

Signal Transducer and Activator of Transcription 1 Regulates Both Cytotoxic and Prosurvival Functions in Tumor Cells

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Abstract

Elsewhere, we reported that multiple serial *in vivo* passage of a squamous cell carcinoma cells (SCC61) concurrent with ionizing radiation (IR) treatment resulted in the selection of radioresistant tumor (nu61) that overexpresses the signal transducer and activator of transcription 1 (Stat1)/IFN-dependent pathway. Here, we report that (a) the Stat1 pathway is induced by IR, (b) constitutive overexpression of Stat1 is linked with failure to transmit a cytotoxic signal by radiation or IFNs, (c) selection of parental cell line SCC61 against IFN- α and IFN- γ leads to the same IR- and IFN-resistant phenotype as was obtained by IR selection, and (d) suppression of Stat1 by short hairpin RNA renders the IR-resistant nu61 cells radiosensitive to IR. We propose a model that transient induction of Stat1 by IFN, IR, or other stress signals activates cytotoxic genes and cytotoxic response. Constitutive overexpression of Stat1 on the other hand leads to the suppression of the cytotoxic response and induces prosurvival genes that, at high levels of Stat1, render the cells resistant to IR or other inducers of cell death. [Cancer Res 2007;67(19):9214–20]

Introduction

Signal transducer and activator of transcription 1 (Stat1) is primarily known as an important upstream regulator of IFN signaling. On activation of IFN receptors, Stat1 is phosphorylated at Tyr⁷⁰¹ by IFN receptor-associated Janus-activated kinase (JAK) 1, JAK2, and Tyk2 kinases and is translocated from the cytoplasm into the nuclei (1, 2). In the nucleus, Stat1 binds to the cognate transcriptional elements and activates IFN-inducible genes (3, 4). Type I receptors responding to IFN- α and IFN- β activate the IFN signaling gene factor 3 complex composed of Stat1, Stat2, and p48 [also known as IFN-regulatory factor (IRF) 9; see ref. 5]. Type II receptors are specific to IFN- γ and activate only Stat1, which forms homodimers, enters the nucleus, and binds to IFN- γ -activated sequences, thereby inducing transcription of genes controlled by IFN- γ (6, 7). Stat1-induced genes do several functions, including antiviral defense (8, 9). For example, 2'-5'-oligoadenylate synthase activates RNase L, which induces degradation of double-stranded RNA formed during viral replication and inhibits viral propagation (10, 11). A second Stat1-dependent function is induction of cell death and growth arrest both in the context of either a viral or a stress response (12, 13). Known examples of genes controlled by

activated Stat1 in this pathway are *IRF1*, *caspase-2*, *caspase-3*, *caspase-7*, *Bcl2*, and *p21/Waf1* (14–16). The third function of Stat1 is modulation of an immune response, mediated by activation of MHC class I and II molecules and immune-related cytokines (17, 18). Although Stat1 was discovered and described in the context of IFN signaling, accumulating evidence indicates that it may participate in other signaling cascades. In addition to the type I and II IFN receptors, Stat1 may be activated by growth factor receptors, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), nonreceptor tyrosine kinases, such as c-Abl (19), and seven-transmembrane receptors (20). Recent data show that Stat1 can be activated by hypoxia/reoxygenation in the myocardium, breast cancer, and endothelial cells (21–23) as well as by EGF and erythropoietin (24, 25). However, the outcome of Stat1 activation may be different depending on the upstream signals (see refs. 8, 26). These data suggest that Stat1 is involved in general stress response pathways and that functions of Stat1-dependent pathways are not restricted by the IFN response but rather have a much broader functional significance.

Previously, we reported that multiple serial *in vivo* passage concurrent with ionizing radiation (IR) treatment of human squamous cell carcinoma cells in nude mice resulted in the generation of a radioresistant tumor, which overexpresses IFN-stimulated genes, including *Stat1*, and genes activated by Stat1 (27). Here, we report that IR activates an IFN-inducible, Stat1-dependent pathway. We also show that *in vitro* selection against IFN- α and IFN- γ leads to an IFN- and IR-resistant phenotype. Finally, we show that suppression of Stat1 in tumor cells constitutively overexpressing this pathway leads to radiosensitization. These results suggest that Stat1 may be potential target for radiosensitization of aggressive tumors that constitutively overexpress the Stat1-dependent pathway.

Materials and Methods

Cell cultures and reagents. All cell lines were maintained in DMEM/F12 (Life Technologies) supplemented with 20% fetal bovine serum, 1% hydrocortisone, and 1% penicillin/streptomycin at 37°C, 100% humidity, and 7% CO₂. IFN-resistant cell lines were produced by serial passage of SCC61 in increasing doses of IFN- α (final concentration, 1,500 ng/mL; R&D Systems) and IFN- γ (final concentration, 30 ng/mL; R&D Systems). IFN- α resistance was quantified by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) according to the manufacturer's instructions, and IFN- γ resistance was quantified by both MTS and clonogenic assays as described previously (28).

Immunoblots. Total cellular protein was extracted in radioimmunoprecipitation assay buffer with protease inhibitors added (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L Na₃VO₄, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride). All samples were normalized by protein concentration using Bradford reagent and standard solution of bovine serum albumin (1 mg/mL). Concentration of all samples was adjusted to 1 mg/mL and equal amount of protein was loaded in each well. For total Stat1 protein, we loaded 10 μg of protein per well, and for

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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phosphorylated Stat1, we loaded 15 μg of protein per well. Proteins were separated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Total Stat1 was detected using the anti-Stat1 p84/p91 (E-23) antibody. For loading control, we used antibodies for actin (I-19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; FL-335). All antibodies were purchased from Santa Cruz Biotechnology. Images were quantified with ImageJ software⁴ by integration pixel values across the area of specific bands.

Real-time PCR analysis. cDNA was synthesized as described previously (29). Primers are described in Supplementary Table S1. For the internal control, we used GAPDH. PCR was done for 40 cycles at 95°C for 15 s and 60°C for 1 min after initial incubations at 50°C for 2 min and 95°C for 10 min using SYBR Green PCR reagent in ABI 7700 System (Applied Biosystems). ΔCt values were calculated according to the manufacturer's instructions. Fold induction relative to GAPDH was calculated as $2^{-\Delta\text{Ct}}$. Fold induction of gene X_{ir} in individual irradiated xenografts relative to gene X_c in the control unirradiated xenografts was calculated as $2^{-(\Delta\Delta\text{Ct})}$, where ΔCt values of all control xenografts were averaged. Number of xenografts per each group varied from three to six.

Mouse xenograft studies. Xenografts were established by injection of 10^7 cells in 100 μL PBS into the right hind limb of 6-week-old female athymic nude mice (Frederick Cancer Research Institute). Experiments were conducted 2 to 3 weeks after injection when tumors reached an average size of 200 to 400 mm^3 . For estimation of tumor sensitivity to anti-IFN antibodies, each group of mice (five animals per group) was irradiated daily with six 5 Gy fractions. At the time of each irradiation, xenografts were injected with a mixture of neutralizing antibodies to IFN- α , IFN- β , and IFN- γ (5 $\mu\text{g}/\text{d}$ each; R&D Systems). Relative tumor volume was measured as described previously (30) and shown in the semilog scale. Data represent mean values for each group of mice with error bars for SE. For estimation of nu61 radiosensitivity after transfection by Stat1 short hairpin RNA (shRNA), we used eight animals per group. Xenografts were grown and irradiated as described above. For transcriptional response, xenografts of cell lines indicated in Fig. 2 were established in nude mice, grown to the average size of 400 mm^3 , and subjected to fractionated irradiation with four 5 Gy fractions daily. Twenty-four hours after IR, tumor animals were sacrificed, tumors were excised, and RNA was purified based on Trizol technique as described previously (29), except column purification. Each point represents from three to six individual xenografts. Experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago.

Irradiation of cells and animals. Irradiation was done using a Philips RT₂₅₀ X-ray generator with dose rate 1.65 Gy/min.

Transfection of cells with Stat1 shRNA. To establish SCC61 and nu61 cell lines stably transfected by shRNA to human Stat1, we used pSIREN-RetroQ-DsRed-Express retroviral vector with inserted Stat1 shRNA sequence (5'-gatccGAAAGAAGCTTCTGCTTGTTCAGAGAAACAGCA-GAAAGTTCCTTTCTTTTCTAGCG-3'). This vector together with control vector (L4) was kindly provided by Dr. Elizabeth Quinn and Baz Smith (Clontech). Vectors were packed using pantropic packaging system (Clontech) with GP-293 as packaging cell line and cotransfection with pVSV-G according to the manufacturer's instructions. Cells were grown in the collagen-coated six-well plates (BioCoat, Becton Dickinson), and supernatants were collected 48 h after transfection and used for infection of SCC61 and nu61. Cycles of infection were repeated twice at the presence of 4 $\mu\text{g}/\text{mL}$ polybrene (Sigma), and infected cells were collected by cell sorting using DakoCytomation MoFlo-HTS cell sorter. Infected cells were maintained in the same medium as the parental cell lines.

Transfected cell lines are designated as S1L4 (SCC61 control), S1shS (Stat1 shRNA), N1L4 (nu61 control), and N1shS (Stat1 shRNA).

Detection of the cytotoxic response in transfected cell lines. Because transfected cells were labeled by DsRed protein, we used fluorescent microscopy and YO-PRO-1 green fluorescent dye (Molecular Probes) for detection of dead cells. Cells were plated in the 96-well clear-bottom plates (Costar) in triplicates in concentration 16,000 per well and, 18 h later,

irradiated at 5 Gy. Forty-eight hours after IR, cells were treated by YO-PRO-1 according to the manufacturer's instructions and incubated at room temperature for 48 h and images were captured using fluorescent microscopy. For that, we used Olympus IX81 wide-field fluorescence microscope with Retiga EXi digital charge-coupled device camera. Green fluorescence was detected with 480 nm exciter/535 nm emission filter, and red fluorescence was detected with band pass 530 nm exciter/590 nm long-pass emission filter. Images were captured with $\times 100$ and $\times 200$ magnification, and green and red images were overlaid using MetaMorph 6.1 software (Universal Imaging Corp.). Five fields containing at least 100 cells for each control and the treated group were captured at $\times 100$ magnification and used for scoring of the cell death. Scoring was based on estimation of the percent of dead (green) to alive (red) cells in each field, calculated as $N(\text{green}) / N(\text{red}) \times 100$. Control values were subtracted from values after treatment and presented as increase of the dead cells relative to control (%). Significance of differences was estimated using paired two-tailed *t* test.

Results

IR induces expression of Stat1 and Stat1-dependent IFN-stimulated genes. Earlier, we reported that multiple serial *in vivo* passage concurrent with IR treatment of a human tumor results in the selection of radioresistant tumor designated nu61 (27). DNA arrays and quantitative reverse transcription-PCR (QRT-PCR) data showed that in nu61 Stat1 was up-regulated on the transcriptional level 4.1-fold compared with SCC61 (27). Western blot analysis showed a 5.2-fold overexpression of Stat1 protein in nu61 tumors compared with SCC61 tumors (data not shown), indicating that up-regulation of Stat1 occurs on both transcriptional and translational levels. In established cell lines, we detected a 2.4-fold increase in Stat1 basal expression in nu61 cells compared with SCC61 cells (see Fig. 1A and B, time 0). These results showed that the constitutive overexpression of Stat1 in nu61 tumors is retained in nu61 cells.

We next asked if exposure to IR up-regulates Stat1 expression. The results presented in Fig. 1A and B show that in both cell lines IR leads to up-regulation of the Stat1 protein. In SCC61, this up-regulation reached 2.5-fold at 36 h after IR compared with untreated controls. In nu61, this up-regulation reached 4.5-fold at 36 h and increased to 5.2-fold by 48 h. In a second series of experiments, we tested the hypothesis that IR also activates downstream genes targeted by Stat1. Genes used by us in these experiments (*IFIT1*, *IFIT3*, *IFITM1*, *MX1*, and *OASL*) have been previously described as coexpressed with Stat1 and/or directly activated by Stat1 (31–33). We analyzed the expression of these genes in tumor xenografts representing seven cell lines corresponding to the four types of cancer, including head and neck, colon cancer, prostate cancer, and breast cancer (see Fig. 1C). The results show that IR induced up-regulation of Stat1 and Stat1-dependent genes in all tumor xenografts, except HCT116 colorectal cancer and PC-3 prostate cancer cell lines (Fig. 1C). We conclude that IR induces Stat1 and downstream Stat1-activated/IFN-stimulated genes in multiple human tumors and is not restricted to the SCC61 cell line we used in our initial selection.

Constitutive overexpression of the Stat1 pathway induces resistance to IR and IFNs. Next, we tested the hypothesis that radioresistance is mediated by a constitutive overexpression of the Stat1-dependent pathway and is associated with resistance to IFNs. In these experiments, mice bearing SCC61 tumors were treated with neutralizing antibodies to IFN- α , IFN- β , and IFN- γ with and without IR (four 5 Gy fractions to a total dose of 20 Gy). Anti-IFN antibodies enhanced regrowth of irradiated SCC61 tumors by day 28 (doubling time reduced from 14.5 ± 0.36 to 10.4 ± 0.80 days; $P < 0.05$; Fig. 2A). In contrast, anti-IFN antibodies had no effect on

⁴ <http://rsb.info.nih.gov/ij/>

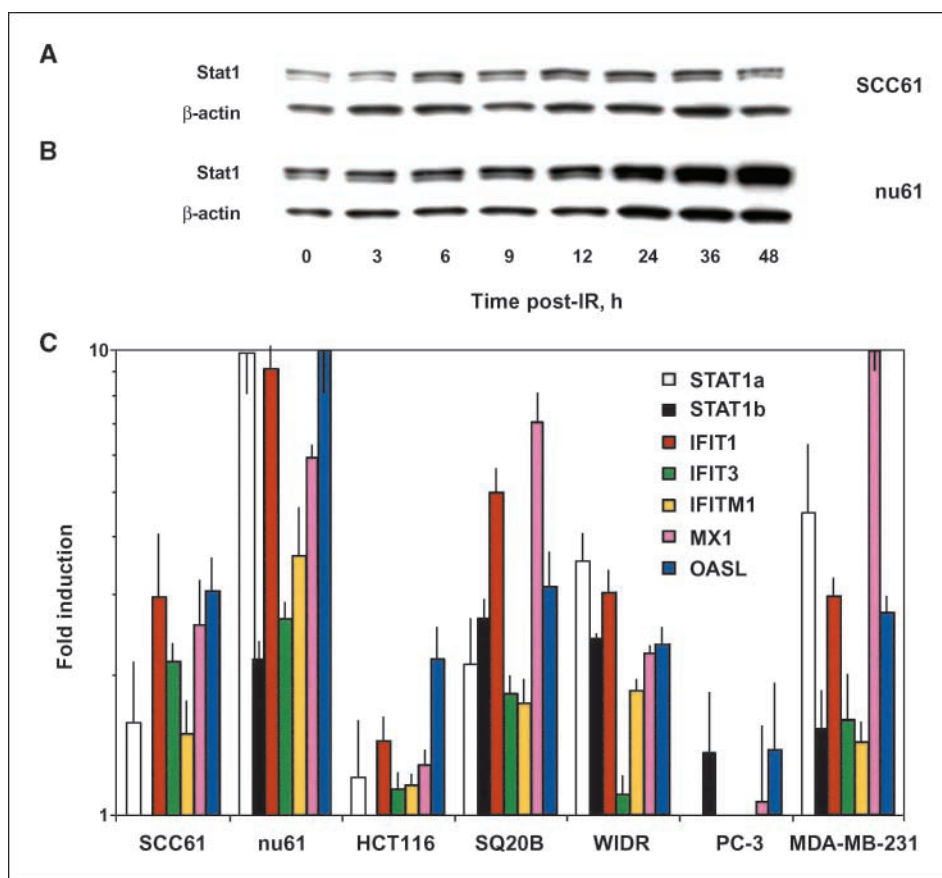


Figure 1. Up-regulation of Stat1 by IR (5 Gy) in SCC61 (A) and nu61 (B) and activation of IFN-Stat1 pathway by IR (C). Western blot analysis (A and B) is described in Materials and Methods. Xenografts of tumor cell lines were established in nude mice and subjected to fractionated irradiation as described in Materials and Methods. Tumors were excised from animals sacrificed 24 h after IR. RNA purification, real-time PCR using SYBR Green in ABI 7700, and calculations of expressional values were done as described in Materials and Methods. Bars, SD.

the regrowth of nu61 tumors (Fig. 2B). These results show that, in parental SCC61 tumors, radiosensitivity can be modulated by IFNs. This tumor is sensitive to IFNs without IR treatment. In contrast, nu61 tumors are resistant to IFNs and its radioresistance cannot be modulated by IFNs.

To test if this phenotype persists *in vitro*, we compared sensitivity of nu61 and SCC61 cell lines to IFN- α and IFN- γ . Figure 3A shows that nu61 cells are more resistant compared with SCC61 cells to IFN- γ at all concentrations tested. At a concentration of IFN- γ of 100 ng/mL (1,000 units/mL), differences in resistance were 1.8-fold ($P = 0.000123$). IFN- α was less cytotoxic to both types of tumor cells. However, at concentrations of IFN- α equal to 25 and 50 ng/mL (7,000 and 14,000 units/mL, respectively), we observed small but significant differences between nu61 and SCC61 ($P = 0.056$ and 0.00022 , respectively). These concentrations are marked by asterisks in Fig. 3A. Taken together, these results show that radioresistant nu61 is also more resistant to IFN- α and IFN- γ compared with SCC61 both *in vivo* (see Fig. 2) and *in vitro* (see Fig. 3A). These results indicate that the association between overexpression of Stat1 and combined resistance to IR and IFNs persists *in vitro*. To confirm a direct role of Stat1 in both IFN and IR resistance, we investigated clones of SCC61 transfected either by a full-size cDNA for Stat1 α or empty vector control, MT4. These clones were described by us previously and showed 2-fold overexpression of Stat1 in transfected clones relative to MT4 and SCC61 (27). In these experiments, we used a clonogenic assay and fitted curves to the linear quadratic model (34). The results were highly significant, with R^2 values for MT4 and Stat1 clones of 0.9929 and 0.9957, respectively. This allowed us to estimate D_0 values for both clones, using data of three independent experiments, presented in Fig. 3B.

These values were equal to 1.12 ± 0.228 for MT4 and 1.80 ± 0.245 for Stat1 clone ($P = 0.0011$). Therefore, mock-transfected and Stat1-transfected SCC61 cells are significantly different and similar to relatively radiosensitive or relatively radioresistant tumor cell lines described previously (35). These results indicate that stable transfection of parental SCC61 cells by a full-size Stat1 confers resistance to both IR and IFNs (see Fig. 3B and C) and recapitulates the phenotype observed by us in *in vivo* studies (see Fig. 2 and ref. 27).

Cells selected to IFN resistance are also radioresistant. To test whether IFN-resistant cells are also resistant to IR, the parental IR- and IFN-sensitive SCC61 cells were serially passaged in medium containing increasing doses of IFNs from 5 to 30 ng/mL for IFN- γ and 1,500 ng/mL for IFN- α (see Materials and Methods). Three IFN- α -resistant clones (aR1, aR5, and aR8) and three IFN- γ -resistant clones (gR8, gR9, and gR13) were chosen as the most resistant (see Materials and Methods). At an IFN- α concentration of 1,500 ng/mL, all three clones selected against IFN- α were significantly more resistant than the parental line SCC61 as measured by MTS assay ($P < 0.05$; Fig. 4A). The IFN- γ -resistant clones were tested in MTS and clonogenic assays. The results of the clonogenic assay (Fig. 4B) show that the IFN- γ -selected clones were 40- to 74-fold more resistant than SCC61. Clonogenic survival analyses done on the six IFN-resistant clones following exposure to 3 or 7 Gy revealed a significant increase in radiation resistance compared with SCC61. For the IFN- α -resistant clones exposed to 3 Gy, the surviving fraction was 50% to 60% greater than SCC61 ($P < 0.008$; see Fig. 5A). At 7 Gy, the differences in surviving fractions were between 1.7- and 3.2-fold ($P < 0.002$). For IFN- γ -resistant clones gR8, gR9, and gR13 at 3 Gy, the increase in the surviving fraction compared with SCC61 varied from 40% to 68% ($P < 0.006$). At 7 Gy,

Stably transfected cell lines (see Materials and Methods) analyzed for Stat1 expression by Western blot analysis (see Fig. 6A) showed that the levels of Stat1 decreased 59% in nu61 and 34% in SCC61 relative to a control vector. In the subsequent experiments, we focused on nu61 cells to investigate the relationships between Stat1 overexpression and radioresistance. To detect the potential effects

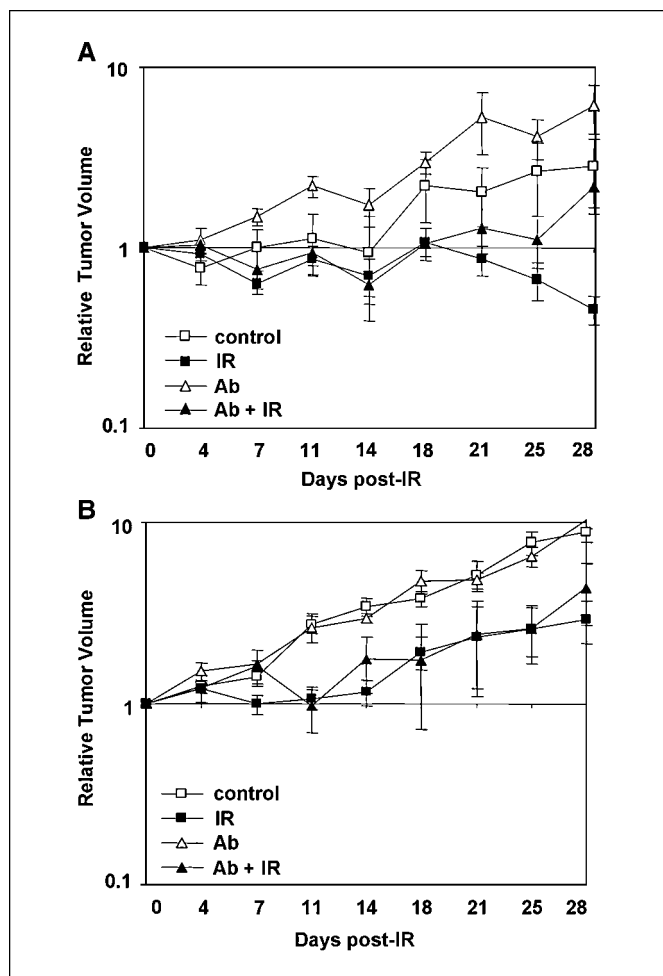


Figure 2. Radioresistant tumor nu61 is also resistant to anti-IFN antibodies *in vivo*. Radiosensitive SCC61 (A) and radioresistant nu61 (B) flank xenografts in nude mice were irradiated (5 Gy) daily for 6 d in the presence or absence of a mixture of neutralizing antibodies (Ab) to IFN- α , IFN- β , and IFN- γ (5 ng/d) given at time of irradiation. Points, mean values for each group of mice; bars, SE.

the differences between SCC61 and IFN- γ -resistant clones ranged between 2- and 3-fold ($P < 0.002$; see Fig. 5B). Western blot analyses revealed an overexpression of Stat1 in IFN- α -resistant and IFN- γ -resistant clones that ranged from 2.0- to 3.5-fold relative to SCC61 using normalization to GAPDH (see Fig. 5C and D). Therefore, all clones selected for IFN resistance overexpressed Stat1 and were also IR resistant compared with the parental cell line SCC61.

Suppression of Stat1 expression reverses tumor radioresistance. Our data indicated that constitutively overexpressed Stat1 leads to resistance to cytotoxic responses of IR and IFNs. To investigate whether suppression of Stat1 leads to radiosensitization, we designed small interfering RNA (siRNA) to silence *Stat1* (si-178). This siRNA suppressed Stat1 mRNA 5.5-fold based on QRT-PCR. Transient transfection with si-178 did not affect the growth and survival of SCC61 cells but significantly (72%; $P = 0.0007$) suppressed nu61 survival (as measured by MTS assay; see Materials and Methods) compared with cells transfected with control "scrambled" siRNA against GAPDH (data not shown). These findings suggest that nu61 survival depends at least in part on constitutive overexpression of Stat1. To investigate the effects of Stat1 suppression in more detail, we used shRNA to establish SCC61 and nu61 clones stably transfected by Stat1 shRNA.

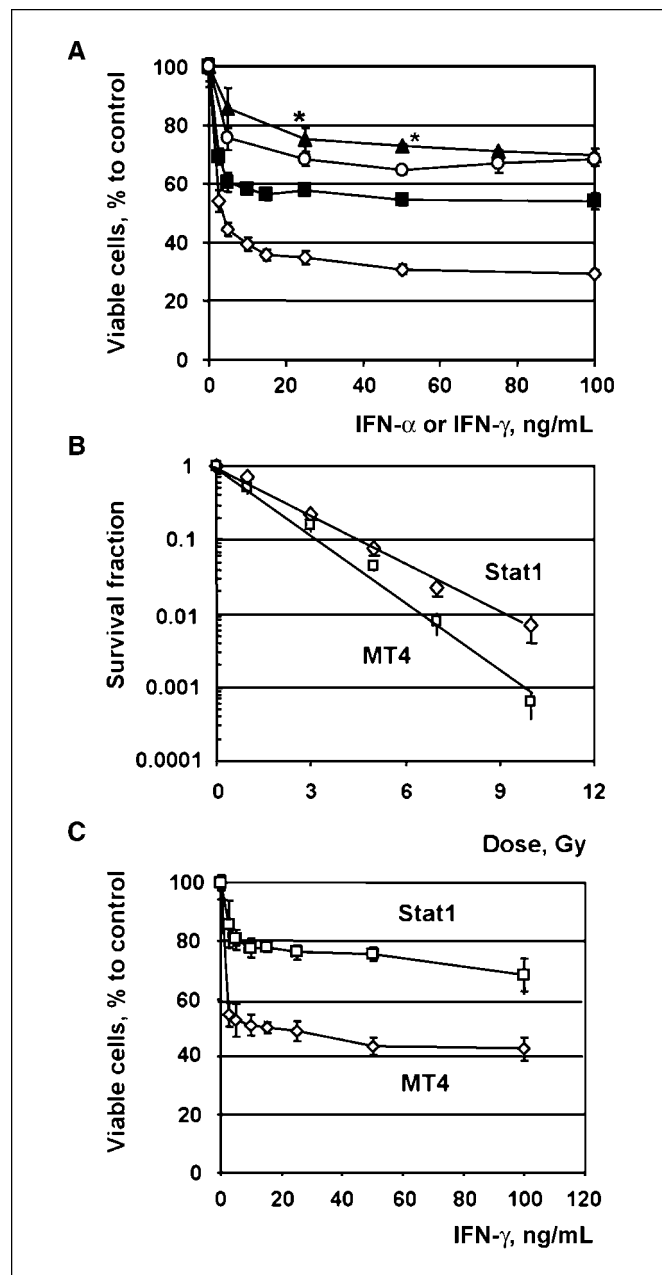


Figure 3. Stat1 overexpression is associated with radioresistance and resistance to IFNs. A, nu61 (filled symbols) and SCC61 (open symbols) were plated in triplicates into the 96-well plates, treated with increasing concentrations of IFN- α (triangles and circles) or IFN- γ (diamonds and squares) and, 48 h later, tested for viability using MTS assay (see Materials and Methods). Bars, SD. B, clonogenic survival of Stat1-transfected clone a16 (see ref. 27) compared with mock-transfected clone MT4. Presented are the data from three independent experiments. Curve fitting is described in the text. Points, mean; bars, SE. C, resistance of Stat1-transfected and mock-transfected clones to IFN- γ . Clones were incubated 72 h in medium containing IFN- γ in concentrations from 1 to 100 ng/mL. Cell viability was assessed by MTS assay. Bars, SD.

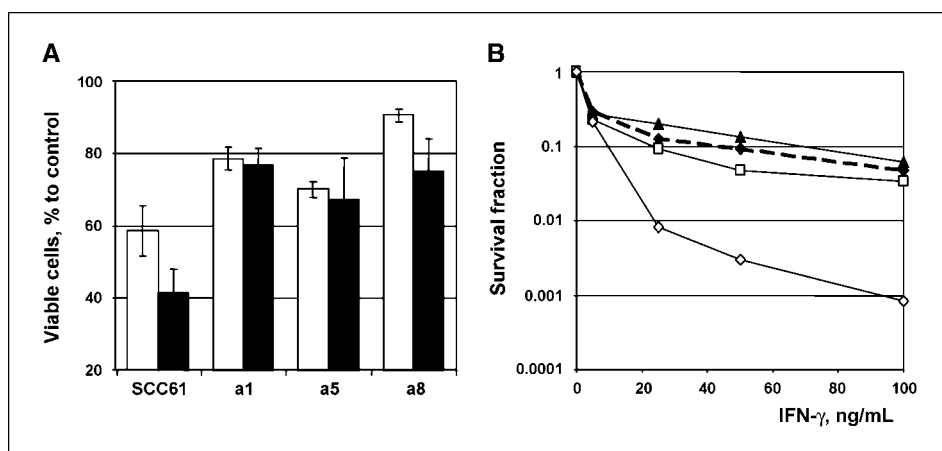


Figure 4. Clones selected against IFN- α and IFN- γ are more resistant to IFNs compared with the parental SCC61. SCC61 was selected by serial passage in medium containing increasing concentrations of IFN- α (up to 1,500 ng/mL; A) or IFN- γ (up to 100 ng/mL; B). Final clones were resistant to 100 and 1,500 ng/mL of IFN- α according to MTS assay (A, white and black columns, respectively) or to the increasing concentrations of IFN- γ according to clonogenic assay (B, open diamonds, SCC61; open squares, clone g8; filled diamonds, clone g9; filled triangles, clone g13; see text and Fig. 5).

of Stat1 suppression on radioresistance, mock-transfected (NIL4) and shRNA-transfected (N1shS) nu61 cell lines were irradiated with 5 Gy and, 48 h after IR, analyzed for the appearance of apoptotic/necrotic cells (see Materials and Methods). Data presented in Fig. 6B and C show that transfection of the shRNA Stat1 constructs into the nu61 cells (N1shS) and irradiation with 5 Gy lead to 6.6-fold increase of the cell death compared with control NIL4 cells with significance of 0.006. We concluded that suppression of Stat1 in nu61 results in radiosensitization of these cells *in vitro*.

To test whether radiosensitization of nu61 can be observed *in vivo*, we established xenografts of NIL4 and N1shS cells in athymic nude mice as described in Materials and Methods. Xenografts were grown to 200 mm³, irradiated, and measured as described previously (see Materials and Methods and ref. 27). The results show that without irradiation N1shS tumors proliferate at a lower rate compared with NIL4 tumors (see Fig. 6D). However, the most striking differences were obtained by comparison of irradiated NIL4 and N1shS tumors. By day 10, the average relative volume of irradiated NIL4 was 1.41 ± 0.17 , whereas N1shS tumors were significantly smaller (0.93 ± 0.36 ; $P = 0.0044$). Thus, anti-Stat1-transfected cells (N1shS) were 1.5-fold more sensitive to irradiation compared with mock-transfected counterparts. By day 14, differences increased by 1.8-fold ($P = 0.004$). We concluded that suppression of Stat1 in tumors overexpressing this protein leads to radiosensitization. The results from *in vitro* and *in vivo* experi-

ments indicate that Stat1 represents a potential target for tumor radiosensitization.

Discussion

In an earlier article, we reported that serial *in vivo* passage of squamous cell carcinoma concurrent with IR resulted in the selection of a population of tumor cells that overexpressed Stat1- and IFN-inducible genes and that the tumor cells exhibited significant levels of resistance to IR compared with the parental cells (27). These findings raised two key questions. First, does IR induce overexpression of Stat1 in tumor cells, and is this phenomenon unique to squamous cell carcinoma or is this a common property of irradiated tumor cells? In this report, we show that induction of Stat1 by IR is a common albeit not a general response of irradiated tumor cells. The second question arising from these data is the role of Stat1 in the development of radiation resistance. Is overexpression of Stat1 a necessary step in the development of resistance to IR or is it a response that is concurrent but not actually related to the development of resistance to IR?

With respect to the first question, two lines of experiments indicate that induction of Stat1 is a common albeit not a general response to IR. In this report, we show that IR induced Stat1 activation in both SCC61 and the radiation-resistant, Stat1-overexpressing nu61 cells (Fig. 1A and B). Our findings are consistent with those of Amundson et al. (36) who showed that

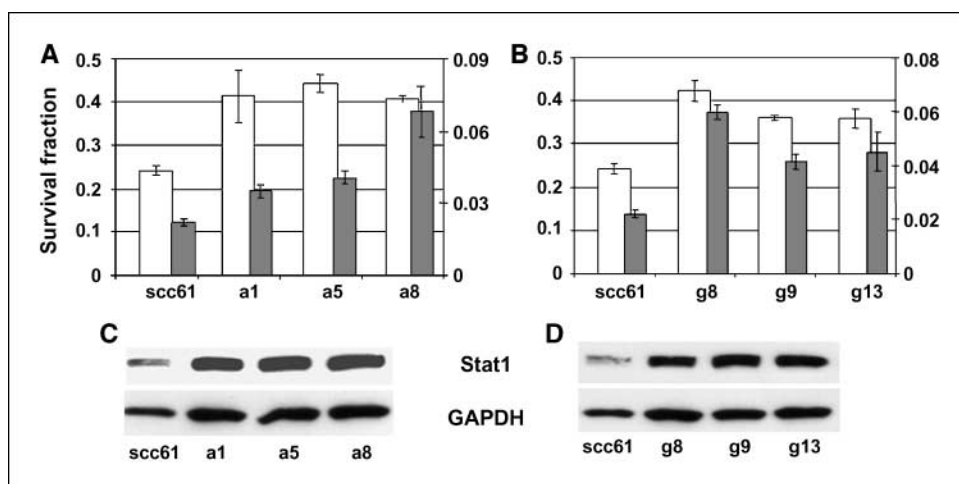


Figure 5. Resistance to IFNs is accompanied by radioresistance and overexpression of Stat1. A and B, resistance of IFN-resistant clones described in the Fig. 4A and B to 3 Gy (white columns, left axis) and 7 Gy (gray columns, right axis) as measured by clonogenic assay. All measurements were done in triplicate. Columns, mean; bars, SE. C and D, Western blot analysis of Stat1 expression in SCC61 and IFN- α -resistant and IFN- γ -resistant clones. GAPDH was used as loading control.

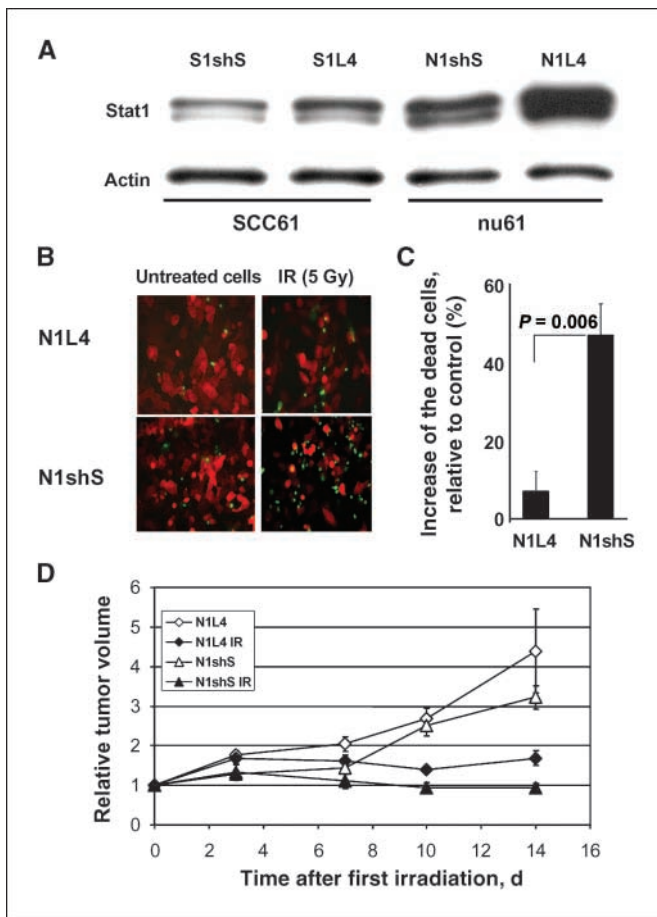


Figure 6. Suppression of Stat1 in radioresistant nu61 leads to radiosensitization. *A*, suppression of Stat1 protein in SCC61 and nu61 by shRNA (see Materials and Methods). *B*, cells, transfected by shRNA and control vector, were stained with 0.5 $\mu\text{g}/\text{mL}$ YO-PRO-1 and visualized and scored as described in Materials and Methods. Magnification, $\times 200$. *C*, significant difference in IR-induced increase of dead cells in shRNA-transfected compared with mock-transfected nu61 cells. Columns, mean; bars, SE. *D*, N1L4 and N1shS cells were injected in flanks of nude mice as described in Materials and Methods. Two groups were left untreated (filled symbols, N1L4 and N1shS controls) and two other groups (open symbols, N1L4 and N1shS irradiated) were subjected to fractionated irradiation (six 5 Gy; total dose, 30 Gy). Last dose was delivered at day 8 and final measurements were done at day 14 after beginning of irradiation. *P* value was estimated by an unpaired two-tailed *t* test. Inset, indication of symbols. Points, mean; bars, SE.

whole-body irradiation leads to the up-regulation of Stat1 and several Stat1-dependent genes in peripheral blood lymphocytes. Jen and Cheung (37) reported that irradiation of lymphoblastoid cell lines leads to the up-regulation of Stat1-dependent genes, which partially overlap with genes, described by us previously (27). Moreover, as illustrated in Fig. 1C, Stat1- and IFN-inducible genes were induced in tumors derived from colon cancer, head and neck cancer, prostate cancer, and breast cancer cells. Up-regulation of Stat1 is readily apparent from QRT-PCR studies of human tumors. We may conclude that Stat1 and the IFN-dependent pathway are up-regulated in a large fraction of diverse human tumors in response to IR.

The second question relates specifically to the role of Stat1 in the development of resistance to IR. Because both SCC61 and nu61 cell cultures secrete IFNs (data not shown), to evaluate the role of IFN in the expression of cytotoxic genes, we tested the growth of SCC61 or nu61 in mouse xenografts treated with anti-IFN antibody. The

results unambiguously showed that anti-IFN antibody enabled SCC61 to grow faster but that the antibody had no effect on nu61 cells (Fig. 2). We can deduce from this experiment that the secreted IFN has a positive therapeutic effect associated with the induction of cytotoxic genes in SCC61 but that this response is not operative in nu61 cells. In these cells, the cytotoxic effects of IFN-inducible genes are blocked and this suppression is also retained *in vitro* (Fig. 3A). In the tumors adapted to continuous exposure to IFNs, Stat1 is overexpressed, and the expected cytotoxic response following exposure to IR or IFNs is also blocked (Figs. 4 and 5). These observations are consistent with published data about overexpression of IFN/Stat1-dependent pathway in primary breast and lung tumors (31, 38). These results suggest that constitutive expression of the cytotoxic Stat1 pathway leads to the selection of tumor clones resistant to the Stat1-dependent cytotoxicity and formation of aggressive tumor clones resistant to IFNs and IR. Although our data show cross-talk between IR- and IFN-inducible pathways and involvement of IFN signaling in the formation of radioresistance, other reports suggest that the Stat1-dependent pathway can be up-regulated by various signaling systems. For example, Pedersen et al. (24) described an ability of ligand-activated EGFR to induce Stat1-dependent genes, which significantly overlapped with the gene pattern we previously reported (27). Jechlinger et al. (39) showed that formation of autocrine PDGF/PDGFR signaling promotes mammary cancer metastasis and involves activation of Stat1. Recent data also show that Stat1 can be activated by erythropoietin (25) and hypoxia (21–23). These data suggest that activation of the Stat1 pathway has more broad significance compared with its traditional link to IFN signaling. Perhaps IFNs and IR may act in concert with other factors of the tumor microenvironment, such as EGF and PDGF. Further experiments are necessary to dissect the role of different signaling systems potentially leading to the constitutive activation of the Stat1-dependent pathway.

The second key experiment was the suppression of Stat1 in nu61 cells with shRNA. Our data show that in this instance we observed that nu61 cells became IR sensitive concurrent with the decrease in the accumulation of Stat1 protein (see Fig. 6A–D).

Based on these data, we propose that Stat1 has a dual function. At levels of activity associated with radiosensitive tumor cells (as SCC61), activation of Stat1 leads to the activation of cytotoxic genes and, ultimately, cell death. At high constitutive levels of Stat1 expression observed in nu61 cells and clones transfected by Stat1 (Fig. 3B and C) or selected against IFN- α and IFN- γ (Figs. 4 and 5), Stat1 activates and maintains the expression of prosurvival genes with simultaneous suppression of cytotoxic effects (Figs. 2 and 3). This may explain the observed switch from proapoptotic to prosurvival functions of Stat1, which is shown by knockdown experiments in nu61 both *in vitro* and *in vivo* (Fig. 6A–D). Indeed, currently, Stat1 is reported to control essential prosurvival genes, such as *MCL-1* (40), *IFITM1*, or *Leu-13* (41), multidrug resistance vault proteins (42), and USP18 (UBP43), which mediates protection from IFN-induced cytotoxicity (43). Importantly, we previously reported that *MCL-1*, *IFITM1*, and USP18 are coexpressed with Stat1 in the IR- and IFN-resistant nu61 tumor (27). Consistent with our observations are recent reports, which indicate that constitutive overexpression of Stat1 and Stat1-dependent genes is associated with protection of tumor cells from genotoxic stress following treatment with fludarabine (44), doxorubicin (45), *cis*-platinum (46), and the combination of IR and doxorubicin (47, 48). The balance between prosurvival and cytotoxic functions of Stat1 may be analogous to the differing effects of tumor necrosis factor superfamily ligands and transforming growth factor- β

on cell growth and death, whereby the outcome of these opposing effects depends on cellular context and environmental factors (49–51).

The most significant finding that bears directly on our model is that suppression of Stat1 accumulation renders the tumor more sensitive to IR. Our data predict that suppression of Stat1 levels in patients overexpressing this protein may lead to a significant benefit to cancer patients undergoing IR therapy.

Addendum

After this manuscript was submitted, Tsai et al. published observations that ionizing radiation induces IFN-related genes including *Stat1*, *Ifit1*, *Ifitm1*, *Oas1*, and *Mx1* in glioma, breast, and

prostate cancer cell lines (52), which is consistent with our current data and explanation of nu61 selection by fractionated IR.

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