOCS3 expression regulates ERK-MAPK activation in GBM cells. We analyzed the effects of SOCS1 and DN STAT3 on ERK-MAPK and Akt phosphorylation in GBM cell lines. As shown in Fig. 5, expression of either DN STAT3 or SOCS1 greatly reduced the levels of phosphorylated ERK1/2 MAPKs in U87 cells (compare *lanes 1* and *2* with *lanes 3* and *4*). This effect was not due to inhibition of ERK protein expression, as ERK1/2 levels were not reduced in U87-DN STAT3 or U87-SOCS1. In contrast to the pronounced effect of SOCS1 on MAPK phosphorylation, we did not observe consistent effects of either DN STAT3 or SOCS1 on Akt phosphorylation (data not shown).

Endogenous SOCS expression regulates ERK-MAPK activation. We showed in Fig. 5A that ectopic expression of SOCS1 or

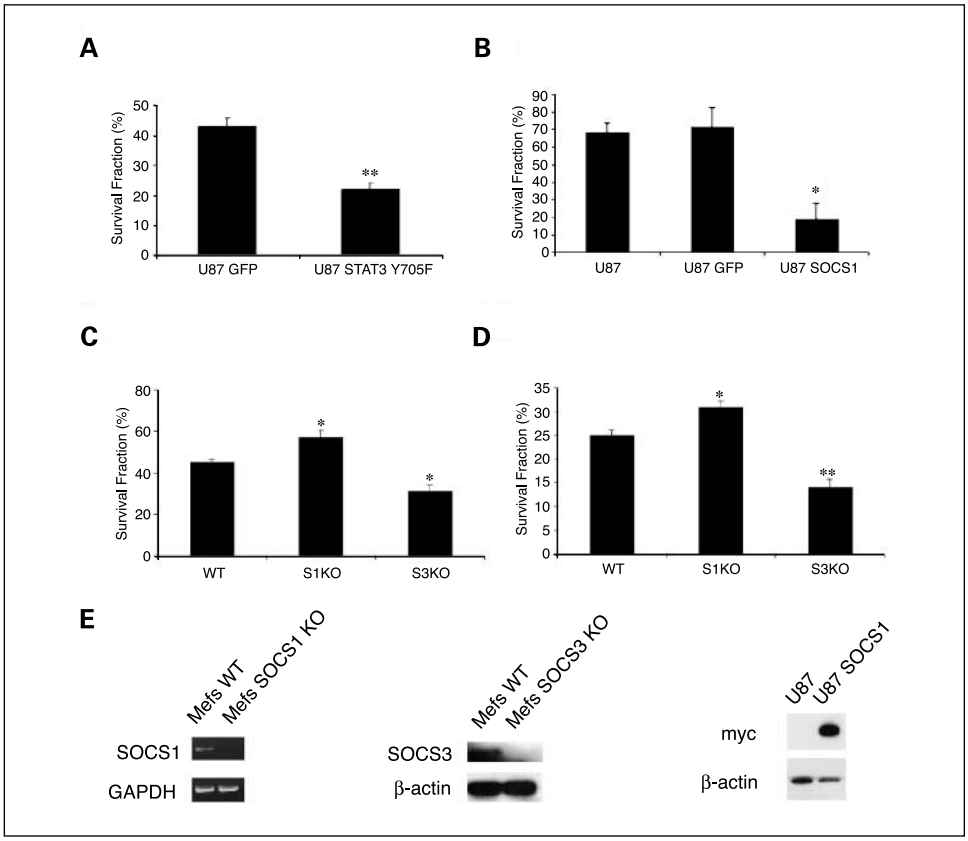

Fig. 4. Ectopic expression of SOCS1 or DN STAT3 sensitizes cells to IR. *A* and *B,* GBM cell line U87 or U87 stably expressing DN STAT3 or SOCS1 was exposed to 4 Gy (*A*) or 2 Gy (*B*) IR from a cesium-137 source and plated in 10-cm dishes at 500 cells per plate. Survival fraction was calculated by dividing the number of colonies in the irradiated samples by the number of colonies in unirradiated controls. Results from four independent experiments. *C* and *D,* MEFs from WT and SOCS1- or SOCS3-deficient mice were exposed to 4 Gy (*C*) or 6 Gy (*D*) IR, and clonogenic survival fraction was determined by the method described in (*A*). Columns, mean; bars, SE. Statistical analysis was done using the Students *t* test. *, $P < 0.05$; **, $P < 0.01$; $n = 3$. *E,* left, RT-PCR showing lack of SOCS1 expression in SOCS1 knockout (*KO*) MEFs; middle, Western blots showing lack of SOCS3 expression in SOCS3 knockout MEFs; right, ectopic expression of Myc-tagged SOCS1.

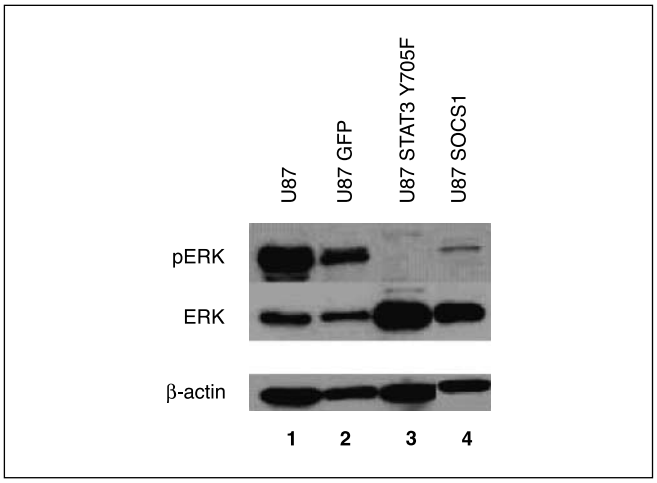

Fig. 5. Expression of SOCS1 or DN STAT3 regulates MAPK activation in GBM cell lines. Top, protein extracts from U87 parental cells (*lane 1*), U87-GFP – negative controls (*lane 2*), U87 cells stably expressing STAT3 Y705F (*lane 3*), or SOCS1 (*lane 4*) were analyzed by SDS-PAGE followed by Western blotting with anti-phosphorylated ERK1/2 (*pERK*) antibodies; middle, control blot with anti-ERK antibodies; bottom, anti-actin blot.

blocking SOCS3 expression markedly reduced phosphorylated ERK levels in U87 GBM cells. However, the role of endogenous SOCS1 in the regulation of ERK-MAPK activation has not been well characterized and is poorly understood. To determine whether endogenous SOCS1 plays a role in the control of MAPK activation, we stimulated WT and SOCS1-deficient MEFs with 50 ng/mL IL-6 for a 6-h time course and determined phosphorylated ERK levels by Western blotting. As shown in Fig. 6A, the amount of phosphorylated ERK in stimulated WT and SOCS1$^{-/-}$ MEFs at 15 and 30 min after stimulation was comparable. However, at later time points, the phosphorylated ERK signal decayed in WT MEFs but remained elevated in the SOCS1-deficient cells (Fig. 6B), suggesting that endogenous SOCS1 controls ERK phosphorylation in response to cytokines. We have previously shown that SOCS3 may sustain ERK-MAPK activation in response to cytokine and growth factor stimulation (32). To determine the role of endogenous SOCS3 in the regulation of ERK activation, we compared ERK phosphorylation in WT and SOCS3-deficient MEFs in response to IL-6. As shown in Fig. 6C, IL-6 strongly activates ERK phosphorylation in WT MEFs (Fig. 6C, *lanes 3* and *5*). In contrast, ERK activation in SOCS3-deficient MEFs is markedly reduced (Fig. 6C, *lanes 4* and *6*). These data agree with our previous observations and support a role for SOCS3 in the activation of ERK MAPKs.

SOCS3 protein is highly expressed in primary tumors from GBM patients. To determine whether the constitutive SOCS3 expression we observed in GBM cell lines reflected molecular defects in primary GBM tumors, we compared SOCS3 protein expression levels in normal brain tissue and GBM tumor samples by immunohistochemistry. As shown in Fig. 7A and B, SOCS3 protein was detected in a relatively small subset of normal brain cells, most of which resembled glial cells by morphology. In contrast, a high proportion of tumor cells from high-grade, malignant GBM primary tissue samples expressed SOCS3 protein (Fig. 7C-H). Interestingly, we found that we consistently detected SOCS3-positive tumor cells clustered around blood vessels (Fig. 7C and D). In other GBM tissue sections, we also found that SOCS3-positive tumor cells clustered near necrotic areas of the tumor (Fig. 7H). A total of 12 primary GBM samples was analyzed by immunohistochemistry and all contained SOCS3-positive tumor cells.

SOCS proteins are differentially distributed in GBM cells. Several signaling proteins have been shown to modulate signal transduction in both the nuclear and cytoplasmic compartments. We determined the subcellular localization of SOCS1 and SOCS3 by immunohistochemistry or Western blotting. In Fig. 7I, SOCS3 immunohistochemistry in primary GBM tumor samples showed almost exclusive SOCS3 localization to the cytoplasm. In contrast (Fig. 7J), c-Myc-tagged SOCS1 protein expressed in U87 cells was evenly distributed between the nuclear and cytoplasmic fractions.

Discussion

In this study, we present evidence that two members of the SOCS family of signal transduction regulators, SOCS1 and

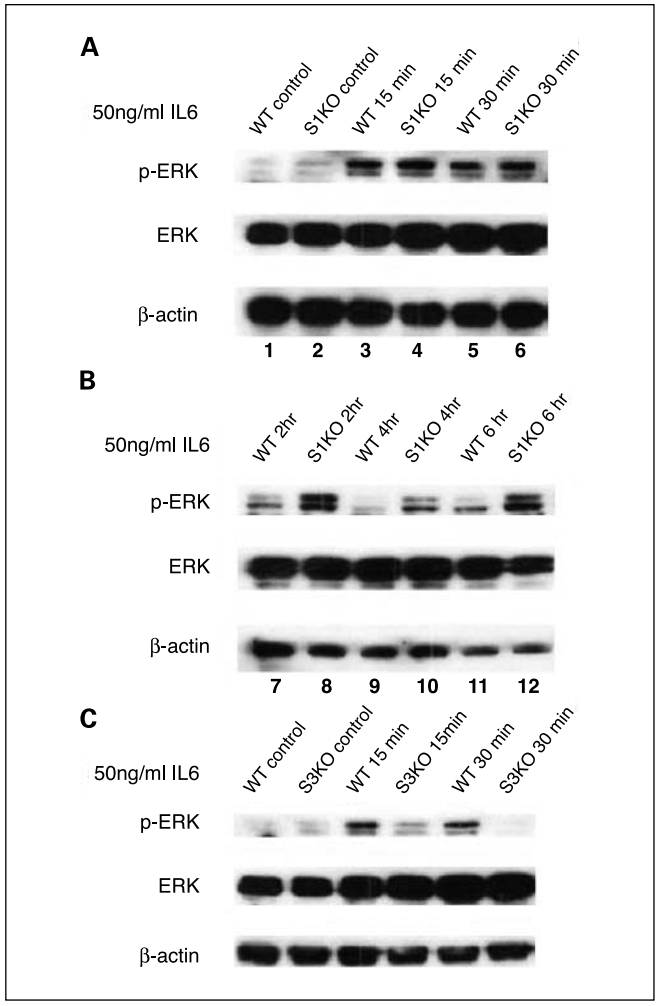

Fig. 6. Endogenous SOCS1 and SOCS3 regulate ERK-MAPK signaling in response to IL-6. MEFs from WT and either SOCS1-deficient (*A* and *B*) or SOCS3-deficient (*C*) mice were incubated in medium containing 0% serum for 18 h and stimulated with 50 ng/mL IL-6 for the indicated times. Whole-cell extracts were prepared at the indicated time points up to 6 h after stimulation. Top, phosphorylated ERK1/2 was detected by Western blot using anti-phosphorylated ERK antiserum. Protein loading controls for ERK (*middle*) and actin (*bottom*) are included.

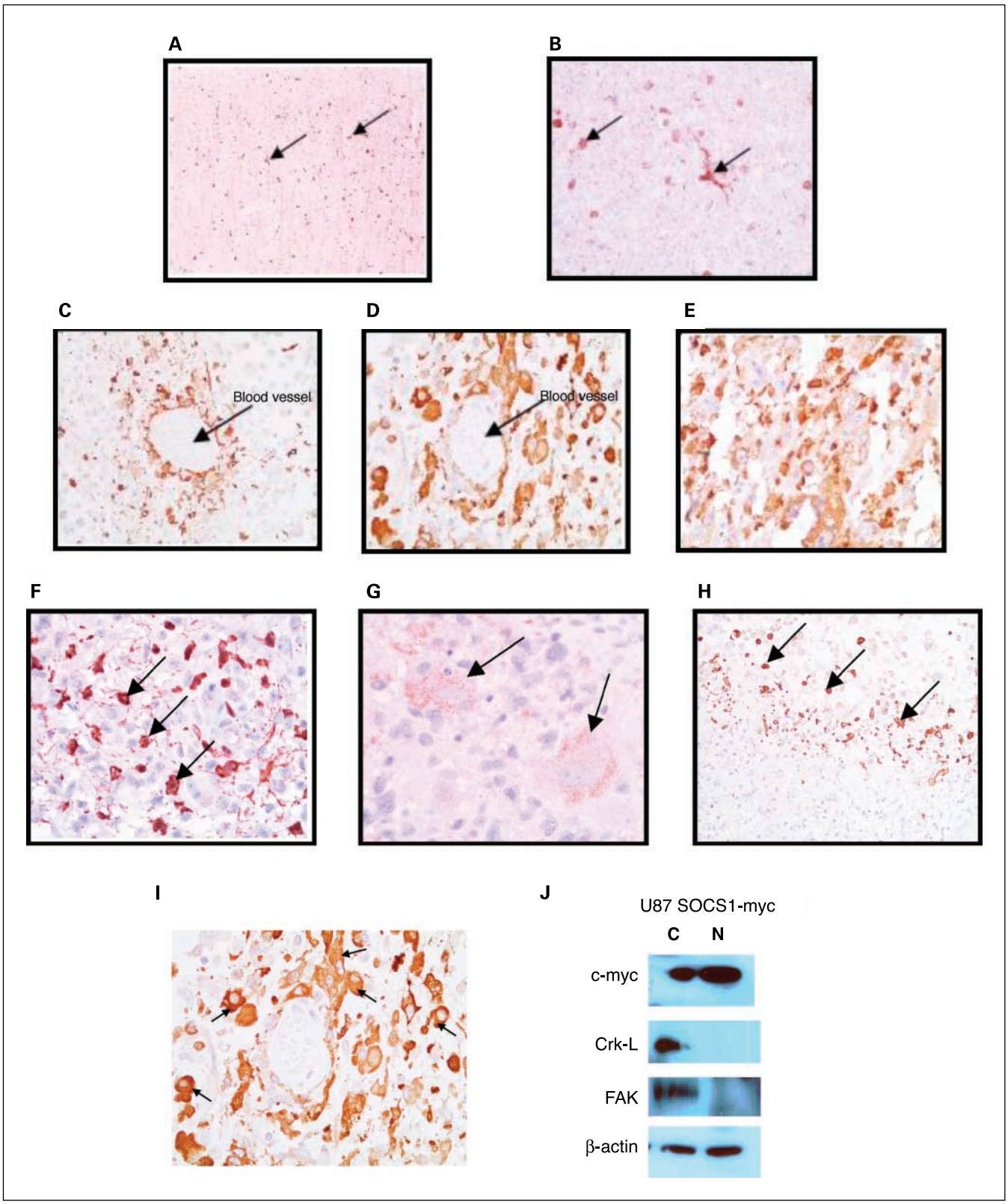

Fig. 7. SOCS3 is highly expressed in primary tumor samples from GBM patients. *A* and *B,* SOCS3 protein expression in normal brain tissue was determined by immunohistochemistry. Isolated glial cells stain positive for SOCS3. *C* and *D,* primary GBM tumor cells express SOCS3 protein. SOCS3-positive cells cluster around blood vessels. *E* and *F,* sections from high-grade, advanced GBM tumors stain extensively for SOCS3. *G,* high-magnification view of a GBM tissue sample showing high levels of SOCS3 expression in tumor cells. *H,* section of a GBM tissue sample showing SOCS3-positive tumor cells in a zone of necrosis. *I,* detail of GBM tissue stained with anti-SOCS3 antibodies shows that SOCS3 protein is expressed almost exclusively in the cytoplasm of tumor cells. *J,* Western blot analysis of cytoplasmic and nuclear fractions of U87 cells stably expressing c-Myc-tagged SOCS1. SOCS1 protein is present in both the cytoplasmic (*C*) and nuclear (*N*) compartments. Blots were stripped and reprobed with anti-β-actin antibody as a loading control.

SOCS3, are reciprocally regulated in human GBM cell lines and primary GBM tissues. We have shown that constitutive SOCS3 expression in GBM is dependent on the STAT3 transcription factor and repression of SOCS1 expression is mediated by epigenetic silencing of the *SOCS1* locus via CpG island methylation. We also present evidence that these defects in SOCS expression affect the biological responses of GBM cells to IR, as repression of SOCS1 and constitutive SOCS3 expression increased radioresistance. Biochemical analysis of these cells suggests that radiosensitization may occur through down-regulation of MAPK signaling. We also present evidence that SOCS3 is highly expressed in primary tissues from GBM patients, clusters to specific areas of tumor architecture (SOCS3 expression is highest in cells surrounding blood vessels), and is highly expressed in the most malignant tumor tissues. Our data argue that SOCS1 and SOCS3 may be biomarkers of aggressive, radio-resistant cancers and that SOCS expression profiling may be of predictive value in assessing tumor cell responses to IR.

SOCS is a large superfamily of proteins that are generally viewed as antagonists of cytokine-induced Janus-activated kinase-STAT signaling. In support of this idea are biochemical and genetic data showing that SOCS1 and SOCS3 block STAT activation in response to multiple cytokines (60–63). However, there is expanding evidence to support a role for SOCS in the regulation of additional signaling pathways, including phosphatidylinositol 3-kinase and ERK MAPK (27, 28, 31, 32). Recent studies have shown that SOCS1 can negatively regulate ERK signaling by targeting p95 Vav degradation (64), and our laboratory has shown that ERK activation is augmented by signaling complexes formed between tyrosine-phosphorylated SOCS3 and the Ras inhibitor p120 RasGAP (32).

SOCS1 seems to function as a tumor suppressor in solid tumors. SOCS1 gene expression is repressed in tumors from multiple tissues, including lung, breast, the immune system, pancreas, and liver. Significantly, the mechanism of SOCS1 gene repression is not via mutation or deletion of the SOCS1 coding sequence but rather by CpG island methylation (33–40). In contrast, SOCS3 may either inhibit or augment tumor cell growth depending on cellular context (41–48).

The role of SOCS in the pathogenesis of GBM is unknown. Many GBM tumors express constitutively active cytokine-dependent and growth factor–dependent signaling pathways, such as those induced by the receptors for IL-6, platelet-derived growth factor, and EGF, which contribute to cell transformation and enhance cellular resistance to anticancer therapies, including IR. Because several of these signaling pathways are potential targets of SOCS regulation, we explored the possibility that SOCS gene expression may be altered in GBM.

In all GBM cell lines we tested, we did not detect SOCS1 mRNA or protein. In contrast, SOCS3 mRNA and protein were constitutively expressed in all of these cells. Previous studies have shown that a high percentage of GBM tumor cells, including U87, display constitutive STAT signaling, which induces SOCS3 expression (57–59). Thus, we introduced a phosphorylation-deficient, DN STAT3 mutant (STAT3 Y705F) into U87 cells. DN STAT3 strongly repressed SOCS3 mRNA and protein expression in U87, suggesting that STAT3 is a major inducer of SOCS3 expression in GBM.

We also determined the mechanism of SOCS1 gene repression in GBM cells. Several previous reports have shown that SOCS1 expression is blocked in solid tumors from a wide range of tissues via CpG island methylation. In agreement with the previous studies, we observed that the *SOCS1* genetic locus is methylated in all GBM cell lines we analyzed, and in 7 of 10 cell lines, we observed high levels of SOCS1 methylation and little or no detectable unmethylated SOCS1 genomic DNA.

SOCS3 overexpression and SOCS1 gene repression were both found to contribute significantly to radiation resistance of U87 cells. GBM cells expressing either SOCS1 or STAT3 Y705F (to repress SOCS3 expression) were markedly more sensitive to radiation than the parental cell line. In support of a role for SOCS1 and SOCS3 in regulation of radiation responses, we showed that MEFs from SOCS3-deficient mice were markedly more sensitive to IR than MEFs from WT mice. In contrast, SOCS1-deficient MEFs were more radioresistant than WT fibroblasts. Although statistically significant, the effects of SOCS1 deficiency on radiation responses in MEFs were not dramatic (Fig. 4C and D). This is not unexpected, as multiple signaling pathways are known to contribute to cellular radiation responses, and SOCS1 is likely to modulate only a subset of these pathways. Our data suggest that SOCS1 and SOCS3 have opposing effects on radiation responses: SOCS1 sensitizes cells to IR, whereas SOCS3 has a radioprotective effect. To the best of our knowledge, this study is the first to show a role for SOCS1 or SOCS3 in the regulation of biological responses of tumor cells to IR.

We have done signal transduction analysis in U87 cells that ectopically express SOCS1 or DN STAT3 to determine a possible mechanism for enhanced radioresistance by aberrant SOCS gene regulation. Constitutive ERK phosphorylation occurs in a high proportion of GBM tumors. In addition, our previous finding that SOCS3 can potentiate MAPK signaling suggested that a likely target of SOCS proteins in GBM may be the ERK pathway. We detected high levels of constitutive ERK1/2 MAPK in the U87 parental cell line (Fig. 5). Interestingly, overexpression of either SOCS1 or DN STAT3 greatly reduced the levels of phosphorylated ERK in U87 but did not reduce ERK protein expression. In contrast, we did not observe consistent changes in phosphorylated Akt levels in U87 cells expressing either SOCS1 or DN STAT3 (data not shown). Further, we present evidence that endogenous SOCS1 negatively regulates MAPK signaling, as IL-6–induced ERK phosphorylation is prolonged in SOCS1-deficient MEFs compared with WT fibroblasts and reduced in SOCS3-deficient MEFs (Fig. 6). Although there may be other signaling pathways involved, our results suggest that sensitization of GBM cells to IR by SOCS1 and DN STAT3 may result from a marked reduction in signals delivered through the MAPK pathway.

Our signaling data agree with several previous studies that implicate Ras-MAPK signaling as a key component of tumorigenesis in brain cancer (10, 22, 23). These findings support an essential role for Ras-MAPK signaling in both initial cell transformation and maintenance of tumor cell survival and proliferation. Our findings suggest that defects in SOCS1 and SOCS3 expression may cooperate with mutated or overexpressed receptors and signaling proteins to activate ERK-MAPK signal transduction in GBM.

Our results suggest that SOCS1 and SOCS3 may also regulate GBM responses to radiation. ERK MAPK is activated by oncogenic EGFR in GBM, and radioprotection by EGFR has been linked to activation of the ERK-MAPK cascade as well as other kinase pathways (17–21). Our finding that SOCS3

enhances ERK signaling and SOCS1 blocks ERK activation is consistent with a model in which reciprocal regulation of SOCS1 and SOCS3 enhances ERK signaling and radioresistance in GBM.

The mechanism by which SOCS1 and DN STAT3 inhibit ERK signaling in GBM is unknown. Recent data have shown that SOCS1 can induce the proteasome-mediated degradation of the guanine nucleotide exchange factor Vav (64). Considering our previous data showing that SOCS3 can sustain MAPK signaling, the inhibitory effects of DN STAT3 on MAPK phosphorylation may be due to inhibition of SOCS3 expression. However, STAT3 activates the expression of other genes that are involved in signal transduction, such as c-Myc and the serine/threonine kinase PIM1. To determine whether the effect of DN STAT3 on GBM signaling and radioresistance is due to loss of SOCS3 expression, it will be necessary to analyze ERK phosphorylation and downstream signaling in GBM cells specifically lacking SOCS3.

To determine whether our observations of SOCS expression defects in established cell lines reflected molecular defects in primary tumors, we analyzed SOCS3 expression in tissue sections from GBM patients. In agreement with our studies in cell lines, we found that all GBM tissue samples we tested contained SOCS3-positive tumor cells, particularly in samples representative of highly malignant tumors (Fig. 7).

Interestingly, SOCS3 expression seemed to concentrate to specific areas of tumor architecture and also correlated with tumor grade. For example, in multiple samples, we detected a high proportion of SOCS3-positive tumor cells clustered around blood vessels. The significance of these findings is unclear, but it may indicate that SOCS3 protein expression is regulated by intracellular oxygen concentration.

Components of cytokine signaling pathways are capable of acting in both the nucleus and cytoplasm. Recently, it has been shown that STAT5 can modulate cytoplasmic phosphatidylinositol 3-kinase activation by forming complexes with the Gab2 protein (65, 66). We have determined the subcellular localization of SOCS1 and SOCS3 in U87 cells or primary GBM tumor samples. SOCS3 immunohistochemistry showed almost exclusive cytoplasmic localization of SOCS3 in primary tumors. In contrast, U87 cells ectopically expressing SOCS1 showed SOCS1 protein equally distributed between the nuclear and cytoplasmic fractions. This finding is consistent with a previous study, which showed nuclear and cytoplasmic localization of a SOCS1-GFP fusion protein (67). Our data suggest that, whereas SOCS3 likely acts on cytoplasmic signaling pathways, SOCS1 may regulate signaling in either the cytoplasm or nucleus. However, these findings do not exclude the possibility that a small fraction of SOCS3, which may be localized to the nucleus, may also affect GBM cell signaling and biological responses to radiation.

Our data suggest that SOCS1 sensitizes cells to radiation, whereas SOCS3 enhances tumor cell survival and radioresistance. The identification of common genetic and signaling defects, including SOCS overexpression or repression, may provide novel biomarkers and effective targets for future therapies that may benefit most patients with GBM or other radioresistant solid tumors. Pharmacologic agents that block SOCS3 expression and function or reactivate SOCS1 transcription may sensitize GBM cells to IR and ultimately improve the effectiveness of radiotherapy.

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