Identification of genes that modulate sensitivity of U373MG glioblastoma cells to *cis*-platinum

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Scatter factor (hepatocyte growth factor) and its receptor c-Met are increasingly expressed during progression from low-grade to high-grade gliomas. Scatter factor/c-Met signaling induces glioma cell motility, invasion, angiogenesis and resistance to DNA-damaging agents. The latter is relevant to the understanding of the resistance of human gliomas to chemotherapy and radiotherapy. The goal of this study was to identify a set of genes that may contribute to scatter factor-mediated protection of U373MG cells against *cis*-platinum, a DNA cross-linking agent. We used DNA microarray assays, confirmatory semiquantitative reverse transcription-polymerase chain reaction analysis and functional assays to identify genes involved in the scatter factor-induced resistance of U373MG to *cis*-platinum. We identified a group of genes that are overexpressed in cells treated with scatter factor plus *cis*-platinum relative to cells treated with *cis*-platinum alone and confirmed some of these gene expression alterations by reverse transcription-polymerase chain reaction. Inhibiting the expression of three of these genes – polycystic kidney disease 1, amplified in breast cancer 1 and DEAD/H box helicase 21 – using small interfering RNAs reduced survival of *cis*-platinum-treated cells and partially reversed the scatter factor protection against *cis*-platinum. Dominant-negative Akt and IκB super-repressor expression vectors inhibited the scatter factor protection, and abrogated the ability of scatter factor to alter the expression of DEAD/H box helicase 21 and polycystin (PKD1) within the context of *cis*-platinum exposure. The Akt and nuclear factor-κB inhibitors had no effect on amplified in breast cancer 1 expression. These studies implicate DEAD/H box helicase 21, polycystin (PKD1) and amplified in breast cancer 1 as novel transcription-dependent regulators of scatter factor-mediated glioma cell protection against cytotoxic death, and identify other potential regulators for future study. *Anti-Cancer Drugs* 17:733–751 © 2006 Lippincott Williams & Wilkins.

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Introduction

Primary malignant brain tumors, including high-grade astrocytomas and glioblastomas, are among the most radioresistant and chemoresistant tumors known. Thus, radiation therapy usually produces only transient responses and chemoresistance occurs *de novo* or is acquired rapidly after the initial attempts at treatment [1,2]. Even with the most aggressive treatment, survival rates for glioblastomas are still less than 10% at 5 years. While the blood–brain barrier is a limiting factor in the delivery of some chemotherapy drugs to brain tumors, disruption of this barrier is common in high-grade gliomas and it is clear that additional intrinsic factors contribute to their chemoresistance. Previously, we showed that the cytokine scatter factor (SF) (also known as hepatocyte growth factor) protects epithelial, carcinoma and glioma cell types against cytotoxicity and apoptosis due to DNA-damaging agents [3,4].

In U373MG human glioblastoma cells, pretreatment with SF reduces the degree of cytotoxicity caused by ionizing (γ)-radiation, *cis*-platinum (CDDP), camptothecin, adriamycin and paclitaxel. The cytotoxic effects of these agents occur through a variety of mechanisms including breakage of the sugar-phosphate backbone of DNA (γ-radiation), cross-linking of DNA strands (CDDP), DNA strand breaks related to inhibition of topoisomerase I (camptothecin) or topoisomerase IIα (adriamycin) and poisoning of the mitotic spindle (paclitaxel). The ability of SF to protect U373MG cells is due, in part, to inhibition of apoptosis mediated through the activation of a phosphatidylinositol-3′-kinase/c-Akt (protein kinase B) cell survival pathway [4]. The ability of SF to protect glioma cells against apoptosis was demonstrable *in vivo*, as decreased radiosensitivity of intracranial 9L gliosarcoma tumors expressing a SF transgene and radiosensitization of glioma xenografts

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due to administration of anti-SF or anti-c-Met receptor-directed ribozymes [4,5].

The importance of understanding the mechanism(s) underlying SF-mediated protection of glioma cells against cytotoxic agents such as radiation and chemotherapy drugs is underscored by earlier findings indicating that SF and its cognate receptor c-Met are highly expressed in human malignant glioma tumors on the basis of immunohistochemistry [6]. In addition, immunoreactive SF was detectable and quantifiable in human glioma tumor extracts, and the levels of SF were significantly higher in high-grade gliomas than in low-grade gliomas [6,7]. Thus, the accumulation of SF within malignant gliomas may contribute to their chemoradioresistance. Immunoreactive SF and c-Met can be detected in most human high-grade glioma tumor cells *in vivo*, but only a subset of cultured human glioma tumor cell lines express SF *in vitro*. On the other hand, 10/10 of such cell lines expressed c-Met and exhibited biologic responsiveness to SF [8]. These findings suggest that glioma cells may lose their ability to produce SF when placed in culture, but retain their ability to respond to SF.

U373MG cells do not produce SF, but do express c-Met, and are biologically responsive to SF in assays of cell proliferation and protection against DNA-damaging agents. While several signaling pathways related to chemoresistance have been identified in cultured glioma cells, the downstream target genes that mediate this chemoresistance are mostly unknown. In this study, we have utilized DNA microarray profiling and a series of follow-up studies to identify target genes in U373MG cells that regulate their sensitivity to CDDP, a chemotherapy agent commonly utilized to treat gliomas.

Materials and methods

Sources of reagents and antibodies

Recombinant human two-chain SF (hepatocyte growth factor) was generously provided by Dr Ralph Schwall (Department of Endocrine Research, Genentech, South San Francisco, California, USA). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) dye was purchased from Sigma (St Louis, Missouri, USA). *Cis*-diaminodichloroplatinum (CDDP, cisplatin), a DNA cross-linking agent, was purchased from Calbiochem (La Jolla, California, USA). MTT dye was obtained from the Sigma. Antibodies directed against phospho-Akt (serine-473) (catalog #9271) and total Akt (catalog #9271) were obtained from Cell Signaling Technology (Beverly, Massachusetts, USA). An antibody against amplified in breast cancer 1 (AIB1) (catalog #61104) was obtained from BD Transduction Laboratories (San Jose, California, USA). Anti-human α-actin antibody I-19 was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell lines and culture

U373MG human glioblastoma cells were obtained from the American Type Culture Collection (Manassas, Vairginia, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (FCS) (5% v/v), nonessential amino acids (100 mmol/l), L-glutamine (5 mmol/l), streptomycin (100 μg/ml) and penicillin (100 U/ml), all from BioWhittaker (Walkersville, Maryland, USA). The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2, with weekly subculture by harvesting with tryspin.

Cis-platinum treatment

Subconfluent proliferating cells in 100-mm plastic dishes or 96-well plates were preincubated in the absence or presence of SF (100 ng/ml × 48 h) in DMEM containing reduced (2.5%) serum and then sham-treated (control) or treated with CDDP (100 μmol/l × 24 h, at 37°C) in DMEM containing 2.5% FCS. The cultures were then harvested for isolation of total cell RNA and cDNA microarray experiments, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assays or MTT assays of cell viability.

Expression vectors and cell transfections

A dominant-negative kinase inactive mutant Akt (K179A) expression vector (DN-Akt) was provided by Dr Michael Quon (National Heart, Lung and Blood Institute, Bethesda, Maryland, USA) [9]. The pCMV4-IκB-α mutant (S32,36A) expression vector has been described earlier [10]. This vector encodes a nonphosphorylatable and nondegradable mutant IκB-α in which serines 32 and 36 are mutated to alanines. IκB-α S32,36A acts as a 'super-active' IκB-α and a 'super-repressor' of nuclear factor-κB (NF-κB) transcriptional activity. An expression vector for AIB1 was provided by Dr Anna Riegel (Lombardi Comprehensive Cancer Center, Georgetown University, Washington, District of Columbia, USA) [11].

Transient transfections

Subconfluent proliferating cells were transfected overnight using Lipofectamine 2000 (Life Technologies, Rockville, Maryland, USA) (10 μg of plasmid DNA per 100-mm dish) and then washed to remove the excess vector and Lipofectamine. To monitor transfection efficiency, cultures were cotransfected overnight with 10 μg of a green fluorescent protein (GFP) expression vector (pcDNA-DEST; Invitrogen, Carlsbad, California, USA) or control vector (pcDNA3; Invitrogen) under parallel conditions. The cultures were postincubated for 24 h and examined by fluorescence microscopy to identify transfected cells.

Isolation of RNA

After cell treatments ± SF and ± CDDP, the total cellular RNA was extracted using TRIzol Reagent (Life

Table 1 **Primer sequences for reverse transcription-polymerase chain reaction analyses**

Gene symbol	Primer sequences (5′→3′)	GenBank accession no.	Position in cDNA sequence	Expected size of product (bp)	PCR cycle parameters	No. of cycles
Fig. 1						
ADK	sense: GCCATGACGTCAGTCAGAGA antisense: CCTGTTGTCACCAGTGATGC	XM_005896	185–583	399	94°C (1 min); 55°C (1 min); 72°C (1 min)	35
AIB1	sense: AGGAACTAATTCTCTGGGTT antisense: TGCTCTTTCGTCACTCTGGCC	NM_006534	2701–3361	661	94°C (30 s); 54°C (30 s); 72°C (30 s)	35
APOD	sense: TGCAGGAGAATTTTGACGTG antisense: GTGCAGCCTCCCTGTAGAAC	XM_003067	151–647	497	94°C (1 min); 55°C (1 min); 72°C (1 min)	32
DDX21	sense: GCCATCCCTTTGATTGAGAA antisense: CTCCATAGGGAGTTCCACCA	NM_004728	782–959	488	94°C (30 s); 55°C (30 s); 72°C (30 s)	28
GAK	sense: CCCTCTTCTCTCCTGGAGGT antisense: CTTGAGTGGGTCCGTGTCTT	XM_004814	3170–3651	481	94°C (1 min); 55°C (1 min); 72°C (1 min)	35
PKD1	sense: CTCCTATCTTGTGACAGTCACCGCG antisense: GTCCAGCTGTAGGAGACGTTGGTGC	NM_000296	4528–5211	684	94°C (30 s); 55°C (30 s); 72°C (30 s)	26
SHC1	sense: TGTGGTTGCTTTATTTTGCAC antisense: TGGATGAATTCAGATGTTTACCA	R52965	6–218	213	94°C (1 min); 55°C (1 min); 72°C (1 min)	32
TSC–22	sense: CCAGTTTCAGGCCCAGCTGC antisense: CATCGCTTCACAACCCCGTG	AJ22270	566–1068	502	94°C (1 min); 57°C (1 min); 72°C (1 min)	35
VEGF	sense: ATGTCTATCAGCGCAGCTACTGCC antisense: CAAGCTGCCTCGCCTTGCAACGCG	XM_004512	150–548	399	94°C (1 min); 60°C (1 min); 72°C (1 min)	32
β_2-microglobulin	sense: CTCGCGCTACTCTCTCTTTC antisense: TGTCGGATGGATGAAACCCAG	XM_007650	41–176	136	94°C (1 min); 54°C (1 min); 72°C (1 min)	28
Figs 2, 5 and 6						
AIB1	sense: ACATGGGAGTCCTGGTCTTG antisense: AAGTCCCCACACCTTCACTG	NM_006534	1606–1811	205	95°C (15 s); 55°C (30 s); 68°C (1 min)	28
DDX21	sense: GCCATCCCTTTGATTGAGAA antisense: CTCCATAGGGAGTTCCACCA	NM_004728	782–959	178	95°C (30 s); 50°C (30 s); 68°C (30 min)	30
PKD1	sense: AGTGGCTGGAGAGGTTCAGA antisense: TCTGAGGAACCTGAGCCCTA	L33243	9014–9206	193	95°C (15 s); 55°C (50 s); 66°C (1 min)	32
β-actin	sense: TAGCGGGGTTCACCCACACTGTGCCC-CATCTA antisense: CTAGAAGCATTTGCGGTGGACC-GAAGGG	XM_004814	541–1201	661	95°C (30 s); 56°C (30 s); 72°C (30 min)	26

Technologies), according to the manufacturer's instructions. The RNA was then treated with DNase and precipitated using 95% ethanol. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA, and the RNA concentrations were determined from absorbance measurements at 260 and 280 nm.

Semiquantitative reverse transcription-polymerase chain reaction analysis

After cell treatments and RNA isolation, aliquots of total cellular RNA (1.0 µg) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Life Technologies) and the cDNA was diluted five times with water. One microliter of the diluted cDNA was used for each PCR reaction. PCR amplifications were performed using a Perkin-Elmer DNA thermal cycler. The PCR primer sets used in this study are shown in Table 1. The PCR reaction conditions were individually optimized for each gene product studied. For each gene product, the cycle number was adjusted so that the reactions fell within the linear range of product amplification. The PCR reaction conditions and cycle numbers are shown in Table 1. The β-actin and β_2-microglobulin genes were used as controls for loading. PCR products were analyzed by electrophoresis through 1.2% agarose gels containing 0.1 mg/ml of ethidium bromide and the gels were photographed under ultraviolet illumination. When appropriate, the amplified cDNA product bands were quantitated by densitometry.

DNA microarray analyses

Cell treatments

Subconfluent proliferating U373MG cells were preincubated ± SF (100 ng/ml × 48 h), treated ± CDDP (100 µmol/l × 24 h) as described above and then harvested for isolation of total cellular RNA (see above). The cells that had been preincubated with SF also had SF present during the postincubation period, irrespective of whether in the absence or presence of CDDP. Three independent sets of cell treatments, RNA isolations and microarray analyses were performed.

cDNA synthesis and microarray hybridization

One hundred microgram of total cellular RNA was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled or Cy5-labeled dUTP (Amersham Pharmacia Biotech, Pisacataway, New Jersey, USA), using 10 000 U/ml of Superscript II reverse transcriptase (Life Technologies). The Cy3-labeled and Cy5-labeled

cDNAs were treated with RNase One (Promega, Madison, Wisconsin, USA) for 10 min at 37°C, combined, purified by using a Centricon-50 filtration spin column (Millipore, Bedford, Massachusetts, USA), and concentrated to a final volume of 6.5 µl. The cDNA was then combined with 12.5 µl of hybridization solution and 1.0 µl of blocking solution to a final volume of 20 µl. The mixture was heated at 94°C for 2 min and centrifuged at 13 000 r.p.m. for 10 min, and the supernatant was transferred to a clean tube and incubated at 50°C for 1 h. Hybridizations were performed on cDNA microarray glass slides prepared at the Albert Einstein College of Medicine microarray facility. Each slide contained 9216 unique human cDNA clones. The hybridization solution was placed on a pretreated microarray slide, covered with Hybri-slip and then incubated in a hybridization chamber overnight at 50°C. The slide was then washed at room temperature, first with 0.2 × SSC, 0.1% sodium dodecyl sulfate for 20 min with gentle shaking, and then with 0.2 × SSC two times (20 min each time). The slide was dried by spinning at low speed in a centrifuge for 5 min.

Scanning, griding and analysis

The slides were scanned using a Microarray Scanner 4000A (Axon Instruments, Foster City, California, USA) at the Albert Einstein College of Medicine microarray facility. The scanner output images were localized by overlaying a grid on the fluorescent images, using the ScanAlyze software by Michael Eisen, Stanford University, and the fluorescent intensities were calculated. The final reported intensity was the difference between average probe intensity and average local background intensity. Both final reported intensities (green and red) were filtered, and the spots with intensity below 1.5 were eliminated. The ratios of the red intensity to the green intensity and green intensity to red intensity were determined for all targets. The cDNA microarray results comparing cells treated with (SF + CDDP) vs. CDDP alone and comparing cells treated with SF alone vs. control (no treatment) are based on three completely independent experiments involving separate cell treatments, separate RNA isolations and separate microarray assays.

Small interfering RNAs

Gene-specific small interfering RNAs (siRNAs) for AIB1, DEAD/H box helicase 21 (DDX21) and polycystic kidney disease 1 (PKD1) were obtained from Ambion (Austin, Texas, USA). The sequences were as follows AIB1 sense: GGACAAGGUCUUACCUGCAtt, antisense: UGCAG GUAAGACCUUGUCCtg; PKD1 sense: GGCUCCUAU CUUGUGACAGtt, antisense: CUGUCACAAGAUAG GAGCCtg; and DDX21 sense: GGAGCCCAUUGAAAA GAAAtt, antisense: UUUCUUUUCAAUGGGCUCCTT. U373MG cells were transfected with siRNAs (100 nmol/l × 48 h) using siPORT Amine reagent (Ambion), according to the manufacturer's instructions. A control-siRNA with no homology to human, mouse or rat DNA sequences was also provided by Ambion and was tested in each experiment.

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assays

MTT assays of cell viability were performed as before [12]. This assay is based on the ability of intact mitochondria to convert MTT, a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], into an insoluble formazan precipitate, which is quantitated by spectrophotometry following its solubilization in dimethyl sulfoxide. For MTT experiments involving plasmid transfection, U373MG cells in 100-mm dishes were transfected overnight with plasmids (see above), washed, allowed to recover for several hours, harvested using trypsin, seeded into 96-well plates (2500 cells per well) in DMEM supplemented with 5% FCS, and incubated overnight. The cells were treated ± SF (100 ng/ml) and then ± CDDP (100 µmol/l) as described above and analyzed for MTT dye conversion. For siRNA experiments, cells were seeded into 96-well plates (2500 cells per well), incubated overnight, treated with siRNA (100 nmol/l) ± SF (100 ng/ml), treated ± CDDP (100 µmol/l) and then analyzed for MTT dye conversion. MTT [5 mg/ml, dissolved in phosphate-buffered saline (PBS)] was added to each well (10 µl per 100 µl of medium) and the plates were incubated for 4 h at 37°C. The medium was then replaced with 100 µl of dimethyl sulfoxide and absorbance readings were taken using a Dynatech 96-well spectrophotometer (Dynatech Laboratories, Chantilly, Virginia, USA). The amount of MTT dye reduction was calculated on the basis of the absorbance readings at 570 nm. Cell viability was expressed as the amount of dye reduction relative to that of non-CDDP-treated control cells. For each assay condition in each experiment, 10 replicate wells were tested. Values shown in the figures in this study are the means ± standard errors of the means of three independent experiments.

Western blot analysis

After the indicated cell treatments, the cells were washed with ice-cold PBS, lysed in radioimmunoprecipitation assay buffer and incubated on ice for 20 min. The lysate was centrifuged at 10 000 *g* at 4°C for 15 min. The supernatant was boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer (Sigma) and electrophoresed on a 4–20% sodium dodecyl sulfate–polyacrylamide Tris–glycine gradient gel (100 µg cell protein per lane). The electrophoresed proteins were then transferred to a nitrocellulose membrane (Millipore) and the membrane was incubated for 1 h at 25°C with 5% milk in PBS containing 0.2% Tween 20 or 5% bovine serum albumin in Tris-buffered saline (0.2% Tween 20). The primary and secondary antibodies (see above) were diluted in 5% milk in PBS containing 0.2% Tween 20 or 5% bovine serum albumin in Tris-buffered saline at 1:200 and 1:1000, respectively. Incubations were performed for

1 h at 25°C. The blotted proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech), with colored markers (Bio-Rad Laboratories, Hercules, California, USA) as size standards.

Statistical analyses

When appropriate, statistical comparisons were made using the two-tailed Student's *t*-test.

Results

Identification of scatter factor-regulated genes in *cis*-platinum-treated U373MG cells

We used DNA microarray analysis to investigate SF-regulated gene expression in the setting of DNA damage induced by CDDP, a DNA cross-linking agent, in U373MG human glioblastoma cells. Briefly, the cells were preincubated ± SF (100 ng/ml) for 48 h, postincubated ± CDDP for 24 h and harvested for microarray experiments. Previous studies revealed that, under these conditions, SF protects U373MG cells against CDDP-induced cytotoxicity on the basis of MTT assays [4,12]. The primary comparison was that of cells treated with (SF + CDDP) vs. CDDP alone. A secondary comparison was that of cells treated with SF alone vs. control (CON). Three independent experiments were performed. On the basis of our prior experience, gene expression changes were considered 'significant' if the ratios were increased by 1.7-fold or more in at least two experiments or decreased by 0.7-fold or less in at least two experiments [13,14]. In our experience, for gene expression changes that meet these criteria, there is a greater than 80% chance that the observed change will be confirmed by independent mRNA analysis.

The results of the microarray experiments are shown in Tables *2* and 3. Tables *2* and 3 show genes that were upregulated and downregulated, respectively, in cells treated with (SF + CDDP) relative to those treated with CDDP alone. Some of these genes may encode proteins that regulate the survival response to CDDP or mediate the ability of SF to protect the cells against CDDP-induced DNA damage. The list of genes upregulated by SF in cells treated with CDDP includes those encoding proteins involved in transcriptional regulation, the response to stress, signal transduction, metabolism, cell structure and adhesion, apoptosis and survival, inflammation and immune responses, differentiation and development, transport and trafficking events, RNA and protein processing, and other processes. The number of genes downregulated by SF in CDDP-treated cells was smaller ($N = 93$) than those upregulated ($N = 280$), but the distribution according to functional categories was equally broad. Only six genes were upregulated by SF alone relative to untreated control cells, while $N = 108$ genes were downregulated in the same setting. It should be remembered that the latter results reflect cells that have been treated with SF (or untreated) for 96 h and differ from the more acute gene expression changes found in cells treated with SF for 24 h [15].

Confirmation of selected microarray results by semiquantitative reverse transcription-polymerase chain reaction analysis

A subset of SF-responsive genes was selected for confirmation and more detailed analyses were carried out on the basis of a collection of criteria that included known function and/or SF responsiveness in other cell lines under similar experimental conditions [13]. To confirm the accuracy of the microarray assays, U373MG cells were treated ± SF ± CDDP as described above and then harvested for rigorously controlled semiquantitative RT-PCR assays, as described before [13,14]. As examples, seven genes found in microarray assays to be upregulated in (SF + CDDP)-treated cells relative to CDDP-treated cells [PKD1, DDX21, TSC-*22* (transforming growth factor-β stimulated gene TSC*22*), VEGF (vascular endothelial growth factor), AIB1, GAK (cyclin G-associated kinase) and SHC1 (SHC transforming protein)] and two genes found to be downregulated in (SF + CDDP)-treated cells [ADK (adenosine kinase) and APOD (apolipoprotein D)] were confirmed by this approach (Fig. 1).

Effect of knockdown of scatter factor-regulated genes on cellular sensitivity to *cis*-platinum

Three of the above-mentioned genes found to be upregulated in (SF + CDDP)-treated cells (AIB1, DDX21 and PKD1) were further studied to determine their contribution to SF-mediated U373MG chemoresistance. All three of these genes were originally identified on the basis of other functions (see Discussion) and two of the three (AIB1 and PKD1) have since been implicated in promoting survival in other cell types. None of these genes is known to modulate glioma chemosensitivity. First, we created specific siRNAs for each of the three genes and confirmed the ability of each siRNA to specifically reduce the levels of the target mRNA, but not a control mRNA (β-actin) (see Fig. 2). On the basis of MTT assays, none of these siRNAs had any effect on the viability of control cells or cells treated with SF only (Fig. 3). As shown in Fig. 3(a–c), pretreatment with SF increased the survival of CDDP-treated cells from a level of 43–50 to 75–80%. This represents about a 30% increase in U373MG survival. Control siRNA had no effect on cell survival. Each gene-specific siRNA significantly reduced the survival of both CDDP-treated cells and (SF + CDDP)-treated cells ($P < 0.01$ to 0.001, two-tailed *t*-tests). Each individual gene-specific siRNA, however, reduced the survival levels of (SF + CDDP)-treated cells to an approximately 2-fold greater extent than for cells treated with CDDP alone (Fig. 3a–c). Specifically, the gene-specific siRNAs reduced survival levels in cells treated with CDDP by only 7–10%.

Table 2 Genes upregulated in U373MG cells treated [scatter factor + *cis*-platinum (CDDP)] relative to CDDP alone

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
Transcription/nuclear proteins			
v-Ets avian erythroblastosis virus E26 homolog 2	H96235	ETS2	3.3 ± 1.5 (2)
high mobility group protein (isoforms I and Y)	AA448261	HMGA1	3.1 ± 0.6 (3)
ETR101 (also called immediate early response 2)	AA496359	IER2	2.9 ± 0.2 (2)
RING finger protein 10 (KIAA0262)	H73586	RNF10	2.7 ± 1.1 (2)
SWI/SNF complex 60 kDa subunit (BAF60b)	AA478436	SMARCD2	2.6 ± 0.0 (2)
TBP-associated factor TAFII80	H12006	TAF6	2.6 ± 1.0 (2)
Drosophila enhancer of split m9/m10 homolog	AA485742	AES	2.5 ± 0.5 (2)
nuclear factor RIP140	AA458503	NRIP1	2.5 ± 0.6 (2)
TGF-β-stimulated protein TSC-22	AA664389	TGFB114	2.4 ± 0.7 (2)
general control of amino-acid synthesis 5-like 2	AA452872	GCN5L2	2.4 ± 0.7 (2)
core-binding factor, β subunit	AA187148	CBFB	2.3 ± 0.1 (2)
SWI/SNF complex 60-kDa subunit (BAF60a)	H91691	SMARCD1	2.3 ± 0.3 (3)
DbpB-like (nuclease sensitive element binding 1)	AA599175	NSEP1	2.2 ± 0.2 (2)
cellular nucleic acid binding protein	AA625995	ZNF9	2.2 ± 0.6 (3)
RNA polymerase II polypeptide A (220 kDa)	AA479052	POLR2A	2.1 ± 0.1 (2)
CAGH1a (trinucleotide repeat containing 1)	AA147043	ZNF384	2.1 ± 0.0 (2)
Transcription factor 3 (also called ITF-1)	AA026102	TCF3	2.1 ± 0.4 (2)
proline, glutamic acid, leucine-rich protein 1	T50139	PELP1	2.1 ± 0.5 (2)
transcription factor ZFM1 isoform B3	AA454673	SF1	2.0 ± 0.4 (2)
GC-rich sequence DNA-binding factor	AA085597	C21orf66	2.0 ± 0.3 (2)
RFG (nuclear receptor coactivator 4)	AA133212	NCOA4	2.0 ± 0.3 (2)
zinc finger protein 482 (ZID protein)	AA397823	ZNF482	2.0 ± 0.2 (2)
PTTG1-interacting protein (surface glycoprotein)	AA156461	PTTG1IP	2.0 ± 0.4 (2)
T3 receptor-associating cofactor-1 (SMRT)	AA400234	NCOR2	1.9 ± 0.2 (2)
DNA-binding protein CROC-1A	R64190	UBE2V1	1.9 ± 0.0 (3)
myeloid/lymphoid or mixed-lineage leukemia 4 (KIAA0304)	AA625915	MLL4	1.9 ± 0.3 (2)
FBJ osteosarcoma viral oncogene homolog B	T62179	FOSB	1.9 ± 0.2 (2)
nuclear factor I/X (CCAAT-binding)	AA406269	NFIX	1.9 ± 0.1 (2)
Siah binding protein 1 (SiahBP1)	AA630094	SIAHBP1	1.9 ± 0.0 (2)
PDGF-associated protein (PAP)	AA490300	PDAP1	1.9 ± 0.2 (2)
amplified in breast cancer 1 (SRC3)	H52110	AIB1	1.8 ± 0.0 (2)
Forkhead box F1 (Forkhead protein FREAC-1)	AA112660	FOXF1	1.8 ± 0.0 (2)
RNA polymerase II subunit hsRPB4	H15431	POLRD	1.8 ± 0.2 (2)
peroxisome proliferator-activated receptor beta	N33331	PPARD	1.7 ± 0.2 (2)
activating transcription factor 3	H21041	ATF3	1.7 ± 0.2 (2)
TFE3 gene, exons 1, 2, 3 (and joined CDS)	AA403035	TFE3	1.7 ± 0.0 (2)
Stress response: oxidative stress and xenobiotic detoxification			
N-acylaminoacyl-peptide hydrolase	AA456408	APEH	2.3 ± 0.1 (2)
malate dehydrogenase precursor, mitochondrial	AI000271	MDH2	2.3 ± 0.3 (3)
glutathione-*S*-transferase, microsomal	AA495936	MGST1	2.2 ± 0.5 (2)
glutathione-*S*-transferase π-1	R33755	GSTP1	2.1 ± 0.2 (2)
transcription factor 11 (basic leucine zipper)	AA496576	NFE2L1	2.0 ± 0.1 (2)
glutathione-*S*-transferase θ-1	H99813	GSTT1	1.9 ± 0.0 (2)
YSK1 (STE20 homolog, yeast)	AA664007	STK25	1.9 ± 0.3 (2)
PGP 9.5 (ubiquitin thiolesterase)	AA670438	UCHL1	1.9 ± 0.2 (2)
INS-1 winged-helix homolog (forkhead box M1)	AA129552	FOXM1	1.7 ± 0.1 (2)
glutathione-*S*-transferase M3 (brain)	R63106	GSTM3	1.7 ± 0.0 (2)
antioxidant enzyme AOE37-2 (periredoxin 4)	AA459663	PRDX4	1.7 ± 0.1 (2)
Replication/cell cycle/DNA repair and metabolism			
cyclin G-associated kinase GAK	AA428959	GAK	3.4 ± 0.6 (2)
cell cycle progression 2 protein (CPR2)	AA676387	TBRG4	2.9 ± 1.3 (2)
prohibitin	R60946	PHB	2.7 ± 1.1 (2)
mitogen-activated protein kinase kinase 5 (MEK5)	AA430035	MAP2K5	2.3 ± 0.6 (2)
quiescin (Q6)	AA464152	QSCN6	2.3 ± 0.7 (2)
RBQ-1 (retinoblastoma binding protein 6)	AA016290	RBBP6	2.2 ± 0.7 (2)
retinoblastoma binding protein 3 (E2F1)	AA424950	E2F1	2.2 ± 0.6 (2)
tyrosine and threonine-specific CDC2-inhibitory kinase	AA478066	PKMYT1	2.1 ± 0.4 (2)
Tob family gene (transducer of ERBB2, 2)	AA879435	TOB2	2.1 ± 0.3 (2)
KIAA0158 (neural precursor developmental gene)	AI025015	NEDD5	2.0 ± 0.4 (2)
DNA ligase IV, ATP-dependent	R54358	LIG4	1.8 ± 0.2 (2)
histone H1x	W81318	H1FX	1.8 ± 0.2 (2)
Shk1 kinase-binding protein 1 (*Saccharomyces cerevisiae*)	AA496357	SKB1	1.7 ± 0.1 (2)
prohibitin	R60946	PHB	1.7 ± 0.1 (2)
Signal transduction			
SHC (Src homology 2 domain containing) transforming protein 1	R52961	SHC1	6.4
lipid-activated protein kinase PRK1	H18068	PKN1	4.9 ± 0.1 (2)
protein tyrosine phosphatase PTPCAAX1	R61674	PTP4A1	3.5 ± 1.9 (2)
human protein tyrosine kinase A6	AA019459	PTK9	2.9 ± 0.9 (2)
PP2A, 65-kDa regulatory subunit, alpha isoform	AA427688	PPP2R1A	2.9 ± 0.8 (2)
insulin-stimulated protein kinase 1 (ISPK-1)	H55921	RPS6 KA3	2.9 ± 1.2 (2)
kinase A anchor protein	AA454947	AKAP1	2.8 ± 1.0 (2)
protein phosphatase 1, catalytic subunit, γ	AI015359	PPP1CC	2.6 ± 0.7 (2)
CDC-like kinase 3 (Clk3)	AA630459	CLK3	2.6 ± 0.7 (2)

Table 2 (continued)

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
protein tyrosine phosphatase, nonreceptor, 11	R45056	PTPN11	2.6 ± 1.0 (2)
integrin-linked kinase	AA148200	ILK	2.3 ± 0.0 (3)
microtubule-associated protein RP3	AA399285	MAPRE3	2.3 ± 0.5 (3)
protein phosphatase 2A (β-type)	AA490696	PPP2CB	2.2 ± 0.3 (2)
nucleolar protein (MSP58)	AA488979	MCRS1	2.1 ± 0.1 (2)
interleukin-1 receptor-associated kinase	AA683550	IRAK1	2.1 ± 0.5 (3)
transducin family protein (putative)	AA188019	FLJ10458	2.0 ± 0.1 (2)
calcium-dependent group × phospholipase A2	T94293	PLA2G4A	2.0 ± 0.4 (2)
G protein, α activating activity polypeptide, olfactory	H49592	GNAO1	2.0 ± 0.2 (2)
glia maturation protein β	H22652	GMFB	1.9 ± 0.1 (2)
enigma (PDZ and LIM domain 7)	AA127096	PDLIM7	1.9 ± 0.2 (2)
annexin-IV (placental anticoagulant protein II)	AA419108	ANXA4	1.8 ± 0.1 (2)
caveolin-2	T89391	CAV2	1.8 ± 0.2 (2)
Plecksrin homology domain protein, family A, 1	AA258396	PHLDA1	1.6 ± 0.1 (2)
KIAA0220 gene	T94293	KIAA0220	1.8 ± 0.2 (3)
Gps2 (G-protein pathway suppressor 2)	AA971634	GPS2	1.8 ± 0.1 (3)
14-3-3 ς (protein kinase C inhibitor protein-1)	AA976477	YWHAZ	1.7 ± 0.1 (3)
PK-A (cAMP-dependent) regulatory subunit I, α	AA630507	PRKAR1A	1.7 ± 0.1 (3)
14-3-3 protein β/α	AA962407	YWHAB	1.7 ± 0.0 (2)
RalGDS-like 2	AA401972	RGL2	1.7 ± 0.1 (2)
faciogenital dysplasia 1	AA902269	FGD1	1.7 ± 0.1 (2)
Biosynthesis and metabolism			
phosphatidylserine synthase I	H28984	PTDSS1	2.8 ± 0.5 (2)
putative glucosamine-6-phosphate isomerase	H48661	GPI	2.8 ± 0.7 (3)
mevalonate pyrophosphate decarboxylase (MPD)	N50834	MVD	2.6 ± 0.9 (2)
phosphatidylinositol transfer protein	R89808	PITPN	2.6 ± 0.5 (2)
phosphoglycerate kinase 1	AA599187	PGK1	2.4 ± 0.1 (2)
lanosterol synthase	AA434024	LSS	2.5 ± 0.5 (3)
apolipoprotein C-III	N53169	APOC3	2.3 ± 0.4 (2)
spermidine/spermine N1-acetyltransferase	AA011215	SAT	2.3 ± 0.4 (2)
mannosidase α-B (lysosomal)	AA427691	MAN2B1	2.3 ± 0.4 (2)
NAD(H)-specific isocitrate dehydrogenase γ	AA459380	IDH3G	2.2 ± 0.5 (2)
liver glutamate dehydrogenase	AA018372	GLUD2	2.1 ± 0.4 (3)
carnitine acetyltransferase	AA621218	CRAT	2.1 ± 0.5 (2)
aldolase C	T77281	ALDOC	2.1 ± 0.4 (2)
pyruvate kinase, liver	R08829	PKLR	2.0 ± 0.2 (2)
hexokinase 1	AA485271	HK1	2.0 ± 0.3 (2)
glucose phosphate isomerase	AA401111	GPI	2.0 ± 0.4 (2)
phosphatidylinositol synthase (PIS)	AA430520	CDIPT	1.9 ± 0.2 (3)
G9 gene encoding sialidase (neuraminidase)	AA401883	NEU1	1.8 ± 0.1 (2)
fetus brain mRNA for vacuolar ATPase subunit F	AA664077	ATP6V0B	1.9 ± 0.1 (2)
mitochondrial ATP synthase, β subunit	AA708298	ATP5B	1.8 ± 0.1 (2)
acyl-CoA dehydrogenase, very long chain	AA464163	ACADVL	1.8 ± 0.2 (2)
retinol dehydrogenase 1 (11-*cis* and 9-*trans*)	AA633882	RDH5	1.8 ± 0.1 (2)
glucuronidase, β	N34827	GUSB	1.8 ± 0.3 (2)
ATP-citrate lyase	AA136054	ACLY	1.8 ± 0.2 (2)
chromosome 17q12-21 mRNA, clone pOV-2	AA482325	COASY	1.8 ± 0.2 (2)
putative fatty acid desaturase MLD	W49667	DEGS	1.7 ± 0.2 (2)
O-linked GlcNAc transferase	AA425655	OGT	1.7 ± 0.2 (2)
hexosaminidase B (β polypeptide)	H71868	HEXB	1.7 ± 0.0 (3)
farnesyl-diphosphate farnesyltransferase	AA679352	FDFT1	1.7 ± 0.1 (2)
Growth factor/cytokine and receptor			
putative progesterone binding protein	AA047567	PGRMC1	3.5 ± 1.2 (2)
interleukin 8	AA102526	IL8	2.4 ± 0.1 (3)
stanniocalcin precursor	AA085318	STC1	2.2 ± 0.0 (2)
hepatoma-derived growth factor	AA453831	HDGF	2.0 ± 0.3 (3)
angiopoietin-2	AA125872	ANGPT2	2.0 ± 0.3 (2)
autocrine motility factor receptor	AA479243	AMFR	1.9 ± 0.2 (2)
vascular endothelial growth factor	R19956	VEGF	1.9 ± 0.1 (2)
triple functional domain (PTPRF interacting)	AA007299	TRIO	1.9 ± 0.3 (2)
phenylalkylamine binding protein	N67038	EBP	1.9 ± 0.2 (2)
IL13 receptor	R52796	IL13RA1	1.8 ± 0.2 (2)
interleukin 6 (B cell stimulatory factor 2)	N98591	IL6	1.8 ± 0.1 (3)
erythropoietin receptor	H15634	EPOR	1.7 ± 0.2 (2)
Cytoskeleton/cell adhesion/celland organelle structure			
gap junction protein, β2, 26 kDa (connexin 26)	AA490688	GJB2	3.4 ± 1.2 (2)
NuMA gene (Clone T33)	W90116	NUMA1	2.6 ± 0.5 (2)
clone 23584 (actin, γ1)	AA430576	ACTG1	2.6 ± 1.0 (2)
KIAA0110 gene	H67988	MAD2L1BP	2.5 ± 0.1 (2)
α-cardiac actin gene, 5' flank and	N90109	ACTC	2.5 ± 0.7 (2)
dynamin 1	AA496334	DNM1	2.4 ± 0.7 (2)
Arp2/3 protein complex subunit p41-Arc	AA188155	ARPC1B	2.3 ± 0.0 (2)
filamin	H15445	FLN	2.3 ± 0.2 (2)
protocadherin 43 (PC43)	R89615	PCDHGC3	2.3 ± 0.4 (2)

Table 2 (continued)

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
junction plakoglobin	R06417	JUP	2.3 ± 0.5 (2)
lamin A	AA489582	LMNA	2.2 ± 0.0 (2)
Arp2/3 protein complex subunit p41-Arc	AA188155	ARPC1B	2.2 ± 0.0 (2)
outer mitochondrial membrane 34-kDa translocase	AA457118	TOMM34	2.2 ± 0.4 (3)
symplekin	W74377	SYMPK	2.1 ± 0.4 (2)
radixin	AA477165	RDX	2.1 ± 0.5 (2)
export protein Rae1	AA504128	RAE1	2.1 ± 0.5 (2)
lysosome-associated membrane protein 1	H29077	LAMP1	2.0 ± 0.4 (2)
angio-associated migratory cell protein	AA452988	AAMP	2.0 ± 0.5 (2)
actin binding LIM protein 1 (KIAA0059 gene)	AA406601	ABLIM1	2.0 ± 0.4 (2)
fetus brain mRNA for vacuolar ATPase subunit F	AA664077	ATP6V0B	1.9 ± 0.1 (2)
torsin family 1, member A [torsin A, dystonia 1 (DYT1)]	AA394148	TOR1A	1.8 ± 0.2 (2)
SMT3A protein (small ubiquitin-like modifier 3)	AA872379	SUMO3	1.6 ± 0.0 (2)
nucleobindin precursor	AA452725	NUCB1	1.8 ± 0.2 (2)
ubiquinol-cytochrome *c* reductase, smallest subunit	AA629862	UQCR	1.7 ± 0.0 (2)
tubulin, α-2	AA426374	TUBA2	1.5 ± 0.0 (2)
Extracellular matrix			
laminin, β2 (laminin S)	AA156802	LAMB2	2.1 ± 0.0 (2)
fibronectin 1	R62612	FN1	1.8 ± 0.0 (2)
Apoptosis/cell death			
chromosome segregation gene homolog CAS	N69204	CSE1L	4.1 ± 2.2 (2)
insulin-like growth factor binding protein 5	T52830	IGFBP5	2.7 ± 0.8 (2)
polycystic kidney disease protein 1	AA448998	PKD1	2.6 ± 0.4 (2)
phosphoprotein enriched in astrocytes 15 (MAT-1 oncogene homolog)	AA293653	PEA15	2.0 ± 0.3 (3)
large proline-rich protein BAT3 (HLA-B associated)	AA598629	BAT3	2.0 ± 0.4 (2)
transcription factor p65 (RelA)	AA443546	RELA	1.9 ± 0.2 (2)
etoposide-induced 2.4 mRNA (Pig8, TP53I8)	AA702548	EI24	1.8 ± 0.0 (2)
peptidyl-prolyl *cis–trans* isomerase, mitochondrial	H05580	PPIF	1.8 ± 0.1 (3)
Inflammation/immune and antiviral response/histocompatibility			
MHC class I polypeptide-related sequence A	N71782	MICA	3.4 ± 1.8 (2)
HLA class I histocompatibility antigen, F α chain	AA988615	HLA-F	2.7 ± 0.6 (2)
TAP binding protein (tapasin, NGS-17)	T69304	TAPBP	2.3 ± 0.4 (3)
TNFAIP3 interacting protein 1 (KIAA0113)	T64626	TNIP1	1.9 ± 0.3 (2)
low-affinity IgG Fc receptor II C precursor	R68106	FCGR2A	1.7 ± 0.1 (3)
Differentiation/development/tissue-specific expression and function			
melanoma-associated antigen 4	AA857809	MAGEA4	2.8 ± 0.4 (2)
basigin (OK blood group)	AA436440	BSG	2.6 ± 0.6 (2)
calponin	AA399519	CNN1	2.4 ± 0.4 (2)
interferon-related developmental regulator 2 (IFN-related protein SM15)	AA454813	IFRD2	2.4 ± 0.6 (3)
KIAA0058 (deleted in azospermia associated 2)	R19889	DAZAP2	2.3 ± 0.4 (3)
myosin 1C (myosin-I β)	AA485871	MYO1C	2.2 ± 0.5 (2)
proliferating cell nucleolar antigen P120	N50854	WBSCR20A	2.1 ± 0.4 (3)
scaffold protein Pbp1 (also called MDA-9)	AA456109	LOC401584	1.9 ± 0.1 (3)
melanoma antigen, family A, 10 (MAGE-10 antigen)	R23773	MAGEA10	1.9 ± 0.3 (2)
Transmembrane/membrane/integrins			
integrin α 3 subunit (subunit of VLA-3 receptor)	AA424695	ITGA3	3.6 ± 1.2 (2)
amyloid β (A4) precursor-like protein 2	H89664	APLP2	2.7 ± 0.2 (2)
laminin receptor (2H5 epitope)	AA629897	LAMR1	2.5 ± 0.2 (3)
integrin, α 7B	AA055979	ITGA7	2.5 ± 0.4 (2)
tumor differentially expressed 1 (Diff33)	H23366	TDE1	2.3 ± 0.6 (2)
placental protein 6 (PL6)	H99532	PL6	2.2 ± 0.5 (2)
CD151 antigen	AA456183	CD151	1.9 ± 0.2 (2)
chromosome 5 open reading frame 18 (polyposis locus protein 1)	H99681	C5orf18	1.8 ± 0.1 (2)
integrin, β2 (antigen CD18 (p95), ITGB2	W68291	ITGB2	1.8 ± 0.1 (2)
Channels/pore structure/transport and trafficking			
creatine transporter (SLC6A10)	AA707453	SLC6A10	3.4 ± 0.8 (2)
annexin-VII (synexin)	H15504	ANXA7	2.6 ± 0.6 (2)
ATPase, H^+ transporting, lysosomal 42kD	H05768	ATP6V1C2	2.6 ± 0.6 (2)
GDP dissociation inhibitor Rab GDI2	R92806	GDI2	2.4 ± 0.4 (2)
solute carrier family 4, anion exchanger, member 1	T86708	SLC4A1	2.4 ± 0.5 (3)
ADP-ribosylation factor 3	AA670422	ARF3	2.4 ± 0.4 (3)
SEC24 related gene family, member C (*S. cerevisiae*) [KIAA0079]	T74846	SEC24C	2.4 ± 0.7 (2)
Golgi SNAP receptor complex member 2 [Golgi SNARE (GS27)]	R78386	GOSR2	2.3 ± 0.4 (2)
solute carrier family 35 (UDP-galactose transporter), member A2	H51549	SLC35A2	2.2 ± 0.5 (2)
Homo sapiens (clone S31i125) mRNA	W94609	LOC391572	2.2 ± 0.0 (2)
AP-3 complex delta subunit	AA630776	AP3D1	2.1 ± 0.4 (2)
ezrin-radixin-moesin binding phosphoprotein-50	AA425299	SLC9A3R1	2.1 ± 0.3 (2)
cullin 5 (also called VACM-1)	AA086475	CUL5	2.1 ± 0.6 (2)
lysosome-associated transmembrane 4α	AA398233	LAPTM4A	2.0 ± 0.3 (3)
N-ethylmaleimide-sensitive factor attachment protein, α (α-SNAP)	AA425754	NAPA	2.0 ± 0.2 (3)
ADP-ribosylation factor 5	AA629584	ARF5	2.0 ± 0.2 (3)
	AA486016	VAMP3	2.0 ± 0.2 (2)

Table 2 (continued)

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
vesicle-associated membrane protein 3 (cellubrevin, synaptobrevin-3)			
nucleoprin-like 1 (KIAA0410)	R43334	NUPL1	2.0 ± 0.4 (2)
B-cell receptor associated protein 31 (6C6-Ag)	AA625628	BCAP31	2.0 ± 0.2 (2)
ER lumen protein retaining receptor 2	AA626867	KDELR2	1.9 ± 0.3 (2)
phosphate carrier, mitochondrial	AA486305	SLC25A23	1.9 ± 0.2 (2)
ADP-ribosylation factor 4	T71316	ARF4	1.9 ± 0.4 (2)
rat translocon-associated protein delta homolog	AA486261	SSR4	1.8 ± 0.1 (2)
dynactin	AA488221	DCTN1	1.8 ± 0.1 (2)
steroidogenic acute regulatory protein	AA679454	STAR	1.8 ± 0.1 (2)
hUNC18a alternatively-spliced mRNA	AA702799	STXBP1	1.8 ± 0.2 (2)
proteolipid protein 2 (intestinal membrane A4)	AA464627	PLP2	1.8 ± 0.1 (2)
putative tumor suppressor protein (101F6)	AA454950	CYB561D2	1.7 ± 0.1 (2)
SEC13-like 1 (*S. cerevisiae*) [Human (chromosome 3p25) membrane protein]	AA496784	SEC13L1	1.7 ± 0.1 (2)
inward rectifier potassium channel 2 (hiGIRK2)	H20547	KCNJ6	1.7 ± 0.0 (2)
clathrin-like protein	R14443	CLTCL1	1.7 ± 0.1 (2)
RNA or protein processing/protein modification and proteolysis			
Gu protein (DEAD box polypeptide 21)	AA465386	DDX21	3.9 ± 1.8 (2)
human spliceosomal protein SAP49	AA699361	SF3B4	3.7 ± 0.3 (3)
fragile X mental retardation syndrome related 2	AA489729	FXR2	3.1 ± 0.8 (2)
histone stem–loop binding protein SLBP	AA629558	SLBP	2.9 ± 0.8 (2)
calpain, small polypeptide	AA676484	CAPNS1	2.8 ± 0.7 (3)
tyrosyl-tRNA synthetase	AA486761	YARS	2.8 ± 1.2 (2)
spliceosome associated protein SAP145	AA633757	SF3B2	2.8 ± 1.2 (2)
eukaryotic translation initiation factor 4 γ, 1	R37276	EIF4G1	2.8 ± 1.2 (2)
ubiquitin conjugating enzyme G2	AA776164	UBE2G2	2.8 ± 0.8 (2)
eukaryotic translation initiation factor eIF3	AA676471	EIF3S9	2.8 ± 0.2 (2)
eukaryotic translation elongation factor 2	R20379	EEF2	2.7 ± 0.7 (3)
proteasome (prosome, macropain) activator subunit 3 (PA28 γ; Ki)	AA486324	PSME3	2.7 ± 0.5 (3)
human (clone E5.1) RNA-binding protein	AA496837	RNPS1	2.6 ± 1.0 (2)
DEAD box polypeptide 5 (P68 protein)	H27564	DDX5	2.6 ± 0.5 (2)
chaperonin-containing TCP1, subunit 8	AA630016	CCT8	2.6 ± 0.9 (2)
FK506-binding protein 1 (12 kDa)	AA625981	FKBP1A	2.5 ± 0.4 (2)
ubiquitin-conjugating enzyme UbcH8	AA292074	UBE2E2	2.4 ± 0.6 (2)
N-acetylgalactosaminidase, alpha	R25825	NAGA	2.4 ± 0.4 (2)
proteasome subunit p58 (also called RPN3)	AA485052	PSMD3	2.4 ± 0.4 (2)
plasminogen activator, tissue type (t-PA)	AA453728	PLAT	2.4 ± 0.2 (2)
eukaryotic translation initiation factor 3, subunit 10 θ, 150/170 kDa	AA916914	EIF3S10	2.3 ± 0.8 (2)
gp25L2 protein (emp24 family protein)	AA489314	HSGP25L2G	2.2 ± 0.5 (3)
Drosophila fat facets related Y protein	AA182680	USP9Y	2.2 ± 0.6 (2)
U3 snoRNP associated 55-kDa protein	AA465355	RNU3IP2	2.1 ± 0.3 (3)
autoantigen p542 mRNA, 3' end of cds	AA504617	RALY	2.1 ± 0.0 (2)
eukaryotic translation initiation factor 5	AA669443	EIF5	2.1 ± 0.1 (2)
ribosomal protein L10 (QM gene)	T67270	RPL10	2.1 ± 0.4 (2)
probable 26S protease subunit TBP-1	AA282230	PSMC3	2.0 ± 0.3 (2)
dolichyl-diphosphooligosaccharide-protein glycosyltransferase	H96850	DDOST	2.0 ± 0.1 (2)
PTB-associated splicing factor	AA425853	SFPQ	1.9 ± 0.0 (2)
coagulation factor X (FX)	AA421687	F10	1.9 ± 0.2 (2)
ribosomal protein L27	AA598582	RPL27	1.9 ± 0.2 (2)
ribosomal protein S5	AA456616	RPS5	1.9 ± 0.1 (3)
proteasome subunit HsC7-I	N73252	PSMB2	1.7 ± 0.0 (3)
alternative splicing factor SF2, P32 subunit	AA485677	SFRS1	1.8 ± 0.1 (2)
ubiquitin-conjugating enzyme L-UBC	AA669526	UBE2L3	1.8 ± 0.1 (2)
ribosomal protein S5	AA456616	RPS5	1.9 ± 0.1 (3)
proteasome subunit HsC7-I	N73252	PSMB2	1.7 ± 0.0 (3)
Ski-W (superkiller viralicidic activity 2-like)	H51554	SKIVL2	1.7 ± 0.1 (2)
Miscellaneous or function unknown			
KIAA0143 gene	AA112057	KIAA0143	3.7 ± 2.2 (2)
human 54-kDa protein	AA056465	–	3.2 ± 1.5 (2)
KIAA0310 gene	AA454812	KIAA0310	2.9 ± 1.1 (2)
human clone 23933	H56918	–	2.9 ± 0.6 (3)
human unknown protein	AA663435	–	2.9 ± 0.4 (3)
KIAA0515 protein	AA481143	KIAA0515	2.9 ± 0.1 (2)
human clone 23933 mRNA sequence	R39465	–	2.4 ± 0.5 (2)
protein XE7	AA496863	DXYS155E	2.3 ± 0.0 (2)
Ro/SSA ribonucleoprotein homolog (RoRet)	AA195036	TRIM38	2.3 ± 0.0 (2)
chromosome 17q12–21 mRNA, clone pOV-3	AA427472	–	2.2 ± 0.2 (2)
human chromosome 19, cosmid R29368	AA157797	–	2.1 ± 0.1 (2)
putatively prenylated protein (CAAX box protein 1)	W72596	CXX1	2.1 ± 0.4 (2)
human clone 23665	AA434504	–	2.0 ± 0.2 (2)
ewing sarcoma breakpoint region 1	R32756	EWSR1	1.9 ± 0.2 (3)
SM22-α homolog (transgelin-2)	H08564	TAGLN2	1.9 ± 0.0 (3)
human BTK region clone ftp-3 mRNA	AA679345	–	1.9 ± 0.1 (2)

Table 2 (continued)

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
human clone 23679	R85562	–	1.8 ± 0.2 (2)
human clone 23851	AA489073	–	1.7 ± 0.1 (2)
chromosome 9, P1 clone 11659	AI024999	–	1.7 ± 0.0 (2)

SEM = standard error of the mean.

Table 3 Genes downregulated in U373MG cells treated with [scatter factor + *cis*-platinum (CDDP)] relative to CDDP alone

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
Transcription/nuclear proteins			
high mobility group nucleosomal binding domain 3 (TRIP7)	AA431611	HMGN3	0.54 ± 0.02 (2)
oigodendrocyte lineage transcription factor 2 (PKC-binding protein RACK17)	R37657	OLIG2	0.56 ± 0.13 (2)
inhibitor of DNA binding 3	AA482119	ID3	0.57 ± 0.06 (2)
inhibitor of DNA binding 2	H82706	ID2	0.59 ± 0.03 (2)
CCR4-NOT transcription complex, subunit 1 (NOT1, CDC39)	AA598611	CNOT1	0.61 ± 0.08 (2)
transcription initiation factor IIF, β subunit	AA479196	GTF2F2	0.62 ± 0.06 (2)
trinucleotide repeat containing 4 (CAGH4)	R53527	TNRC4	0.64 ± 0.09 (2)
transcriptional activation factor TAFII32	AA608514	TAF9	0.64 ± 0.10 (2)
histone acetyltransferase 1	AA625662	HAT1	0.65 ± 0.04 (2)
transcription factor 20 (KIAA0292 gene)	AA857407	TCF20	0.70 ± 0.04 (2)
Stress response: oxidative stress and xenobiotic detoxification			
homolog of mouse tumor rejection antigen gp96	AA598758	TRA1	0.42 ± 0.07 (2)
heat shock protein HSP90-α	H88540	HSPCA	0.47 ± 0.04 (2)
metallothionein-IE (functional)	AA872383	MT1E	0.55 ± 0.05 (2)
thymidine kinase 2, mitochondrial	AA029737	TK2	0.63 ± 0.09 (2)
ATP synthase, H^+ transporting, mitochondrial F1 complex, γ polypeptide 1	AA644234	ATP5C1	0.67 ± 0.05 (2)
Replication/cell cycle/DNA repair and metabolism			
adenosine kinase (ADK)	R12473	ADK	0.48 ± 0.23 (2)
proliferating cell nuclear antigen	AA450265	PCNA	0.58 ± 0.12 (2)
minichromosome maintenance deficient (*Saccharomyces cerevisiae*) 3	AA455786	MCM3	0.60 ± 0.04 (2)
putative M phase phosphoprotein 1	AA282936	MPHOSPH1	0.61 ± 0.02 (2)
SCP-1 (synaptonemal complex protein 1)	AA609655	SYCP1	0.63 ± 0.11 (2)
polymerase (DNA directed), δ 1, catalytic (125 kDa)	AA429661	POLD1	0.65 ± 0.03 (2)
silver-stainable protein SSP29	AA489201	ANP32B	0.65 ± 0.05 (2)
mRNA expressed in osteoblast	AA410567	C1orf29	0.70 ± 0.04 (2)
adenosine kinase	R12473	ADK	0.71 ± 0.00 (2)
BRCA1-associated RING domain 1 protein	AA678295	BARD1	0.71 ± 0.02 (2)
Signal Transduction			
β3-endonexin mRNA, long form and short form	AA043806	ITGB3BP	0.45 ± 0.01 (2)
guanine nucleotide-binding regulatory protein (Go-α)	R43320	GNAO1	0.56 ± 0.11 (2)
protein tyrosine phosphatase, receptor-type, ς, 1	AA476460	PTPRZ1	0.58 ± 0.09 (2)
Ca^{+2}/calmodulin-dependent serine kinase	AA045965	CASK	0.71 ± 0.03 (2)
glomulin, FKBP-associated protein (48-kDa FKBP-associated protein FAP48)	N95144	GLMN	0.71 ± 0.04 (2)
guanine nucleotide exchange factor (Son of sevenless 2)	R78735	SOS2	0.72 ± 0.02 (2)
Biosynthesis and metabolism			
fatty acid binding protein, heart	W72051	FABP3	0.44 ± 0.12 (2)
rTS β protein	N66132	HSRTSBETA	0.59 ± 0.13 (2)
acetylcholinesterase precursor	N63940	ACHE	0.64 ± 0.04 (2)
hormone-sensitive lipase	W96325	LIPE	0.65 ± 0.00 (2)
serine palmitoyltransferase (LCB2)	AA160852	SPTLC2	0.70 ± 0.04 (2)
apolipoprotein D	H15842	APOD	0.71 ± 0.02 (2)
Growth factor/cytokine and receptor			
chemokine (C-C motif) ligand 13 (monocyte chemoattractant protein-4 precursor)	T64134	CCL13	0.49 ± 0.06 (2)
human Jagged 1 (HJ1)	R70685	JAG1	0.60 ± 0.06 (2)
transforming growth factor, α	AA933077	TGFA	0.60 ± 0.13 (2)
endothelin 1 {alternative products}	H11003	EDN1	0.61 ± 0.08 (2)
patched (*Drosophila*) homolog	AA169807	PTCH	0.68 ± 0.03 (2)
Protein-tyrosine kinase RON (MST1 receptor, CDw136)	AA173453	MST1R	0.71 ± 0.01 (2)
Cytoskeleton/cell adhesion/cell and organelle structure			
catenin (cadherin-associated protein), δ 1 (δ-catenin)	H04985	CTNND1	0.37 ± 0.14 (2)
NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18 kDa	AA055101	NDUFS4	0.56 ± 0.03 (2)
dystrophin-related protein, utrophin (dystrobrevin-ς)	H09172	UTRN	0.57 ± 0.07 (2)
bicaudal-D	AA504478	BICD1	0.72 ± 0.01 (2)
Extracellular matrix			
high endothelial venule precursor (Hevin)	AA490694	SPARCL1	0.44 ± 0.27 (2)
EGF-containing fibulin-like extracellular matrix protein 1 (S1-5)	AA875933	EFEMP1	0.45 ± 0.20 (2)
biglycan	R77226	BGN	0.64 ± 0.11 (2)
collagen, type IV, α2	AA430540	COL4A2	0.71 ± 0.02 (2)

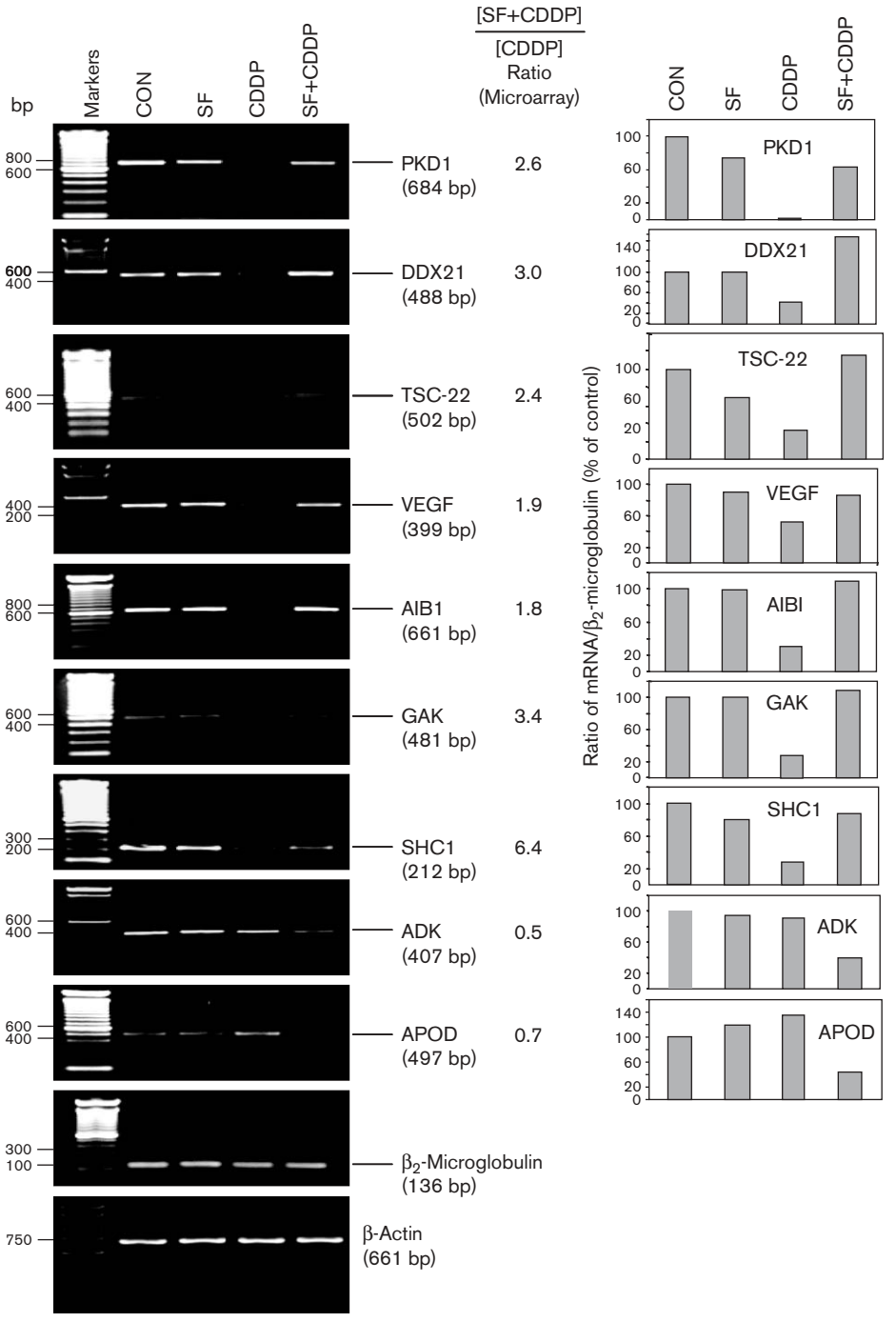
Table 3 (continued)

Gene name	GenBank accession no.	Symbol	Ratio mean ± SEM or range (*N*)
Apoptosis/cell death			
pleckstrin homology-like domain, family A, member 2	H68885	PHLDA2	0.62 ± 0.09 (2)
E1B 19K/Bcl-2-binding protein Nip3	AA063521	BNIP3	0.67 ± 0.04 (2)
Inflammation/immune and antiviral response/histocompatibility			
immunoglobulin μ	H73590	IGHM	0.57 ± 0.10 (2)
CD1c antigen (thymocyte antigen), c polypeptide	AA002086	CD1C	0.57 ± 0.00 (2)
indole 2,3-dioxygenase	AA478279	INDO	0.57 ± 0.12 (2)
monocyte/macrophage Ig-related receptor MIR-10)	H54023	LILRB2	0.60 ± 0.13 (2)
Differentiation/development/tissue-specific expression and function			
cysteine and glycine-rich protein 2	T59334	CSRP2	0.42 ± 0.03 (2)
enhancer of zeste homolog 2	AA430744	EZH2	0.58 ± 0.03 (2)
homeo box A9	AA497085	HOXA9	0.65 ± 0.08 (2)
LUCA14 (hyaluronoglucosaminidase 3)	R44982	HYAL3	0.55 ± 0.07 (2)
Transmembrane/membrane/integrins			
thrombomodulin	H59861	THBD	0.51 ± 0.23 (2)
human membrane-associated protein (HEM-1)	AA668726	HEM1	0.61 ± 0.13 (2)
kinectin 1	AA459106	KTN1	0.67 ± 0.07 (2)
peripheral myelin protein 22	R26960	PMP22	0.68 ± 0.04 (2)
Channels/pore structure/transport and trafficking			
endosome-associated protein 1	N66043	EEA1	0.61 ± 0.07 (2)
synaptotagmin XI (KIAA0080 gene)	AA446147	SYT11	0.68 ± 0.00 (2)
RNA or protein processing/protein modification ands proteolysis			
small nuclear riboprotein SM D1	H16454	SNRPD1	0.43 ± 0.03 (2)
heat shock 10-kDa protein 1 (chaperonin 10)	AA448396	HSPE1	0.50 ± 0.06 (2)
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3	AA398883	SERPINB3	0.51 ± 0.07 (2)
splicing factor, arginine/serine-rich 3 (pre-mRNA splicing factor SRP20)	AA598400	SFRS3	0.53 ± 0.12 (2)
multifunctional aminoacyl-tRNA synthetase	AA599158	EPRS	0.59 ± 0.14 (2)
PI3K-kinase-related kinase SMG-1 (KIAA0421 gene)	H42894	SMG1	0.61 ± 0.03 (2)
KIAA0020 gene (also called XTP5, HBxAg)	AA454662	KIAA0020	0.64 ± 0.11 (2)
exosome component 9 [75-kDa autoantigen (PM-Scl)]	AA458994	EXOSC9	0.66 ± 0.05 (2)
splicing factor, arginine/serine-rich 7, 35 kDa (9G8)	H54020	SFRS7	0.69 ± 0.03 (2)
Sm protein F (U6 small nuclear RNA associated)	AA668189	LSM6	0.69 ± 0.01 (2)
type 1 TNF receptor shedding aminopeptidase regulator (KIAA0525)	AA490894	ARTS1	0.69 ± 0.06 (2)
cathepsin O precursor	H70866	CTSO	0.71 ± 0.01 (2)
asparaginyl-tRNA synthetase (clone 23693 mRNA sequence)	AA668520	NARS	0.72 ± 0.00 (2)
ribosomal protein L10	AA434088	RPL10	0.72 ± 0.02 (2)
Miscellaneous or function unknown			
KIAA0409	AA453701	KIAA0409	0.61 ± 0.04 (2)
KIAA0346 gene (Jumongi domain containing 3)	AA159068	JMJD3	0.64 ± 0.08 (2)
KIAA0101 gene	W68220	KIAA0101	0.64 ± 0.01 (2)
human clone 23 798 and 23 825 mRNA sequence	R41996	–	0.65 ± 0.04 (2)
complement factor-like protein DOWN16	T74567	FHR-3	0.66 ± 0.03 (2)
clone 121711 defective mariner transposon Hsmar2	T53022	–	0.67 ± 0.02 (2)
KIAA0282 gene (tripartite motif-containing 9)	R44936	TRIM9	0.67 ± 0.09 (2)
human clones 23920 and 23921 mRNA sequence	AA161465	–	0.68 ± 0.07 (2)
KIAA0097 gene (ch-TOG)	AA598942	KIAA0097	0.68 ± 0.06 (2)
KIAA0082 gene	AA504534	KIAA0082	0.68 ± 0.03 (2)
human clone 23892 mRNA sequence	R61821	–	0.70 ± 0.03 (2)
human clone 23574 mRNA sequence	AA113331	–	0.74 ± 0.02 (2)

SEM = standard error of the mean.

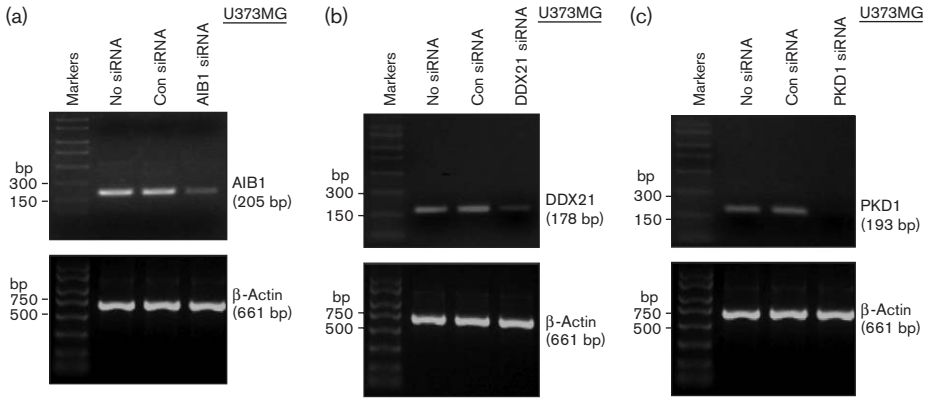
Protection by SF was calculated according to the following formula: protection(%) = $\{[S_{(SF+CDDP)} - S_{CDDP\ alone}]/[S_{SF\ alone} - S_{CDDP\ alone}] \times 100$. For AIB1, DDX21 and PDK1, the percent protection values in the presence of control siRNA (or no siRNA) were 56, 60 and 61%, respectively, while the corresponding values in the presence of gene-specific siRNAs were 38, 32 and 39%. Thus, each siRNA reduced SF-mediated protection by 18–28%. A combination of all three siRNAs used at 33 nmol/l each (i.e. 1/3 of the concentration used when each siRNA was tested individually) did not have a greater effect on the SF-mediated cell protection than did the individual siRNAs (Fig. 3d). A combination of the three siRNAs used at 100 nmol/l each yielded a greater reduction in the survival of CDDP-treated cells (20%) than did the siRNAs tested individually (7–10%) Fig. 3(e). The reduction in survival, however, was similar in cells treated with CDDP (20%) vs. cells treated with (SF + CDDP) (22%), suggesting that, under the conditions tested in Fig. 3(e), the combination of gene-specific siRNAs' significantly increased cell sensitivity to CDDP had little or no effect on the degree of SF-mediated cell protection. Here, we note that the siRNAs probably produce a greater reduction in protein levels for a longer period of time than does CDDP alone (see below) (as gene expression levels are already reduced at the

Fig. 1



Semiquantitive reverse transcription-polymerase chain reaction (RT-PCR) assays of genes identified in DNA microarray experiments: [(scatter factor (SF) + *cis*-platinum (CDDP)] vs. CDDP alone. Subconfluent proliferating U373MG cells in 100-mm plastic dishes were treated under the same conditions used for DNA microarray assays. The cells were preincubated in the absence or presence of SF (100 ng/m1) for 48 h, then sham-treated (control) or treated with CDDP (100 μmol/l) for 48 h, and then harvested for the isolation of total cell RNA and semiquantitative RT-PCR assays, as described in Materials and methods. β_2-Microglobulin was used as the control gene. The panels on the right show densitometric quantitation of amplified cDNA bands (expressed relative to β_2-microglobulin). For comparison, the ratios of gene expression in cells treated with (SF + CDDP) relative to (CDDP alone) on the basis of the microarray experiments are shown. See text for full names of the genes studied.

Fig. 2



Knockdown of gene expression by gene-specific vs. control small interfering RNAs (siRNAs). Subconfluent proliferating U373MG cells were treated with siRNAs against amplified in breast cancer 1 (AIB1) (a), DEAD/H box helicase 21 (DDX21) (b) or polycystic kidney disease 1 (PKD1) (c), or control siRNA (100 nmol/l × 48 h) and harvested for semiquantitative reverse transcription-polymerase chain reaction analysis, as described in Materials and methods. β-Actin was used as a control gene.

beginning of the CDDP treatment). Thus, the absence of three potential survival-promoting proteins might disproportionately lower survival in CDDP-treated and abrogate SF-mediated protection.

For one of the three genes (AIB1), we tested the effect of overexpression (using an AIB1 expression vector) on the cell survival response. Here, we observed a significant increase in cell survival levels from 43 to 57% (+ 14%) in the cells treated with CDDP in the absence of SF, relative to cells transfected with an empty vector or no vector (Fig. 3f). Figure 3(g and h) shows the levels of AIB1 protein in U373MG cells corresponding to the different treatment conditions tested in Fig. 3(a and f, respectively). These figures indicate several findings: (1) consistent with the mRNA data (Fig. 1), CDDP causes a reduction in AIB1 protein levels that is attenuated by SF; (2) the AIB1-siRNA (but not the control-siRNA) reduces AIB1 protein levels under all experimental conditions; and (3) the AIB1 expression vector increased the protein levels of AIB1, as compared with empty pcDNA3 vector. Together, these data are consistent with a role for AIB1, DDX21 and PKD1 in the survival response of U373MG glioma cells to CDDP, and they suggest that these gene products contribute to SF-mediated protection against CDDP.

Roles of c-Akt and nuclear factor-κB signaling in scatter factor-mediated protection against *cis*-platinum

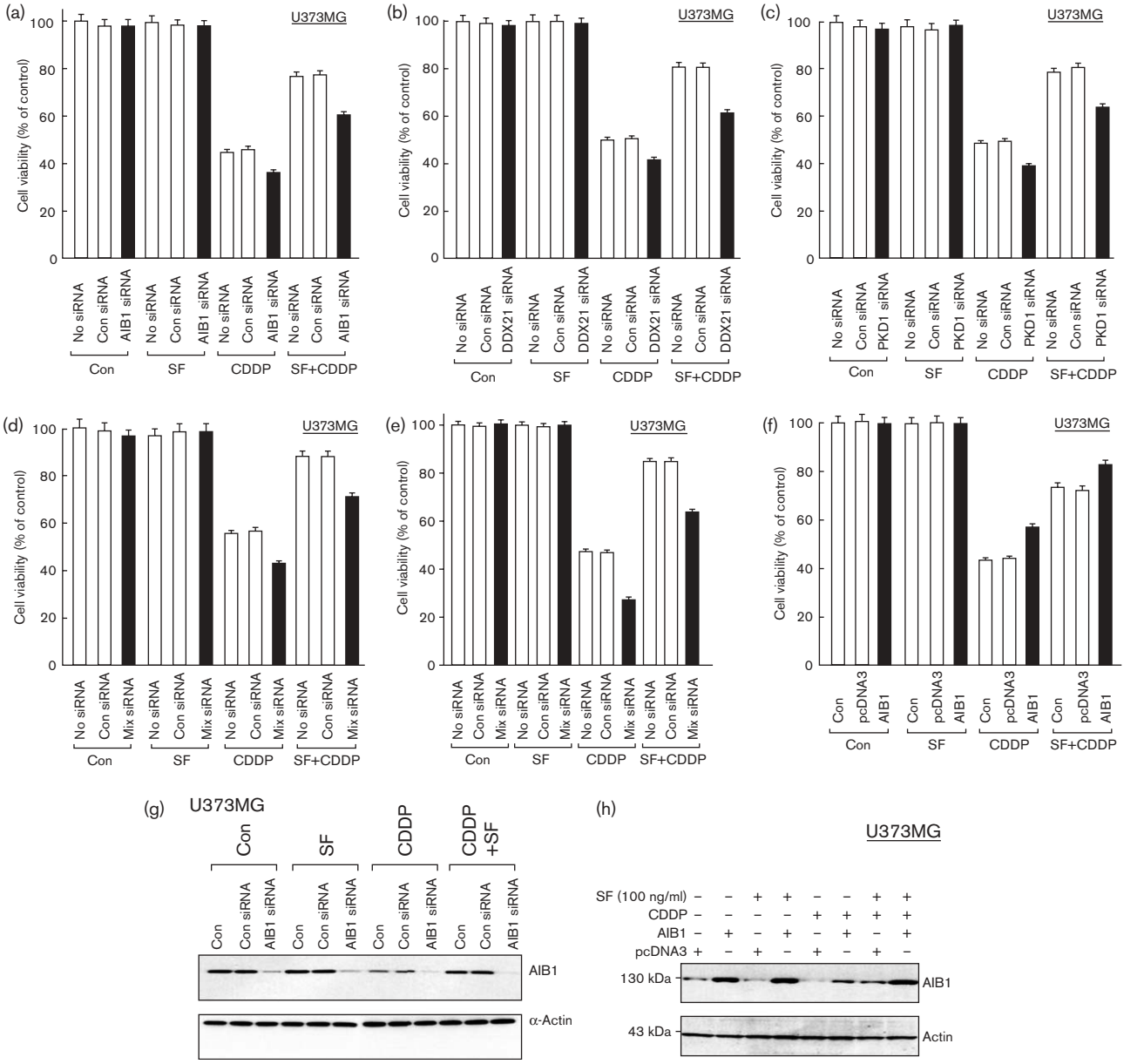
We next tested the roles of two signaling pathways previously implicated in SF-mediated cell protection (c-Akt and NF-κB) in the SF-mediated protection of U373MG cells against CDDP. In the experiments shown in Fig. 4(a), transient expression of a dominant-negative (kinase-dead) Akt expression vector (DN-Akt) significantly attenuated, but did not abrogate, the SF protection against CDDP. Specifically, SF-mediated protection was 67% in the absence of DN-Akt and was decreased to only 17% in the presence of DN-Akt ($P < 0.001$). We also used a mutant IκB-α expression vector [S(32,36)A] that functions as a 'super-repressor' to inhibit NF-κB activity [10]. The IκB mutant vector reduced SF-mediated protection to a degree similar to that by DN-Akt (Fig. 4b). DN-Akt and the mutant IκB super-repressor had little or no effect on the survival of CDDP-treated cells in the absence of SF nor did they alter the viability of non-CDDP-treated cells.

Cotransfection with a GFP expression vector demonstrated that the transfection efficiencies were around 60–70% under the conditions of these experiments (Fig. 4c). To confirm the efficacy of the DN-Akt expression vector in inhibiting c-Akt signaling, we determined the effect of DN-Akt on SF-induced Akt activation using an antibody against Akt phosphorylated at serine-473, normalized to either total Akt or actin. As shown in Fig. 4(d), the DN-Akt blocked SF-induced c-Akt phosphorylation at both 20 min and 24 h following stimulation with SF. On the basis of the transfection studies, the inability of the DN-Akt and IκB mutant vectors to completely abrogate SF-mediated protection against CDDP could still be due to incomplete expression of the mutant proteins. These studies, however, do not rule out a contribution of SF-inducible pathways not related to c-Akt and NF-κB in cell protection.

Roles of c-Akt and nuclear factor-κB in scatter factor inhibition of downregulation of gene expression by *cis*-platinum

We examined the roles of c-Akt and NF-κB signaling in the SF-induced effects on AIB1, DDX21 and PKD1 expression in U373MG cells exposed to CDDP. For these experiments, cells were transfected with DN-Akt or

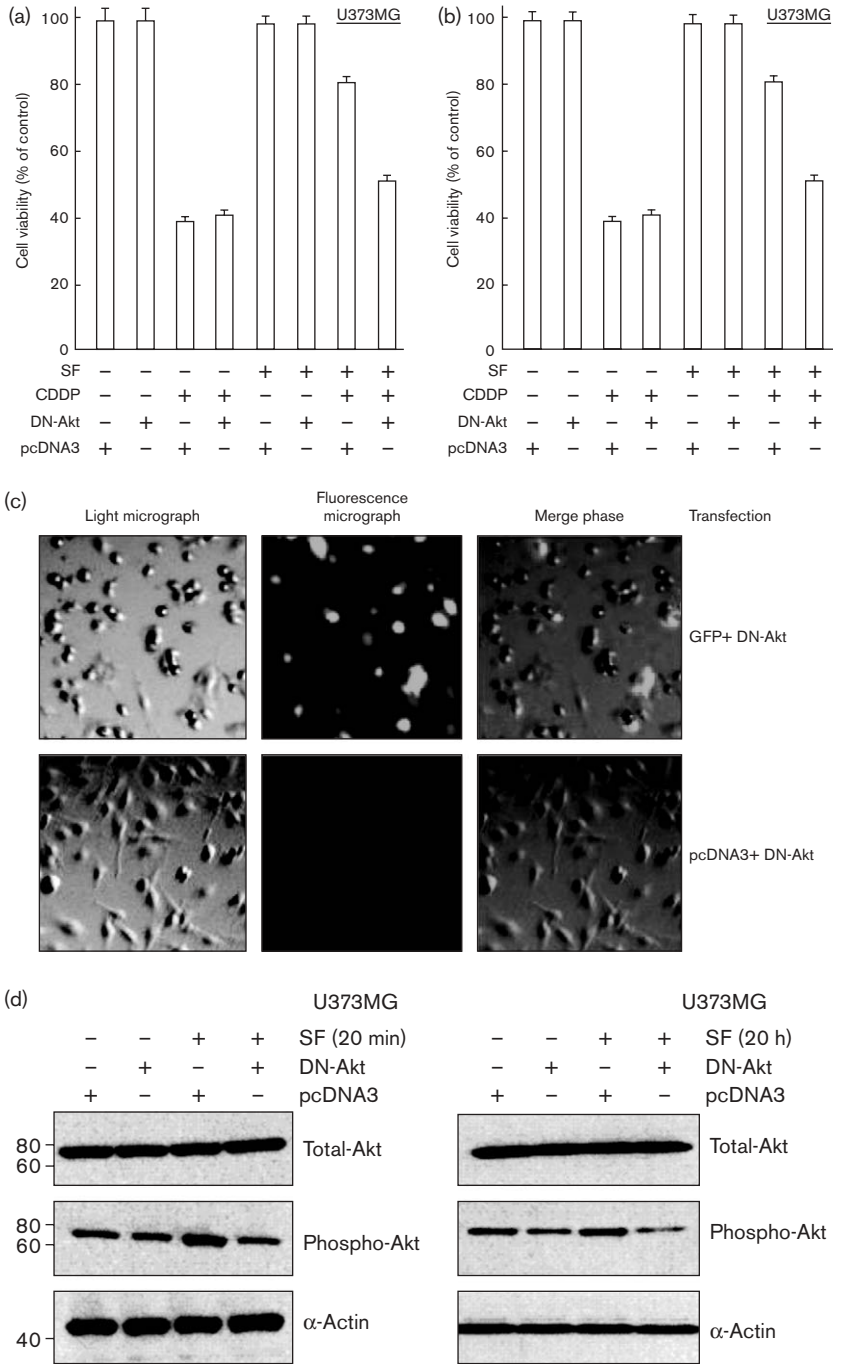
Fig. 3



Effects of experimental manipulation of gene expression on cell survival response to *cis*-platinum (CDDP). (a–c). Subconfluent proliferating U373MG cells in 96-well dishes were treated with no small interfering RNA (siRNA) (vehicle only), control siRNA or gene-specific siRNA (100 nmol/l) in the absence or presence of scatter factor (SF) (100 ng/ml) for 48 h. The cells were then treated ± CDDP (100 μmol/l × 24 h) and analyzed for cell viability using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assays. The effects of individual knockdown of amplified in breast cancer 1 (AIB1) (a), DEAD/H box helicase 21 (b) and polycystic kidney disease 1 (c) are shown. The data plotted represent the cell viability values (relative to the non-CDDP-treated controls) and are the means ± standard errors of the means (SEMs) of three independent experiments per gene. Each experiment used 10 replicate wells per assay condition. (d and e) The effects of a combination of three gene-specific siRNAs on the cell survival response to CDDP. In (d), each of the three gene-specific siRNAs was used at 33 nmol/l and the control siRNA was used at 100 nmol/l. In (e), the concentration of each gene-specific siRNA was 100 nmol/l and the concentration of control siRNA was 300 nmol/l. (f) Subconfluent proliferating cells in 100-mm dishes were transfected with no vector (vehicle only), empty pcDNA3 vector or AIB1 expression vector overnight, washed, allowed to recover for several hours, seeded into 96-well plates and postincubated for 24 h. They were then treated ± SF (100 ng/ml × 48 h), treated ± CDDP (100 μmol/l × 24 h) and assayed for viability. The values plotted are means ± SEMs of three independent experiments, each of which used 10 replicate wells per assay condition. (f and g) Cells were treated under conditions parallel to those used in (a) and (f), respectively, and then harvested and subjected to Western blotting to detect AIB1 and α-actin (control for loading and transfer).

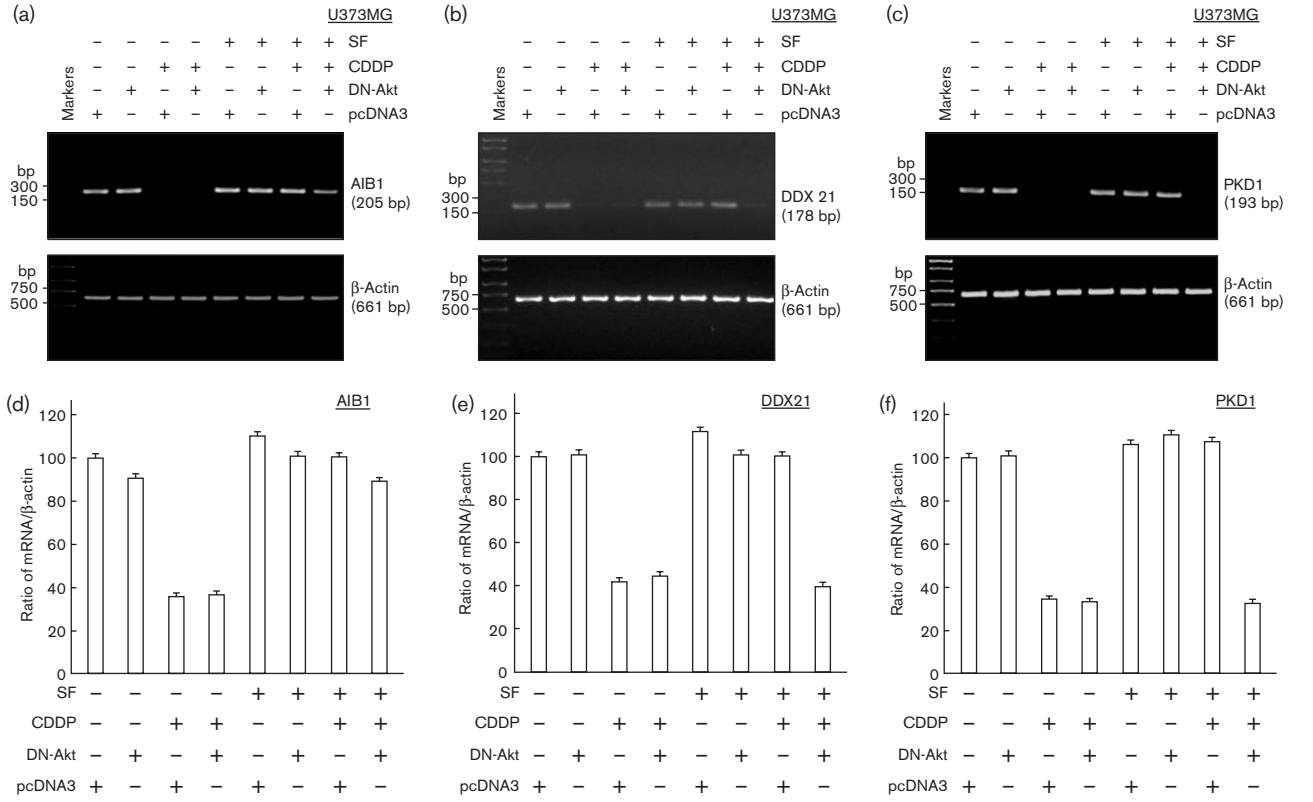
control vector, treated ± SF and ± CDDP, and then harvested for semiquantitative RT-PCR analysis, using β-actin as an internal control gene. The results are shown in Fig. 5. While SF had a relatively small effect on gene expression by itself, it clearly blocked the CDDP-induced downregulation of AIB1, DDX21 and PKD1 mRNA levels

Fig. 4



Effects of inhibition of c-Akt or nuclear factor-κB (NF-κB) signaling on the cell survival response to *cis*-platinum (CDDP). Subconfluent proliferating U373MG cells in 100-mm plastic dishes were transfected overnight using DN-Akt to inhibit c-Akt signaling (a) or a mutant IκB (super-repressor) to inhibit NF-κB signaling, as described in Materials and methods. Controls included no vector (vehicle only) or an empty pcDNA3 vector. The cells were then washed, allowed to recover for several hours, seeded into 96-well plates, postincubated for 24 h, treated ± scatter factor (SF) (100 ng/ml × 48 h), treated ± CDDP (100 μmol/l × 24 h) and assayed for viability using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assays. The cell viability values plotted are means ± standard error of the means of three independent experiments, each of which used 10 replicate wells per assay condition. (c) To assess the transfection efficiency, the cells were cotransfected overnight with DN-Akt plus a green fluorescent protein (GFP) expression vector or an empty pcDNA3 vector, postincubated for 24 h, and visualized by light microscopy and/or fluorescence microscopy. (d) To verify the activity of the DN-Akt expression vector, U373MG cells were transfected overnight with a DN-Akt or empty pcDNA3 vector, postincubated for 24-h to allow gene expression and treated ± SF (100 ng/ml) for $T = 20$ min or $T = 24$ h. The cells were then harvested and Western blotted to detect phospho-Akt total Akt or α-actin.

Fig. 5



Role of c-Akt signaling in scatter factor (SF)/*cis*-platinum (CDDP)-regulated expression of amplified in breast cancer 1 (AIB1), DEAD/H box helicase 21 (DDX21) and polycystic kidney disease 1 (PKD1). Subconfluent proliferating U373MG cells in 100-mm dishes were transfected overnight using DN-Akt (to inhibit c-Akt signaling) or the empty pcDNA3 vector (control). The cells were then washed, allowed to recover for several hours, treated ± SF (100 ng/ml × 48 h), treated ± CDDP (100 μmol/l × 24 h) and harvested for semiquantitative reverse transcription-polymerase chain reaction assays to detect AIB1 (a), DDX21 (b) or PKD1 (c). The data shown in (a)–(c) are representative of three independent experiments. β-Actin was used as a control gene. Panels (d)–(f) show densitometric quantitation of AIB1, DDX21 and PKD1, respectively. The values plotted are ratios of the amplified cDNA bands relative to β-actin, normalized to the control condition (pcDNA3, no SF, no CDDP) and are the means ± standard errors of the means of three independent experiments.

($P < 0.001$, two-tailed *t*-tests). DN-Akt abrogated the ability of SF to block the downregulation of DDX21 and PKD1 by CDDP. In contrast, DN-Akt only modestly altered the ability of SF to prevent CDDP-induced downregulation of AIB1.

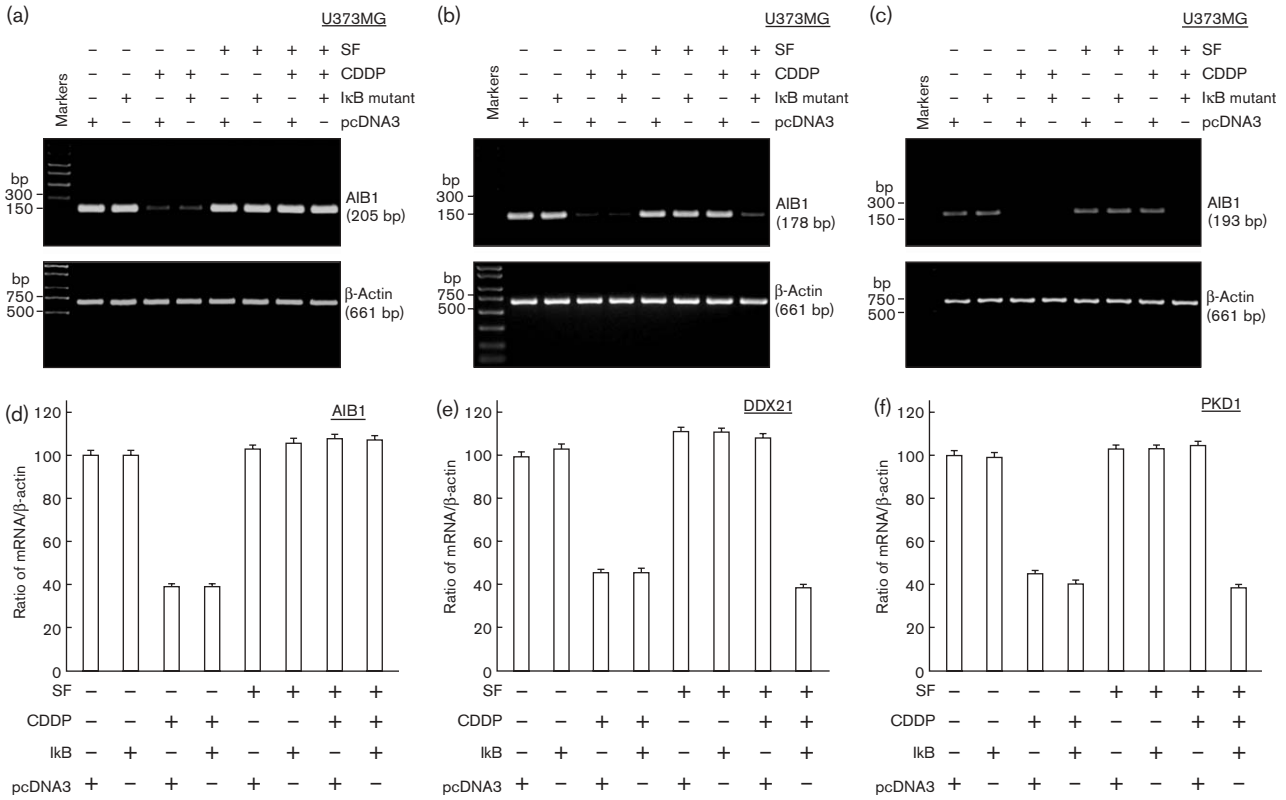
The mutant IκB super-suppressor showed very similar inhibitory activity to DN-Akt. The mutant IκB had no effect on the expression of AIB1 but essentially fully prevented the SF-mediated inhibition of DDX21 and PKD1 expression downregulation by CDDP (Fig. 6). These findings suggest that a c-Akt/NF-κB signaling pathway activated by SF acts to prevent downregulation of DDX21 and PKD1 in CDDP-treated cells. The ability of SF to block the CDDP-induced downregulation of AIB1, however, does not require c-Akt/NF-κB signaling. Thus, the SF protection of U373MG cells against CDDP appears to require both c-Akt/NF-κB-dependent and c-Akt/NF-κB-independent pathways.

Discussion

Overcoming the chemoresistance of malignant gliomas and glioblastomas is a major clinical problem, one that is hindered by an incomplete understanding of the mechanisms that render these tumors so resistant to therapy. Such chemoresistance is also observed in primary brain tumor cell cultures [16]. Several signal transduction pathways have been identified that may contribute to glioma cell survival, including those involving PI3K/c-Akt, PTEN (phosphatase and tensin homolog), NF-κB and mTOR (mammalian target of rapamycin) [4,17,17–21]. Manipulation of these pathways has led to the inhibition of cell survival in cultured glioma cells and in experimental glioma tumor models, but the significance of these pathways in promoting clinical chemoresistance remains to be proven.

Despite the identification of signal transduction pathways contributory to glioma cell chemoresistance, the

Fig. 6



Role of nuclear factor-κB (NF-κB) signaling in scatter factor (SF)/*cis*-platinum (CDDP)-regulated expression of amplified in breast cancer 1 (AIB1), DEAD/H box helicase 21 (DDX21) and polycystic kidney disease 1 (PKD1). Subconfluent proliferating U373MG cells in 100-mm dishes were transfected overnight using the mutant IκB (super-repressor) (to inhibit NF-κB signaling) or the empty pcDNA3 vector. The cells were then washed, allowed to recover, treated ± SF (100 ng/ml × 48 h) and then ± CDDP (100 μmol/l × 24 h), and harvested for semiquantitative reverse transcription-polymerase chain reaction assays for AIB1 (a), DDX21 (b) or PKD1 (c). Panels (d)–(f) show densitometric quantitation for AIB1, DDX21 and PKD1, respectively, relative to β-actin, normalized to the control condition (pcDNA3, no SF, no CDDP), and expressed as the means ± standard error of the means of three independent experiments.

downstream target genes responsible for this chemoresistance are mostly unknown. These genes may include hypoxia-inducible factor-1 (a transcriptional factor that plays a key role in the response to hypoxia) [22], DNA repair genes [those involved in mismatch repair (e.g. hMSH2) or repair of alkylated DNA (MGMT)] [23,24], and the drug transport pump multidrug resistance 1 (or P-glycoprotein), which mediates resistance to multiple structurally distinct drug compounds [25]. Within the last few years, DNA microarray technology has been applied to investigate the biology of gliomas and other types of brain tumors. These studies are aimed at identifying genes or sets of genes that are overexpressed in glioblastoma multiforme or are associated with specific histopathologic classifications of tumors (e.g. high-grade gliomas), tumor recurrence and survival time [26–34]. Such genes reflect the aggressive behavior of malignant gliomas and some of these genes might contribute to this behavior. Our study does not address the issue of genes linked to aggressive tumor behavior. As chemoresistance is, however, linked to poor survival, we compared our lists of genes altered in U373MG owing to treatment with (SF + CDDP) with published studies of biologically aggressive glioma tumors. This comparison identified 10 genes upregulated both in (SF + CDDP)-treated U373MG cells and in high-grade, recurrent or rapidly progressive gliomas, including FOXM1, VEGF, BMI1, IGFBP5, FN1, IL13RA1, TRIO, STC1, IL6 and PGK1. The significance of this finding requires further study.

The gene lists in Tables 2 and 3 undoubtedly contain a number of genes that contribute to the SF-mediated protection of U373MG glioma cells against CDDP, as well as many genes that are irrelevant to cell protection. Our studies suggest that the three genes we have selected for more detailed study (AIB1, DDX21 and PKD1) contribute to SF-mediated cell protection to a modest, but significant degree. Knockdown of each of these three genes significantly reduced protection by SF but also sensitized cells to CDDP in the absence of SF. We have

not been able to locate published information linking any of these genes to the biology of gliomas, so their identification as potential modifiers of glioma chemosensitivity is, to our knowledge, novel. AIB1, a putative breast cancer oncogene, encodes a nuclear receptor coactivator. AIB1 mediates some of the effects of insulin-like growth factor 1 and promotes activation of the PI3K/Akt survival pathway [35,36]. DDX21 is a member of the DEAD box family of RNA helicases that mediate various functions including rRNA biogenesis, RNA editing, RNA transport and transcription. Its specific function is unknown, but it was shown that autoimmune antibodies from a patient with watermelon stomach disease were reactive against the DDX21 protein (also called 'Gu' protein) [37]. PKD1 (polycystin 1), a gene linked to autosomal dominant polycystic kidney disease, is expressed highly in the brain, especially in astrocytes [38,39], suggesting a central nervous system function. It is a high molecular weight (around 460 kDa) membrane-associated protein that interacts with cadherin/catenin complexes that may mediate cell adhesion, calcium channel activity, kidney tubulogenesis and resistance to apoptosis [40,41]. Additional work will be required to establish whether AIB1, DDX21 and/or PKD1 contribute to the clinical chemoresistance of gliomas.

For all three genes (AIB1, DDX21 and PKD1), we showed that CDDP causes downregulation of mRNA expression that was blocked by SF. SF by itself, however, had little or no effect on the basal expression levels. For DDX21 and PKD1, but not for AIB1, DN-Akt and IκB super-repressor expression vectors abrogated the ability of SF to block the downregulation of gene expression. These findings suggest that c-Akt and NF-κB signaling pathways are required for SF to prevent the downregulation of DDX21 and PKD1 caused by CDDP. Interestingly, while DN-Akt and IκB super-repressor completely abolished the ability of SF to prevent downregulation of DDX21 and PKD1, they did not completely abolish SF-mediated protection of U373MG cells against CDDP. These findings suggest that another non-Akt/non-NF-κB related signaling pathway may contribute to the SF-mediated protection of U373MG cells, and they are consistent with the finding that neither DN-Akt nor IκB super-repressor had any effect on the ability of SF to block the downregulation of AIB1 by CDDP. Alternatively, the inability of DN-Akt and IκB super-repressor to fully block SF-mediated cell protection may be due to an incomplete transfection efficiency. Our results suggest that AIB1 modulates SF-regulated chemosensitivity via a c-Akt and NF-κB-independent mechanism. Additional studies are needed to identify this mechanism.

Previously, we showed that c-Akt acts as an upstream activator of NF-κB signaling in the SF-mediated cell protection pathway in DU-145 prostate cancer and MDCK epithelial cells [21]. Thus, DN-Akt or pharmacologic inhibitors of PI3K blocked the ability of SF to stimulate the activity of an NF-κB responsive luciferase reporter. The similarity of the effects of DN-Akt and the IκB super-repressor on U373MG cell survival and gene expression (AIB1, DDX21 and PKD1) in this study suggests that c-Akt and NF-κB operate within the same cell survival pathway in U373MG glioma cells. It also appears likely that a parallel as yet unidentified SF-activated pathway is operative.

It has been established that human glioblastomas often exhibit genomic alterations, including epidermal growth factor receptor DNA amplification, epidermal growth factor receptor activating mutations p53 inactivating mutations, PTEN mutations and p16INK4A deletions ([31] and references therein). U373MG cells are mutant for PTEN, which functions as an upstream inhibitor of c-Akt signaling. Previously, we showed that, in other cell types (DU-145 and MDCK cells), exogenous PTEN inhibits SF-mediated cell protection and SF-mediated stimulation of NF-κB transcriptional activity [21,42]. We also showed that restoration of PTEN modulated c-Met-regulated gene expression in U373MG cells by substantially reducing the number of c-Met-inducible genes [15]. The implication is that the loss of PTEN may enhance the ability of SF to mediate chemoresistance within human gliomas.

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