ARTICLE
Journal of Cellular Biochemistry 112:3685–3694 (2011)

Journal of Cellular Biochemistry

Signal Transducer and Activator of Transcription 1 (Stat1) Maintains Basal mRNA Expression of Pro-Survival Stat3-Target Genes in Glioma C6 Cells

Alicja Adach-Kilon, Karolina Swiatek-Machado, Bozena Kaminska, and Michal Dabrowski*

Laboratory of Transcription Regulation, Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland

ABSTRACT

The STAT proteins (signal transducers and activators of transcription) are transcription factors mediating cytokine/growth factor signaling, which play important role in controlling cell cycle progression and apoptosis. In many cancer cell lines and tumors (including gliomas) the STAT proteins (in particular Stats 1, 3, and 5) are persistently activated. In this study, we employed DNA decoys, siRNAs, and protein overexpression, to elucidate the role of Stat1 and Stat3 in regulation of expression of endogenous Stat3-target genes (Bcl2l1, Myc, Ccnd1) and a Stat-driven reporter plasmid, in rat C6 glioma cells. The results obtained with the decoys and siRNA suggest that in proliferating C6 cells, Stat1 supports the basal expression of Bcl2l1, while the decoy and chromatin immunoprecipitation results suggest it also plays a similar role for Myc. In the Stat-driven reporter system, overexpression of Stat1 stimulated, while overexpression of Stat3 inhibited the reporter gene expression. The level of Stat1 phosphorylation observed under basal conditions in proliferating glioma C6 cells is very low. Therefore, we speculate that it is the activity of the unphosphorylated Stat1 that is inhibited by Stat1 decoy or Stat1 siRNA. Taken together, our results demonstrate that it is Stat1 not Stat3 that maintains the expression of Bcl2l1 and possibly Myc in proliferating glioma C6 cells. An established paradigm is that Stat3 exerts a pro-survival and potentially oncogenic effects, while Stat1 is mainly associated with the immune response. Our results add to a number of reports that challenge this paradigm. J. Cell. Biochem. 112: 3685–3694, 2011.

KEY WORDS: Stat1; Stat3; C6 GLIOMA; DNA DECOY; STAT OVEREXPRESSION; STAT SILENCING

Signal transducer and activator of transcription 3 (Stat3) is a transcription factor constitutively activated in diverse human tumors, including gliomas, and contributing to malignant transformation, tumor progression and resistance to apoptosis—for a review see Brantley and Benveniste [2008]. It is believed that the phosphorylated active Stat3 (P-Stat3), or its constitutively active mutant, exerts a pro-proliferative and anti-apoptotic effect, acting as a potent oncogene in many tumors [Bromberg et al., 1999; Bowman et al., 2000]. A number of studies indicate that P-Stat3 promotes cell proliferation—by regulating the expression of key genes controlling cell-cycle progression, such as Myc [Kiuchi et al., 1999; Bowman et al., 2001], Ccnd1 [Sinibaldi et al., 2000]; and inhibits apoptosis—by up-regulation of pro-survival genes such as Bcl2l1 [Zushi et al., 1998]. However, effects opposite to the paradigm for Stat3 were repeatedly reported, such as a negative effect of phosphorylated Stat3 on expression of Myc and Ccnd1 in MCF7 cells stimulated with LIF [Zhang et al., 2003], inhibition of Myc-induced transformation of p53-deficient primary mouse fibroblasts [Ecker et al., 2009], or influence of unphosphorylated Stat3 on expression of Met or Mras [Yang et al., 2005].

Activation of Stat1 has been also observed in a number of tumors [Bowman et al., 2000], but it was considered unlikely that activated Stat1 directly contributes to oncogenesis—rather that it plays a role in anti-tumor immune response [Adamkova et al., 2007]. However, in addition to its established role in the immune surveillance, Stat1 can also play a direct role in oncogenesis [Kovacic et al., 2006].

The optimal binding sites for Stat1 and Stat3 have the same consensus TTCC(C/G)GGAA [Horvath et al., 1995], and the experimentally verified binding sites in genes also share a consensus $TTC(N)_3GAA$ [Ehret et al., 2001]. While regulation by both Stats via the same binding site was demonstrated for a few genes, including Socs3 [Ehret et al., 2001], the expression of a larger number of genes, including Myc [Ramana et al., 2000; Zhang et al., 2003], Bcl2l1 [Fujio et al., 1997; Catlett-Falcone et al., 1999], Icam1 and Ccl2 [Fujio et al., 1997; Naik et al., 1997; Valente et al., 1998] was differentially affected by treatments that activate either Stat.

Grant sponsor: Polish Ministry of Science and Higher Education; Grant number: N N401 234934.
*Correspondence to: Michal Dabrowski, Laboratory of Transcription Regulation, Nencki Institute of Experimental Biology, Pasteur 3 Str., 02-093 Warsaw, Poland. E-mail: m.dabrowski@nencki.gov.pl
Received 24 January 2011; Accepted 25 July 2011 • DOI 10.1002/jcb.23305 • © 2011 Wiley Periodicals, Inc.
Published online 3 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

Therefore, it can be expected that regulation of the same genes by Stat1 and Stat3 is likely a rule, rather than exception. The situation is further complicated by the fact that unphosphorylated Stat1 and Stat3 also enter the nucleus [Lodige et al., 2005; Brown and Zeidler, 2008] and regulate gene expression—reviewed in Yang and Stark [2008].

The similarity of Stat1 and Stat3 consensus binding sites does not preclude the existence of binding sites specific for a particular Stat or a subset of them. In particular, the GAS (IFN-γ activation site) element of mouse Ly6E gene [Khan et al., 1993] was shown to bind preferentially Stat1 homodimer, but not Stat3 homodimer or Stat1-Stat3 heterodimer [Horvath et al., 1995]. The M67 variant of the human FOS SIE [Wagner et al., 1990] was shown to bind Stat1 and Stat3 [Horvath et al., 1995], but not Stat4 or Stat5 [Leong et al., 2003]. Note that GAS element denotes a class of similar sites, most of which are not specific for Stat1. In particular, the GAS element in the reporter plasmid pGAS-TA-Luc used in this work contains the sequence TTCCGGGAA binding both Stat1 and Stat3.

Oligodeoxynucleotide decoys are short double-stranded DNA fragments harboring binding sites for a particular transcription factor [Bielinska et al., 1990]. Transcription factor decoy strategy has been used to block the transcriptional activity of many transcription factors—reviewed in Cutroneo and Ehrlich [2006]. This study aims to further verify the inhibitory effect of Stat1 and 3 decoy oligodeoxynucleotides on mRNA expression of selected genes, and on viability of C6 glioma cells. We employed decoys bearing sequences used previously in various studies: the sequence from the Ly6E gene containing the GAS element used in a number of studies as Stat1-specific decoy [Khan et al., 1993; Krzesz et al., 1999; Wagner et al., 2002; Holschermann et al., 2006; Huckel et al., 2006] and the sequence containing M67 FOS SIE used in a number of studies as a decoy against Stat3 [Leong et al., 2003; Xi et al., 2005; Sun et al., 2006], although it binds also Stat1 [Lui et al., 2007]. The results obtained with the decoys were compared to the results of siRNA silencing or overexpressing either Stat1 or Stat3.

MATERIALS AND METHODS

CELL CULTURE

The rat C6 glioma cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (NCS, Gibco, Bethesda, MD) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37°C (Heraeus, Hanau, Germany) as described in Ciechomska et al. [2003].

OLIGODEOXYNUCLEOTIDE DECOYS

All the decoys used in this study were synthetic, double-stranded, phosphorothioate, 5′-FITC-labeled on both strands, HPLC purified oligodeoxynucleotides. The decoys containing Stat1 or Stat3 binding sites, and the respective control decoys were purchased, as single-stranded oligonucleotides, from Sigma–Aldrich (Sigma Life Science). Double-stranded oligomers were prepared in an annealing buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA) by melting at 95°C followed by cooling to room temperature. The sequences (5′ → 3′) of the decoys were the following, with the other strand fully complementary: Stat1 consensus CATGTTATGCATATTCCTGTAAGTG and Stat1 scrambled TGTCATACTCGTACACAC, both from Holschermann et al. [2006]; Stat3 consensus CATTTCCCGTAAATC and Stat3 mutated CATTTCCCTTAAATC, both from Xi et al. [2005].

FLUORESCENCE MICROSCOPY

FITC labeling of the decoys permitted studying internalization and subcellular localization of the decoys. Four hours after transfection, the efficiency of uptake of the decoys and their cellular distribution was estimated using Olympus IX70 fluorescence microscope. The images were acquired with Viewfinder Lite Version 1.0.125 software.

PLASMIDS

Plasmid pGAS-TA-Luc from Clontech (Cat. No. 631915), coding for the firefly luciferase reporter gene driven by two copies of a GAS element, was used to track the Stat-dependent gene expression. The plasmid pCMV6-XL4 encoding Stat1 was purchased from OriGene (Cat. No. SC128145), and the plasmids (kindly provided by Dr. Yuxin Wang, Cleveland Clinic Lerner Research Institute, Ohio, USA) pLEGFP-STAT3-WT and pLEGFP-STAT3-Y705F, were used to analyze the effect of Stat1 or Stat3 overexpression and of Stat3 tyrosine 705 phosphorylation on endogenous or reporter gene expression.

TRANSFECTION OF THE DECOYS

Rat C6 glioma cells were plated in 12-well plates in a complete medium (DMEM, 10% NCS, 1% P/S) at a density of 5×10^5/well and incubated for 24 h. The next day, the cells were transfected with the indicated decoy at the final concentration of 15 nM, using Lipofectamine2000 (Invitrogen Paisley, UK) in the medium containing 2% NCS without antibiotics, according to manufacturer's instruction, or treated with the transfection reagent only (mock transfection). The DNA and Lipofectamine2000 were diluted in OptiMem (Gibco/BRL, Bethesda, MD). Total RNA was isolated at 4 h after the transfection. Cell viability was determined by a MTT metabolism assay performed at 48 h after the transfection as described [Ciechomska et al., 2003].

ELECTROPORATION OF SIRNA OR PLASMIDS

Rat C6 glioma cells (2×10^6) were transfected by electroporation with the indicated siRNA at the final concentration of 150 nM, or with 2.5 μg/well of the indicated plasmid, using the Amaxa and Cell Line Nucleofector Kit V (both from Lonza), according to the manufacturer's protocol. Total protein and RNA were isolated at 48 h after the transfection. The MTT metabolism assay was performed at 48 h after the transfection.

REPORTER GENE ASSAY

C6 cells were plated into 24-well plates at a density of 2×10^5/well and incubated for 24 h. To study the effects of the decoys, or Stat1 or Stat3 overexpression on the reporter gene activity, the cells were co-transfected with 1 μg/well of the reporter plasmid, and a given decoy at the final concentration 15 nM, or 1 μg/well of Stat-overexpressing plasmid, using Lipofectamine2000, as described for

the decoys. Four hours later, the medium was changed to the complete one. Twenty hours later, the cells were lyzed in Passive Lysis Buffer and subjected to luciferase activity assay. To study the effects of siRNA, 24 h after the electroporation with siRNA, the cells were transfected with 2.5 μg of the reporter plasmid using Lipofectamine2000. After 4 h the medium was changed and the cells were cultured for further 20 h and lyzed as described above.

CELL LYSIS AND RNA ISOLATION

Four hours after transfection, the cells were lysed in solution containing β-mercaptoethanol in RLT buffer (Qiagen). Cells were scraped with a cell scraper and cell lysates were collected. RNA was isolated using Rneasy Kit (Qiagen) according to the manufacturer's protocol and eluted in Rnase-Free Water. RNA was analyzed using Agilent 2100 Bioanalyzer that generates the RNA Integrity Number (RIN) and quantity estimation.

RT-PCR AND REAL-TIME PCR

Quantification of gene expression was done with real time PCR. First, 1.8 μg of RNA was reverse-transcribed by extension of oligo $(dT)_{15}$ primers with the M-MLV reverse transcriptase using Mastercycler (Eppendorf). The obtained cDNA was diluted 32× in ultra pure H_2O (Qiagen). Real-time PCR was performed in duplicate in 25 μl reaction volume containing Sybr Green PCR Master Mix (Applied Biosystems) and two pairs of primers: one for 18S rRNA, and the other for a particular target gene, designed using Primer Express software. For each particular target gene, master mixes containing Sybr Green Master Mix, forward and reverse primers and water were prepared. Then, appropriate cDNA templates were added. Amplification was performed with 7500 Real-Time PCR System (Applied Biosystems) using SDS v1.2 software. Relative quantification (ΔCt) method was used, with 18S rRNA as an endogenous control.

ANALYSIS OF CELL VIABILITY—MTT METABOLISM ASSAY

C6 glioma cells were plated into 24-well plates. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution was added to each well to a final concentration of 0.5 mg/ml. MTT and all other reagents were purchased from Sigma–Aldrich. After 4 h of incubation at 37°C water-insoluble dark blue formazan crystals that formed from MTT cleavage in actively metabolizing cells were dissolved in lysis buffer containing 20% SDS and 50% DMF. Optical densities were measured at 570 nm using a scanning multiwell spectrophotometer.

CHROMATIN IMMUNOPRECIPITATION ASSAY

For ChIP assay, we used ChIP-It Express Kit (Active Motif) according to manufacturer's instruction. The chromatin was sheared by enzymatic digestion for 10 min at 37°C and immunoprecipitated for 4 h at 4°C with constant rotation with the following antibodies (Santa Cruz Biotechnology): Stat1 p84/p91(E23)—sc-346, P-Stat1 (Tyr 701)—sc-7988, Stat3 (C20)—sc-482, P-Stat3 (Tyr 705)—sc-7993. The positive control antibody—against RNA polymerase II, and the negative control—normal goat IgG, were both from Active Motif.

Immunoprecipitated DNA fragments were used as templates for PCR with following primers: Beta-actin forward GGCCTTGGAGTGTGTATTCAGTAG, and reversed GGGGTGTTGAAGGTCTCAAA (Active Motif), Myc promoter forward GTTTACACCCCGAGTCGGAGTA, and reversed CAGACCCCCGGATTATAAAGG. The PCR reaction was started with an initial melt step at 94°C for 4 min and then 40 cycles of [94°C for 20 s, 55°C for 30 s, and 72°C for 30 s] and a hold cycle at 4°C were performed. All PCR products were analyzed by resolving on 1.5% agarose gel. A 100 bp ladder (Fermentas) was used as a migration standard.

WESTERN BLOT

Whole-cell protein extract from C6 glioma cells were resolved on polyacrylamide gel before electrophoretic transfer to nitrocellulose membrane as described [Ciechomska et al., 2005; Ellert-Miklaszewska et al., 2005]. Membranes were incubated overnight at 4°C with Stat1, P-Stat1, Stat3, and P-Stat3 (Cell Signaling) antibodies diluted 1:1,000 in TBS-T (Tris-Buffered Saline-Tween-20: 50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6). The primary antibody reaction was followed by 1-h incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Vector Laboratories). Immunocomplexes were visualized using the enhanced chemiluminescence detection system (ECL; Amersham Bioscience).

STATISTICAL ANALYSIS OF THE RESULTS

The values of ΔCt (target gene − 18S rRNA) exported from the SDS v1.2 software were converted to ΔΔCt by subtracting from them the mean ΔCt of the reference samples in the same experiment. In the decoy or siRNA experiments, the samples from the cells transfected with the respective control decoy or with the negative control siRNA were used as the reference. The resulting ΔΔCt values were analyzed by Student's *t*-test. In the analysis of effects of Stat overexpression on the reporter activity or gene expression, the samples from the cells transfected with the control pEGFP plasmid were used as the reference. The resulting ΔΔCt values for each target gene were analyzed by one-way ANOVA followed by the Duncan post hoc test. The values of luciferase activity were first were normalized to the total protein in each sample, then divided by the mean of protein-normalized values in the reference samples. In the analysis of effects of siRNA on cell viability, the samples from the mock-transfected cells were used as the reference. The resulting reference normalized values were analyzed by *t*-test for the decoys, or by one-way ANOVA for siRNA and overexpression. Data normalization was performed in Excel, and the statistical analysis in Mathematica 8 (Wolfram Research). Results with P-value <0.05 were considered significant.

RESULTS

TRANSFECTED DECOYS ENTER THE NUCLEI OF THE CELLS

Decoys to be effective should be localized in the cell nuclei [Bene et al., 2004]. Therefore, we first studied the cellular uptake and subcellular distribution of the decoys. The cells were transfected with fluorescein-labeled phosphorothioate decoys. Transfection efficiency, estimated at 4 h by comparing the number of cells

exhibiting green fluorescence with the total number of cells in five independent fields, was ~60% for all tested decoys and their respective controls (data not shown). The green fluorescent signal was intranuclear (Fig. 1), where it persisted for several hours. The intensity of fluorescence was reduced 24 h after transfection and its distribution started to become extranuclear. Our observations demonstrated that the decoys containing nuclease-resistant phosphorothioate linkages were stable within cells and had nuclear distribution; therefore, they may bind Stat proteins rendering them incapable of binding to the promoters of their target genes.

Stat1-SPECIFIC DECOY REDUCES, WHILE Stat3 DECOY INCREASES, THE EXPRESSION OF GAS-DRIVEN REPORTER GENE

The reporter plasmid pGAS-TA-luc contains two copies of the GAS element, which matches the common consensus of Stat1 and Stat3 optimal binding site. In order to check the influence of Stat1 or Stat3 decoy on the transcriptional activity driven by this element, the glioma C6 cell were co-transfected with the decoys and pGAS-TA-luc. We observed that Stat1 decoy significantly inhibited GAS-driven gene expression (Fig. 2A), in comparison to its control—Stat1 scrambled decoy, whereas Stat3 decoy increased the GAS-driven expression (Fig. 2B), as compared to its control—Stat3 mutated decoy. The changes were highly statistically significant and strongly indicated antagonistic effects of Stat1 and Stat3 on GAS-driven transcription in glioma C6 cells.

Stat1-SPECIFIC DECOY REDUCES THE BASAL EXPRESSION OF Bcl2l1

Having established that Stat1-specific and Stat3 decoys enter the nuclei and exert opposing effects on expression of GAS element-driven reporter gene expression, we sought to study their effects on expression of endogenous Stat-target genes. For this study, we focused on three well-known Stat3 target genes: Bcl2l1, Myc, and Ccnd1, because of their important role in tumor biology. The levels of mRNA of two genes, namely: Bcl2l1 and Myc, were significantly down-regulated following the transfection of Stat1 decoy, as compared to Stat1 scrambled decoy (Fig. 2C). A similar but not statistically significant reduction in the expression of Ccnd1 was also observed.

Transfection of Stat3 decoy slightly increased the expression of Bcl2l1 (Fig. 2D, $P = 0.07$), as compared to Stat3 mutated decoy. A minor (but not non-significant) increase in the expression of Myc was also observed. Neither Stat1 nor Stat3 decoy had any effect on cell viability of C6 glioma cells as demonstrated using MTT metabolism assay (Fig. 2E).

Stat3 siRNA INCREASES THE GAS-DRIVEN TRANSCRIPTION

We first checked the effectiveness and specificity of Stat1 or Stat3 silencing with specific siRNAs in transfected glioma cells by Western blotting. The effects of siRNA against Stat1 or Stat3 were compared to the effect of the negative control siRNA. Stat1 siRNA effectively silenced the expression of Stat1 (Fig. 3A), while transfection with

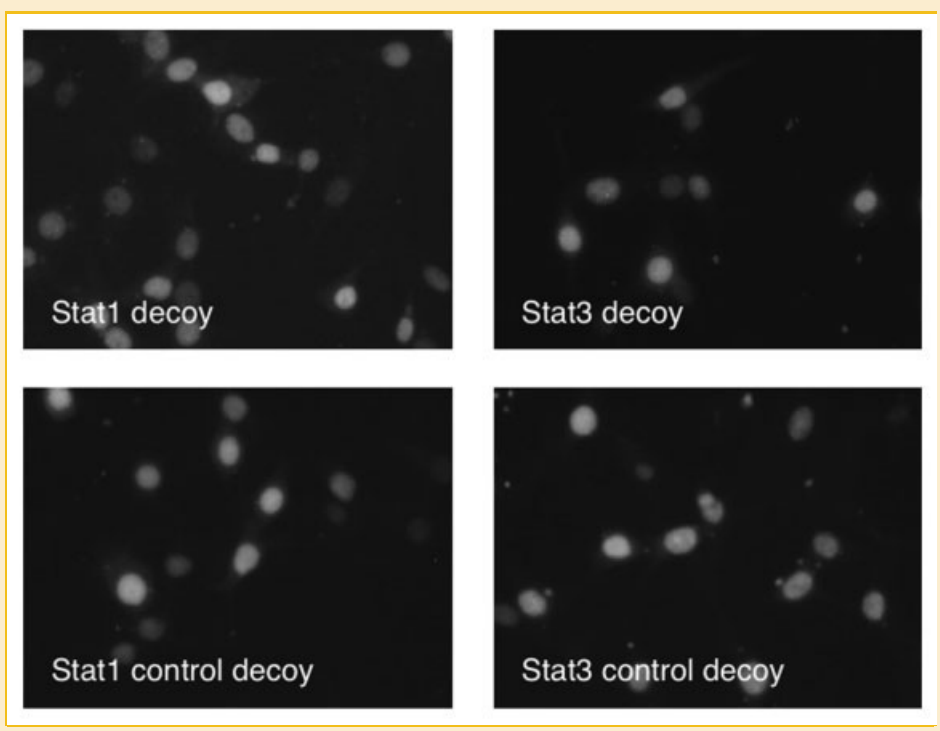

Fig. 1. Decoys uptake by glioma C6 cells. Representative pictures show cells at 4 h after transfection with the indicated decoy. FITC-labeled decoys were visualized using Olympus IX70 fluorescence microscope. For the figure, the green fluorescence was converted to the grayscale. The nuclear localization of the fluorescence is well visible over the weak fluorescence of the cytoplasm.

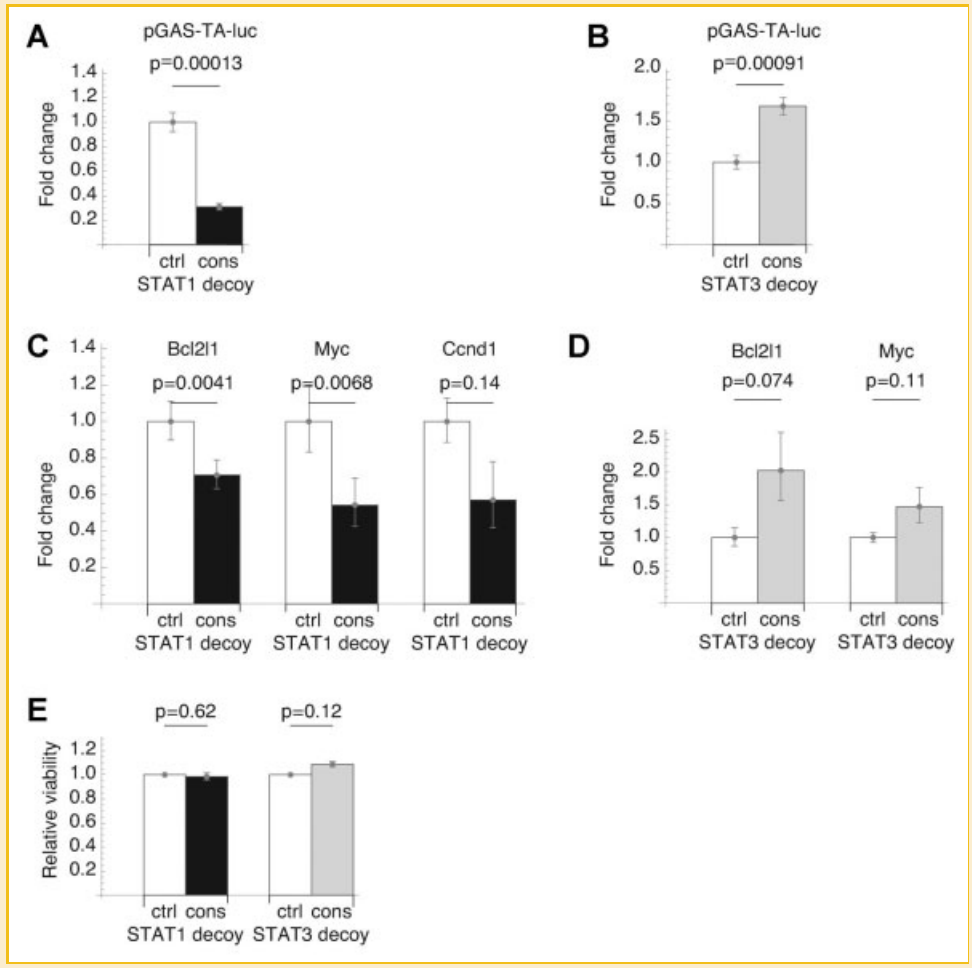

Fig. 2. Effects of the Stat1 or Stat3 decoys on gene expression and cell viability. A,B: The luciferase activity was measured at 24 h after co-transfection pGAS-TA-luc with the indicated decoy. The bars indicate mean values of luciferase activity, relative to the activity in the cells transfected with the respective control decoy, with error bars marking ± 1 standard deviation. Data are from representative experiments, each performed in triplicate. C,D: mRNA expression of the indicated genes was measured by quantitative PCR at 4 h after the transfection of the indicated decoy. The bars represent average fold change (FC) of expression, relative to the average expression in the cells transfected with the respective control decoy. Error bars mark ± 1 standard deviation of $\Delta\Delta$Ct, converted to the FC scale. Data are from two experiments, each in duplicate. E: Cell viability was measured at 48 h after the transfection of the indicated decoy using the MTT metabolism assay; data are from two experiments, each in duplicate. In all panels "STAT1 decoy" and "STAT3 decoy" indicate pairs of specific/control decoy, with "cons" marking the active decoy and "ctrl"—the control decoy. *P*-values are from the Student's *t*-test.

Stat3 siRNA led to a several-fold reduction of Stat3 expression (Fig. 3B). Stat3 is constitutively phosphorylated in proliferating glioma C6 cells, whereas phosphorylated Stat1 is expressed at a very low level (data not shown). The silencing of Stat3 induced by siRNA was accompanied by a similar reduction in P-Stat3 level (Fig. 3B). We were not able to detect P-Stat1 in the cells transfected with the negative or Stat1 siRNA. Each Stat siRNA was specific for its intended target, that is, Stat1 siRNA had no effect on Stat3, and vice versa (data not shown).

In order to check the influence of Stat1 or Stat3 siRNA on the GAS element-driven transcriptional activity, the C6 glioma cells were co-transfected with the reporter plasmid pGAS-TA-luc and a given siRNA. Stat3 siRNA significantly increased the reporter expression, as compared to the control siRNA (Fig. 3C). Unexpectedly, Stat1 siRNA, despite effectively silencing Stat1 expression, had no effect on the GAS element-driven expression.

Stat1-SPECIFIC siRNA DECREASES EXPRESSION OF Bcl2l1

Transfection of Stat1 siRNA led to a small but significant reduction in the expression of Bcl2l1 (Fig. 3D), as compared to its expression in the cells transfected with the control siRNA, with no effect ($P > 0.5$) on expression of either Myc or Ccnd1. Notably, the effect of Stat1 silencing on mRNA expression of Bcl2l1 was in the same direction as after blocking Stat1 activity with the decoy. Following the transfection of Stat3 siRNA, we observed a slight but statistically non-significant increase in expression of Bcl2l1 and Myc (Fig. 3E). Neither Stat1 nor Stat3 siRNA had any effect on viability of C6 glioma cells (Fig. 3F).

OVEREXPRESSED Stat1 INCREASES, WHILE OVEREXPRESSED Stat3 DECREASES THE EXPRESSION OF GAS-DRIVEN REPORTER GENE

To elucidate a crosstalk between the Stat proteins, we studied how overexpression of Stat1 or Stat3 influences Stat-dependent gene

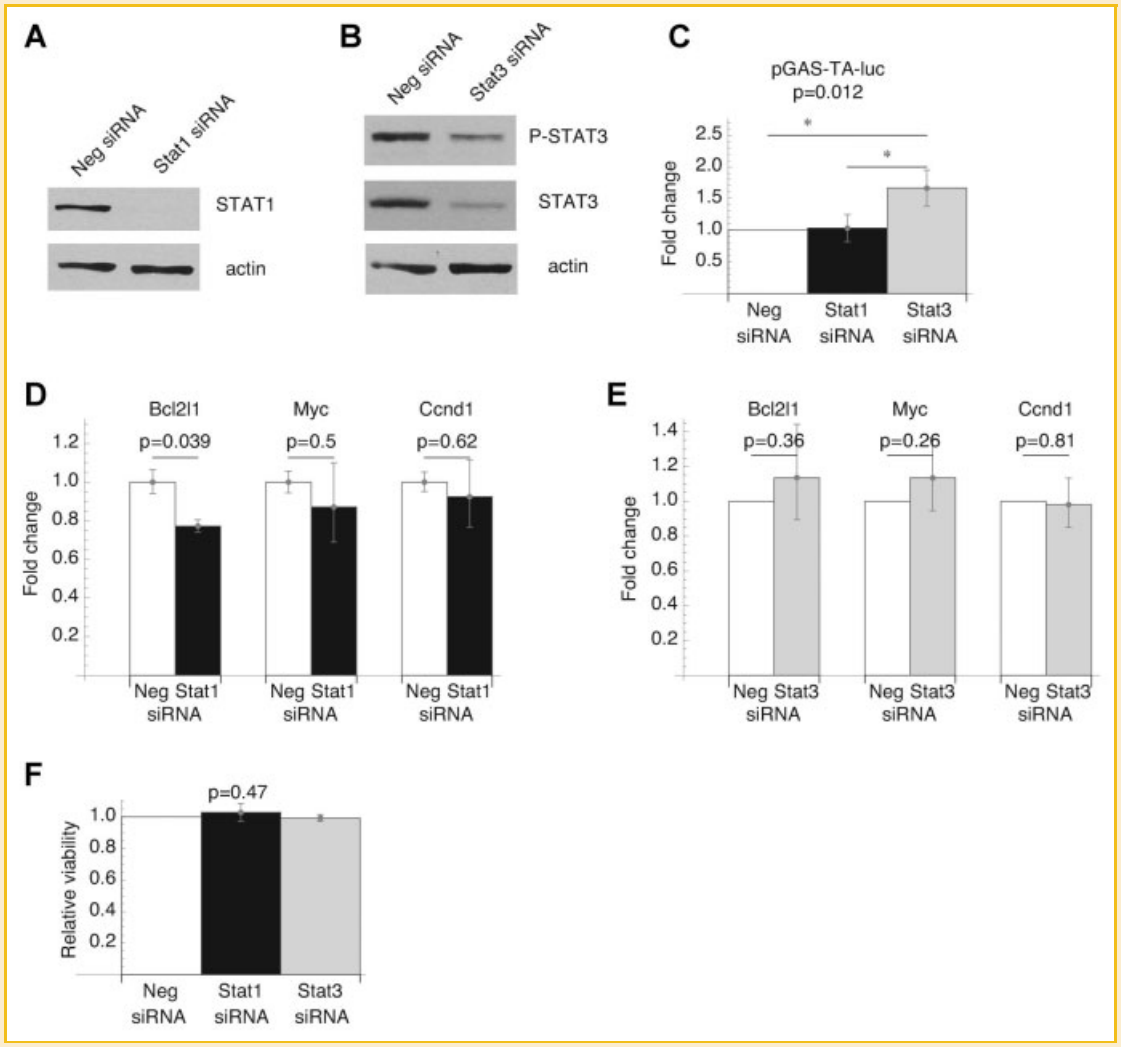

Fig. 3. Effects of Stat1 or Stat3 silencing on gene expression and cell viability. A,B: Western blots confirm complete silencing of Stat1 (A) and partial silencing of Stat3 (B) by the respective siRNA. C: The cells were electroporated with the indicated siRNA, at 24 h transfected with pGAS-TA-luc and cultured for additional 24 h. The bars indicate mean values of luciferase activity, relative to the activity in the cells transfected with the control negative siRNA, with error bars marking the intervals of ± 1 standard deviation; data are from three experiments, each in triplicate. $^{*}P < 0.05$ (Duncan post hoc test). D,E: mRNA expression of the indicated genes was measured by quantitative PCR 24 h (Stat3) or 48 h (Stat1) after the transfection of the indicated siRNA. The bars represent average fold change of expression, relative to the average expression in the cells transfected with the control negative siRNA. Error bars mark the intervals of ± 1 standard deviation of $\Delta\Delta$Ct, converted to the FC scale. Data are from 2 (Stat1) or 4 (Stat3) experiments, each in triplicate. F: Cell viability was measured 48 h after transfection of the indicated siRNA by the MTT metabolism assay; data are from three experiments, each in triplicate. In all panels "Stat1" and "Stat3" indicate siRNA specific for the given Stat, and "Neg" indicates the negative control siRNA. *P*-values from one-way ANOVA (A,F) or Student's *t*-test (D,E).

expression. The expression constructs encoding Stat1, Stat3 (Stat3-WT), or phosphorylation deficient mutant Stat3-Y705F [Yang et al., 2005] were employed. We first checked that their transfection into the cells led to overexpression of the encoded Stat protein (Fig. 4A,B). In the case of Stat1 and Stat3-WT, but not the Stat3-Y705F, the overexpressed protein become phosphorylated (Fig. 4A,B).

In co-transfection experiments, we found that GAS-driven transcription was highly significantly increased when Stat1 was overexpressed, while it was equally significantly decreased when Stat3 was overexpressed (Fig. 4C). The inhibitory effect of overexpression of the phosphorylation deficient Stat3 was less pronounced than in the case of the Stat3-WT, but it was in the same direction and highly statistically significant. Thus, the effects of overexpressing either Stat1 or Stat3 on GAS-element driven expression were opposite to each other, with Stat1 inducing and Stat3 inhibiting the reporter gene expression. For either Stat, the effect of its overexpression was opposite to the effect of the respective decoy.

Stat3 overexpression resulted in a significant increase in the expression of Myc (Fig. 4E), whereas had no effect on Bcl2l1 and Ccnd1 (Fig. 4D,F). Overexpression of Stat1, or of the phosphorylation-deficient Stat3-Y705F mutant, had no effect on the expression of three studied genes. Unexpectedly, the direction of the change in expression of Myc induced by Stat3 overexpression was opposite to the changes observed for the GAS-driven reporter construct.

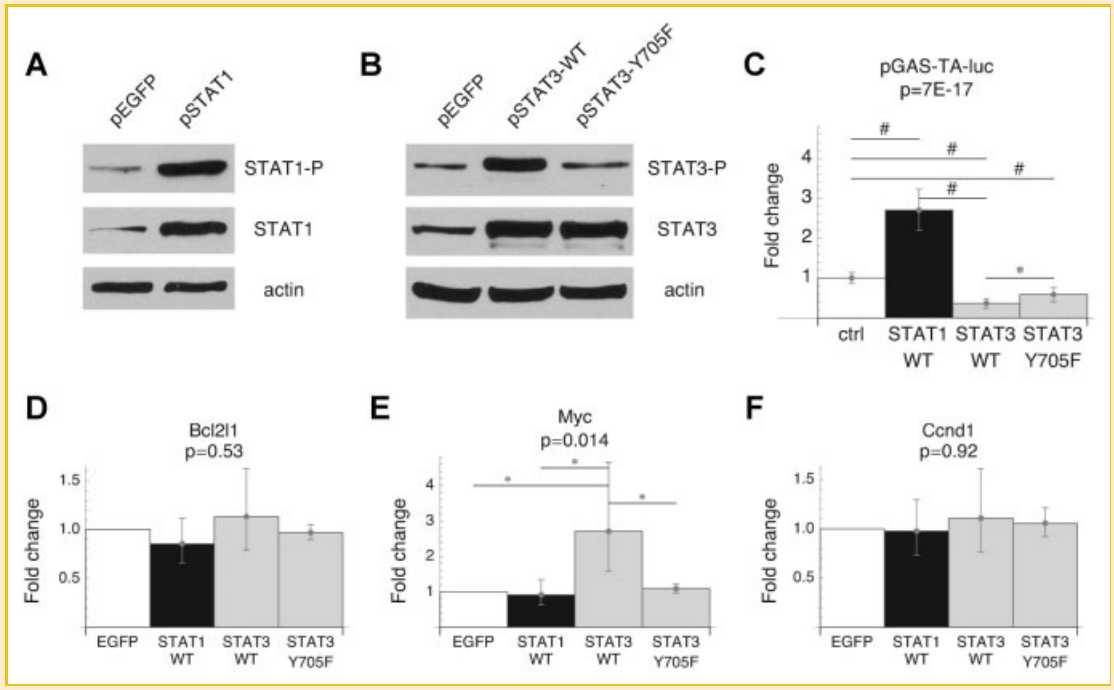

Fig. 4. Effects of overexpression of Stat1, Stat3, or phosphorylation-deficient Stat3-Y705F on gene expression. A,B: Western blots confirm overexpression of Stat1 (A), Stat3 or pSTAT3-Y705F (B) in transfected cells. C: The luciferase activity was measured at 24 h after the co-transfection of pGAS-TA-luc and the indicated expression plasmid. The bars indicate mean values of luciferase activity, relative to the activity in the cells transfected with the control plasmid pEGFP, with error bars marking the intervals of ± 1 standard deviation. Data are from four experiments, each in triplicate. $^{\#}P < 0.0001$, $^{*}P < 0.05$ (Duncan post hoc test). D–F: mRNA expression of the indicated genes was measured at 24 h after the transfection of the expression plasmid by quantitative PCR. The bars represent average fold change of expression, relative to the activity in the cells transfected with the control plasmid pEGFP. Error bars mark the intervals of ± 1 standard deviation of $\Delta\Delta$Ct, converted to the FC scale. Data are from three experiments, each in triplicate.

BOTH Stat1 AND Stat3 BIND TO THE Myc PROMOTER

Chromatin immunoprecipitation (ChIP) with antibodies specific for total Stat1 (p84/p91), P-Stat1, total Stat3, and P-Stat3, was employed to study recruitment of Stat proteins to the P1–P2 promoter region of Myc—a gene known to be regulated (in different cells and under different conditions) by both Stat1 and Stat3. The ChIP showed that under basal conditions the Myc gene promoter in glioma C6 cells was occupied by both Stat1 and Stat3, both at least partially phosphorylated (Fig. 5).

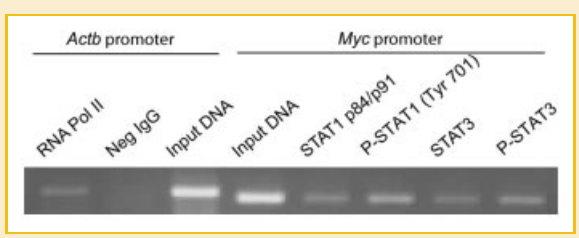

Fig. 5. Binding of Stat1 and Stat3 to the Myc gene promoter. ChIP analysis of Stat1 and Stat3 binding to Myc gene promoter. Chromatin complexes from C6 cells were immunoprecipitated with antibodies specific for: total Stat1, P-Stat1, total Stat3, P-Stat3, RNA polymerase II (positive control), or the pre-immune IgG (negative control). The DNA binding was analyzed by PCR, using primers specific for promoter regions of Myc or Actb (a control region).

DISCUSSION

In this study, we employed DNA decoys, siRNAs, and protein overexpression, to elucidate the role of Stat1 and Stat3 in regulation of expression of endogenous Stat3-target genes (Bcl2l1, Myc, Ccnd1) and a GAS-driven reporter gene. For the ease of discussion, the results obtained with these approaches are summarized and compared in Figure 6.

The results obtained with the decoys and siRNA suggest that in the proliferating C6 glioma cells, Stat1 participates in maintaining the basal expression of at least one Stat3 target gene, namely Bcl2l1. We demonstrate moreover, using DNA decoys and overexpression, that Stat1 and Stat3 have opposite effects on GAS element dependent gene expression, with Stat1 stimulating and Stat3 (also thyrosine 705 phosphorylation deficient Stat3) inhibiting GAS-element driven expression. In the light of these results, it seems plausible that the small and non-significant increases in expression of Bcl2l1 and Myc, induced by both Stat3 decoy and Stat3 siRNA, may reflect down-regulatory effects of Stat3 on these genes. Participation of both Stats in the regulation of Myc is corroborated by demonstration of binding of both Stat1 and Stat3 to the Myc gene promoter. It is unclear why silencing of Stat1 had no effect on expression of the GAS-element driven reporter gene. Treatments of the cells with the decoys or with the siRNA did not affect their viability, possibly because the induced changes of transcription

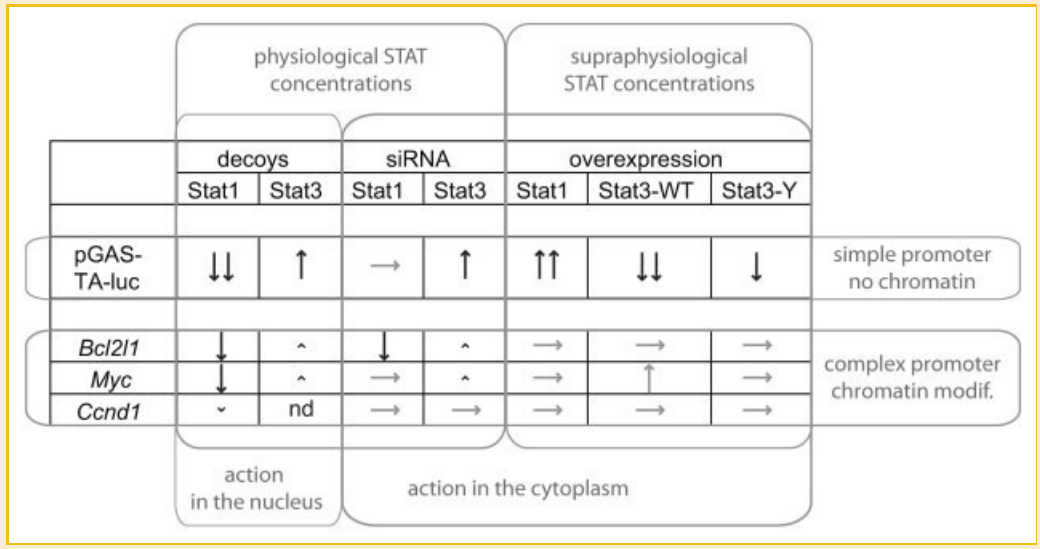

Fig. 6. Summary of the results. The results of using decoys, siRNA and overexpression are summarized, with the treatments in columns and measured targets in rows. For every pair of treatment and target, the vertical arrows indicate the direction of the significant ($P < 0.05$) changes in expression/reporter activity. Vertical arrowheads indicate the direction of the changes in expression that did not reach significance ($0.05 < P < 0.5$). Horizontal arrows indicate no change in expression ($P > 0.5$). The results that support the hypothesis that Stat1 maintains expression of the target genes are marked black. Results against this hypothesis are marked gray, "nd" indicates no data. The side panels highlight some of the differences between the approaches and targets.

factor activity or expression were too small or transient. The observed lack of the effects of Stat inhibition or silencing on cell viability argues against an important role of Stat1 or Stat3 in the regulation of cell survival.

Stats overexpression, while clearly showing regulation for the co-transfected GAS-driven reporter construct, did not produce changes in the expression of the endogenous target genes. The notable exception was Stat3-WT stimulating the expression of Myc, which suggests that the regulation of this gene in C6 glioma cells differs from the regulation of the other two genes, in particular of Bcl2l1. The two genes also responded differently to Stat1 siRNA, which down-regulated Bcl2l1 but not Myc. Notably, only the Stat3-WT, but not the Stat3-Y705F mutant, stimulated the expression of Myc, while both proteins down-regulated the expression of pGAS-TA-luc. This highlights the difference between a simple reporter system and the complexity of the regulation of an endogenous gene, or alternatively, limitations of our experimental approaches.

Our results obtained in C6 glioma cells with Stat3 decoy are in apparent disagreement with the results obtained by Gu et al. [2008] in two glioma cell lines: U251 and A172. Upon transfecting those cells with the same Stat3 decoy, the authors observed down-regulation of Bcl2l1, Myc, and Ccnd1, as well as decreased cell viability. However, their and our data can be re-conciliated by noting that Stat3 effects may be cell-type specific. Furthermore, Gu et al. used higher concentration (50 vs. 15 nM) of the decoys, more prolonged (24 vs. 4 h) lipofectamine-mediated transfection, and a different control decoy (scrambled vs. mutated), which could also contribute to the differences in the results.

Cuevas et al. [2006b] reported that dobesilate—a synthetic FGF inhibitor—reduces the level of Stat3 phosphorylation in C6 glioma cells, with a concurrent reduction in expression of Bcl2l1 and Ccnd1 assessed by immunocytochemistry. These results appear contradictory to our results suggesting that Stat1, and not Stat3, maintains the expression of Bcl2l1 in the C6 glioma cells. However, dobesilate also inhibits ERK1/2 pathway [Cuevas et al., 2006a]. Therefore, the reduction in P-Stat3 level may be not causative of the down-regulation of Bcl2l1 and Ccnd1.

Stat3 decoy inhibited the expression of Bcl2l1 and cell proliferation in head and neck squamous carcinoma cells [Lui et al., 2007]. In C6 glioma cells the same decoy did not significantly changed expression of Bcl2l1 or cell viability. In MCF7 cells, phosphorylated Stat3 down-regulated the expression of Myc and Ccnd1 [Zhang et al., 2003]. These and other results support the notion that effects of Stat3 may be cell-type specific [Zhang et al., 2003], with the status of Pten, Egfr, and NFkb signaling as important co-factors [de la Iglesia et al., 2008a,b].

The inhibitory effect of Stat3 decoy on proliferation of head and neck squamous carcinoma [Lui et al., 2007] was dependent on inhibition of Stat3 (and not Stat1), as it was lost following siRNA mediated silencing of Stat3, but not Stat1. An additional, unexpected finding from that experiment, not discussed by the authors, was that effective silencing of either Stat with siRNA had no effect on proliferation and survival of those cells. Similarly, in our study Stat1 siRNA had no effect on pGAS-TA-luc or Myc, while Stat1 decoy down-regulated their expression. This demonstrates the importance of using several differing tools (Fig. 6), including DNA decoys, to study the intricate mechanisms of Stat signaling. The ability of Stat1 decoy to down-regulate the GAS-driven expression was reproduced in breast (MCF7) and lung (A549) cancer cell lines (data not shown).

In proliferating glioma C6 cells the level of phosphorylated Stat1 is very low. Therefore, we speculate that it is the activity of the

unphosphorylated Stat1 (uStat1), which is inhibited by Stat1 decoy or Stat1 siRNA. The observed role of Stat1 in maintaining the basal expression of genes Myc and Bcl2l1, is reminiscent of the role of uStat1 in maintaining the basal expression of LMP2 gene [Chatterjee-Kishore et al., 2000]. In our study, Bcl2l1 was down-regulated by Stat1 decoy, suggesting a role of Stat1, possibly uStat1, in maintaining its basal expression. This is in contrast to the previous result [Cheon and Stark, 2009] obtained in a different cell type showing that Bcl2l1 was down-regulated by overexpression of phosphorylation-deficient Stat1, again suggesting cell type specific effects.

The established paradigm is that Stat3 exerts a pro-survival—potentially oncogenic effects, while Stat1 is mainly associated with the immune response. Our results, demonstrating that Stat1 not Stat3 maintains the expression of Bcl2l1 and possibly Myc, add to a number of the reports challenging this paradigm, in the context of glioma [de la Iglesia et al., 2008a,b] or glioma-initiating cells [Yuki et al., 2009].

ACKNOWLEDGMENTS

This work was supported by the grant N N401 234934 from the Polish Ministry of Science and Higher Education. Plasmids pLEGFP-STAT3-WT and pLEGFP-STAT3 Y705F were kindly provided by Dr. Yuxin Wang.

REFERENCES

Adamkova L, Souckova K, Kovarik J. 2007. Transcription protein STAT1: Biology and relation to cancer. Folia Biol (Praha) 53:1–6.

Bene A, Kurten RC, Chambers TC. 2004. Subcellular localization as a limiting factor for utilization of decoy oligonucleotides. Nucleic Acids Res 32:e142.

Bielinska A, Shivdasani RA, Zhang LQ, Nabel GJ. 1990. Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. Science 250:997–1000.

Bowman T, Garcia R, Turkson J, Jove R. 2000. STATs in oncogenesis. Oncogene 19:2474–2488.

Bowman T, Broome MA, Sinibaldi D, Wharton W, Pledger WJ, Sedivy JM, Irby R, Yeatman T, Courtneidge SA, Jove R. 2001. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. Proc Natl Acad Sci USA 98:7319–7324.

Brantley EC, Benveniste EN. 2008. Signal transducer and activator of transcription-3: A molecular hub for signaling pathways in gliomas. Mol Cancer Res 6:675–684.

Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr. 1999. Stat3 as an oncogene. Cell 98:295–303.

Brown S, Zeidler MP. 2008. Unphosphorylated STATs go nuclear. Curr Opin Genet Dev 18:455–460.

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R. 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10:105–115.

Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR. 2000. How Stat1 mediates constitutive gene expression: A complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. EMBO J 19: 4111–4122.

Cheon H, Stark GR. 2009. Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes. Proc Natl Acad Sci USA 106:9373–9378.

Ciechomska I, Pyrzynska B, Kazmierczak P, Kaminska B. 2003. Inhibition of Akt kinase signalling and activation of Forkhead are indispensable for upregulation of FasL expression in apoptosis of glioma cells. Oncogene 22: 7617–7627.

Ciechomska I, Legat M, Golab J, Wesolowska A, Kurzaj Z, Mackiewicz A, Kaminska B. 2005. Cyclosporine A and its non-immunosuppressive derivative NIM811 induce apoptosis of malignant melanoma cells in in vitro and in vivo studies. Int J Cancer 117:59–67.

Cuevas P, Diaz-Gonzalez D, Garcia-Martin-Cordova C, Sanchez I, Lozano RM, Gimenez-Gallego G, Dujovny M. 2006a. Dobesilate diminishes activation of the mitogen-activated protein kinase ERK1/2 in glioma cells. J Cell Mol Med 10:225–230.

Cuevas P, Diaz-Gonzalez D, Sanchez I, Lozano RM, Gimenez-Gallego G, Dujovny M. 2006b. Dobesilate inhibits the activation of signal transducer and activator of transcription 3, and the expression of cyclin D1 and bcl-XL in glioma cells. Neurol Res 28:127–130.

Cutroneo KR, Ehrlich H. 2006. Silencing or knocking out eukaryotic gene expression by oligodeoxynucleotide decoys. Crit Rev Eukaryot Gene Expr 16:23–30.

de la Iglesia N, Konopka G, Lim KL, Nutt CL, Bromberg JF, Frank DA, Mischel PS, Louis DN, Bonni A. 2008a. Deregulation of a STAT3-interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness. J Neurosci 28:5870–5878.

de la Iglesia N, Konopka G, Puram SV, Chan JA, Bachoo RM, You MJ, Levy DE, Depinho RA, Bonni A. 2008b. Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. Genes Dev 22:449–462.

Ecker A, Simma O, Hoelbl A, Kenner L, Beug H, Moriggl R, Sexl V. 2009. The dark and the bright side of Stat3: Proto-oncogene and tumor-suppressor. Front Biosci 14:2944–2958.

Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P. 2001. DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem 276:6675–6688.

Ellert-Miklaszewska A, Kaminska B, Konarska L. 2005. Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. Cell Signal 17:25–37.

Fujio Y, Kunisada K, Hirota H, Yamauchi-Takihara K, Kishimoto T. 1997. Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. J Clin Invest 99:2898–2905.

Gu J, Li G, Sun T, Su Y, Zhang X, Shen J, Tian Z, Zhang J. 2008. Blockage of the STAT3 signaling pathway with a decoy oligonucleotide suppresses growth of human malignant glioma cells. J Neurooncol 89:9–17.

Holschermann H, Stadlbauer TH, Wagner AH, Fingerhuth H, Muth H, Rong S, Guler F, Tillmanns H, Hecker M. 2006. STAT-1 and AP-1 decoy oligonucleotide therapy delays acute rejection and prolongs cardiac allograft survival. Cardiovasc Res 71:527–536.

Horvath CM, Wen Z, Darnell JE, Jr. 1995. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. Genes Dev 9:984–994.

Huckel M, Schurigt U, Wagner AH, Stockigt R, Petrow PK, Thoss K, Gajda M, Henzgen S, Hecker M, Brauer R. 2006. Attenuation of murine antigen-induced arthritis by treatment with a decoy oligodeoxynucleotide inhibiting signal transducer and activator of transcription-1 (STAT-1). Arthritis Res Ther 8:R17.

Khan KD, Shuai K, Lindwall G, Maher SE, Darnell JE, Jr., Bothwell AL. 1993. Induction of the Ly-6A/E gene by interferon alpha/beta and gamma requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. Proc Natl Acad Sci USA 90:6806–6810.

Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, Hibi M, Hirano T. 1999. STAT3 is required for the gp130-mediated full activation of the c-myc gene. J Exp Med 189:63–73.

Kovacic B, Stoiber D, Moriggl R, Weisz E, Ott RG, Kreibich R, Levy DE, Beug H, Freissmuth M, Sexl V. 2006. STAT1 acts as a tumor promoter for leukemia development. Cancer Cell 10:77–87.

Krzesz R, Wagner AH, Cattaruzza M, Hecker M. 1999. Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor kappaB and signal transducer and activation of transcription-1. FEBS Lett 453:191–196.

Leong PL, Andrews GA, Johnson DE, Dyer KF, Xi S, Mai JC, Robbins PD, Gadiparthi S, Burke NA, Watkins SF, Grandis JR. 2003. Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci USA 100:4138–4143.

Lodige I, Marg A, Wiesner B, Malecova B, Oelgeschlager T, Vinkemeier U. 2005. Nuclear export determines the cytokine sensitivity of STAT transcription factors. J Biol Chem 280:43087–43099.

Lui VW, Boehm AL, Koppikar P, Leeman RJ, Johnson D, Ogagan M, Childs E, Freilino M, Grandis JR. 2007. Antiproliferative mechanisms of a transcription factor decoy targeting signal transducer and activator of transcription (STAT)3: The role of STAT1. Mol Pharmacol 71:1435–1443.

Naik SM, Shibagaki N, Li LJ, Quinlan KL, Paxton LL, Caughman SW. 1997. Interferon gamma-dependent induction of human intercellular adhesion molecule-1 gene expression involves activation of a distinct STAT protein complex. J Biol Chem 272:1283–1290.

Ramana CV, Chatterjee-Kishore M, Nguyen H, Stark GR. 2000. Complex roles of Stat1 in regulating gene expression. Oncogene 19:2619–2627.

Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R. 2000. Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: Role of activated STAT3 signaling. Oncogene 19:5419–5427.

Sun Z, Yao Z, Liu S, Tang H, Yan X. 2006. An oligonucleotide decoy for Stat3 activates the immune response of macrophages to breast cancer. Immunobiology 211:199–209.

Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. 1998. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. J Immunol 161:3719–3728.

Wagner BJ, Hayes TE, Hoban CJ, Cochran BH. 1990. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J 9:4477–4484.

Wagner AH, Gebauer M, Pollok-Kopp B, Hecker M. 2002. Cytokine-inducible CD40 expression in human endothelial cells is mediated by interferon regulatory factor-1. Blood 99:520–525.

Xi S, Gooding WE, Grandis JR. 2005. In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: Implications for cancer therapy. Oncogene 24:970–979.

Yang J, Stark GR. 2008. Roles of unphosphorylated STATs in signaling. Cell Res 18:443–451.

Yang J, Chatterjee-Kishore M, Staugaitis SM, Nguyen H, Schlessinger K, Levy DE, Stark GR. 2005. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. Cancer Res 65:939–947.

Yuki K, Natsume A, Yokoyama H, Kondo Y, Ohno M, Kato T, Chansakul P, Ito M, Kim SU, Wakabayashi T. 2009. Induction of oligodendrogenesis in glioblastoma-initiating cells by IFN-mediated activation of STAT3 signaling. Cancer Lett 284:71–79.

Zhang F, Li C, Halfter H, Liu J. 2003. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene 22:894–905.

Zushi S, Shinomura Y, Kiyohara T, Miyazaki Y, Kondo S, Sugimachi M, Higashimoto Y, Kanayama S, Matsuzawa Y. 1998. STAT3 mediates the survival signal in oncogenic ras-transfected intestinal epithelial cells. Int J Cancer 78:326–330.