INTERNATIONAL JOURNAL OF ONCOLOGY 34: 377-389, 2009

Dominant-negative inhibition of Ets 1 suppresses tumor growth, invasion and migration in rat C6 glioma cells and reveals differentially expressed Ets 1 target genes

AYGUEN SAHIN[1,5], CHANTAL VERCAMER[2], ANNETTE KAMINSKI[1], TANJA FUCHS[1], ALEXANDRA FLORIN[1], JENS CLAUS HAHNE[1], VIRGINIE MATTOT[2], ALBIN POURTIER-MANZANEDO[3], TORSTEN PIETSCH[4], VÉRONIQUE FAFEUR[2] and NICOLAS WERNERT[1]

[1]Institute of Pathology, University of Bonn, 53011 Bonn, Germany; [2]CNRS UMR 8161, Institut de Biologie de Lille, Institute Pasteur de Lille, B.P. 447, 59021 Lille; [3]CNRS UMR 8161, Universite de Lille II, Institut Pasteur de Lille, 1 rue Calmette, 59021 Lille Cedex, France; [4]Department of Neuropathology, University of Bonn, 53105 Bonn, Germany

Received September 2, 2008; Accepted October 20, 2008

DOI: 10.3892/ijo_00000161

Abstract. We previously reported that the inactivation of the Ets 1 transcription factor by a specific decoy strategy reduces rat C6 glioma cell proliferation and *mmp-9* expression. In the present study, we analysed the effects of the dominant-negative form of Ets 1 (Ets-DB) on rat C6 glioma cell proliferation, migration, invasion, *in vivo* tumor growth on the chicken chorioallantoic membrane (CAM) and *mmp-9* expression. In addition, we examined differences in gene expression between Ets-DB expressing and control cells using suppression subtractive hybridization (SSH). We found that retrovirus mediated expression of Ets-DB inhibited cellular proliferation, migration, invasion, *mmp-9* expression, cellular growth in soft agar, and *in vivo* growth in the chicken chorioallantoic membrane assay. SSH analysis revealed expression of different genes in Ets-DB expressing cells involved in basic cellular processes. Each of these genes contained binding sites for different Ets-factors within their promoters. Finally, we found that, in addition to Ets 1, Elk-1, Elf-1, Fli-1 and Etv-1 are further Ets family members expressed in rat C6 glioma cells. Our results indicate that Ets transcription factors play important roles for basic properties of rat C6 glioma cells. Targeting of these factors might therefore become a useful experimental tool for therapeutic strategies against malignant gliomas.

Correspondence to: Dr Nicolas Wernert, Institute of Pathology, University of Bonn, D-53011 Bonn, Germany
E-mail: nicolas.wernert@ukb.uni-bonn.de

[5]*Current address:* MGH-HMS Center for Nervous System Repair, Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

Abbreviations: Ets-DB, Ets 1-DNA binding domain; CAM, chicken chorioallantoic membrane; SSH, suppression subtractive hybridization; MMP, matrix metalloproteinase; SPARC, secreted protein acidic and rich in cysteine; FCS, fetal calf serum; Neo, neomycine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *rpL13*, ribosomal protein L13; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; DNA, deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; RNA, ribonucleic acid; PCR, polymerase chain reaction; EDTA, ethylene diamine tetraacetic acid; $MgCl_2$, magnesium chloride; dNTP, deoxyribonucleotide triphosphate; DTT, dithiothreitol

Key words: glioma, Ets 1, Ets family, dominant-negative mutant, *mmp-9*, tumor growth, suppression subtractive hybridization

Introduction

Malignant gliomas are the most common tumors of the human nervous system (reviewed in ref. 1). Unfortunately, conventional treatment of these neoplasms through surgery, radiotherapy and/or chemotherapy yield unsatisfactory results (reviewed in refs. 2,3). Therefore, alternative strategies such as gene therapeutic approaches are currently being studied in pre-clinical models (reviewed in ref. 3). A number of transgenes coding for cell death inducing proteins (expressed through viral or non-viral vectors), modulators of immune response, or inhibition of tumor angiogenesis have been used in experimental therapeutic approaches.

Since transcription factors are central regulators of many cell functions, they appear to be suitable molecular targets in experimental tumor therapy. In this report, we targeted Ets 1, the prototype of the Ets transcription factor family, which has been demonstrated to be expressed in human astrocytomas (4,5) as well as in human astrocytoma cells lines, such as CCF and 1321N1 (6,7). We recently demonstrated that rat C6 glioma cells (which imitate many characteristics of malignant gliomas *in vivo* and which are a commonly used model system) likewise express Ets 1 (8). This transcription factor has been studied in different tumors, but its role in gliomas is not yet well described. A known role of Ets 1 is participation in tumor invasion involving both the tumor stroma and the neoplastic cells. We have shown that Ets 1 and several matrix degrading proteases are expressed together

within the fibroblastic stroma of different invasive tumors, non invasive lesions being negative (9-11). Genes encoding different proteases [particularly matrix metalloproteinases, MMPs, which play a major role in invasion (10,11)] are actually among the target genes of Ets 1 (12-14) and we have confirmed transcriptional regulation of MMP-genes through Ets 1 using fibroblasts from Ets 1 knock-out and control mice (15). In addition we have linked Ets 1 expression to invasive behaviour of different neoplastic cells (such as HeLa and melanoma cells) using an Ets 1 antisense approach (16,17). In these cells, MMP-1, MMP-3, uPA and integrin ß3 (16,17) were encoded target genes of Ets 1. Expression of the gelatinases MMP-2 and MMP-9, in particular, closely correlates with invasive and metastatic potential of various cancers, including gliomas (18,19). MMP-9 is actively secreted by glioma cells (18,20,21) and we have shown the *mmp-9* gene to be an Ets 1 target gene in these cells (8). Other molecules implicated in glioma invasion include SPARC [secreted protein acidic and rich in cysteine (22)] or the extracellular matrix protein fibronectin (23).

Expression of Ets 1 correlates with poor prognosis (10,11,24-27) and a high risk for lymph node metastases (28-30) in several cancer types. We have recently shown that blockade of Ets 1 through Ets 1 specific double stranded oligonucleotides reduced proliferation and *mmp-9* expression in rat C6 glioma cells (8). As an extension of our previous study, we evaluated here a blockade of Ets 1 in rat C6 glioma cells through a retroviral expression of the Ets DNA-binding domain (Ets-DB). This domain has no transactivation properties and is a complete dominant negative form of Ets 1. The same retroviral approach has already been applied in the mouse mammary tumor cell line MMT (31), in different endothelial cell lines (32), and in an *in vivo* model of the mouse ear (33).

In the present study, we investigated whether Ets-DB expression in rat C6 glioma cells affects major tumor cell properties, such as proliferation, anchorage-independent growth in soft-agar, migration, invasion, as well as *in vivo* tumor development on the chicken chorioallantoic membrane (CAM). In addition, we searched for Ets 1 target genes by comparing gene expression between Ets-DB expressing and control cells using suppression subtractive hybridization (SSH). We finally identified further Ets family members which are expressed in rat C6 glioma cells.

Materials and methods

Cell culture. Rat C6 glioma were obtained from ATCC (Rockville, USA). Cells were cultured in RPMI-1640 medium (Invitrogen, Breda, The Netherlands), complemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). NIH3T3 cells were used for assessment of virus titration. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% Hyclone bovine serum (HBS, Invitrogen) and 1% penicillin/streptomycin. Ets-DB or Neo-producing GP^+E86 cells were routinely cultured in DMEM, containing 10% FCS, 800 μg/ml geniticin (Invitrogen) and 1% penicillin/streptomycin. Rat C6 glioma and NIH3T3 cells infected with Ets-DB or Neo were selected by 800 μg/ml geniticin. Cells were incubated for different times at 37˚C in a humidified atmosphere containg 5% CO_2.

Viral construction and production. The establishment of MFG-Ets1-DB-Ires-Neo^R-, also called Ets-DB, or MFG-Ires-Neo^R-, also called Neo, producing GP^+E86 cells have been described previously (32). Briefly, the cDNA sequence encoding the Ets 1-DB (the DNA-binding domain of mouse Ets 1, corresponding to amino acids 306-423) was cloned upstream of an internal ribosomal entry site and a neomycin-resistance sequence. A second vector carrying the IRES and Neo sequences was constructed and used as control in the entire study. These Ets-DB and Neo sequences were inserted into a retroviral MFG vector. Virus packaging murine fibroblasts [GP^+E86 (34)] were then transfected with either Ets-DB or Neo-MFG retroviral vectors (32). The GP^+E86-virus producing cells were then obtained following selection in the presence of 800 μg/ml geniticin. After overnight incubation of subconfluent cells with fresh medium, the supernatant containing viral particles was collected, filtered and used for infection. Viral titration was performed by counting the number of geniticin-resistant clones after infection and selection of NIH3T3 cells for 10 days.

C6 glioma cell infection and characterisation. Rat C6 glioma (80,000 cells/well) or NIH3T3 (100,000 cells/well) cells were incubated for 8 h with 1 ml of filtered virus solution. Selection started two days after infection by addition into the culture medium of 800 μg/ml geniticin. After 10 days, cell colonies were pooled. The resulting infected cells were maintained in their specific media complemented with 800 μg/ ml geniticin. These infected cells referred to as C6-DB or DB and C6-Neo or Neo. The expression of the Ets-DB protein was assessed by immunoprecipitation. For metabolic labelling, DB cells (100,000 cells/100 mm plate) were cultured for 24 h in DMEM-10% FCS. The medium was replaced by methionine- and cysteine-free modified Eagle's medium and cells were further incubated for 3 h. Then incubation was performed for 3 h in methionine- and cysteine-free MEM containing 250 μCi/3 ml of ^{35}S methionine and -cysteine (trans ^{35}S-Label, 1,066 Ci/mmol, ICN). At the end of the experiments cells were lysed and processed for immunoprecipitation as described by Gilles *et al* (35). Briefly, cell extracts containing identical amounts of ^{35}S-labelled material were incubated in the presence of an anti-Ets-DB rabbit polyclonal antibody (35) and immunoadsorbed onto Protein-A-agarose beads. Proteins were then separated onto 15% SDS-polyacrylamide gels and the gels were fixed, treated with Amplify (Amersham Biosciences, Buckinghamshire, UK) and exposed to Hyperfilm (Amersham Biosciences).

Proliferation and anchorage-independent growth assays. Cell proliferation was assessed by a modified MTT Assay (EZ_4U; Biomedica, Vienna, Austria). Cells/well (1,000) (DB, Neo or C6) were seeded into 96-well plates and incubated in RPMI medium with 10% FCS at 37˚C. Cell proliferation was assessed in triplicate 9, 24, 48 and 72 h after seeding according to the manufacturer's instructions. Anchorage-independent proliferation was examined with a small modification as described previously. Briefly, cells (DB, Neo

or C6) were diluted to 2x10^5 cells/ml in 0.4% noble agar (Seakem, Rockland, Maine, USA) solution (in PBS, Invitrogen). Cell suspension (1x10^5 cells/0.5 ml/well) was added to each well of a 35-mm tissue culture plate with an underlayer of 1.4% noble agar (in PBS) at 37˚C, and then were cultured at 37˚C for 24 days. Twice weekly, RPMI with 10% FCS was added (0.3 ml/well) to provide additional nutrients and growth factors. Colonies were evaluated and documented under x35 magnification at day 1, 11 and 24. Colonies in each triplicate investigation were counted and presented as a graphic.

Wound and quantitative migration assays. Wound-induced migration assay was performed as described before (36). Briefly, DB, Neo or C6 cells resuspended in RPMI with 10% FCS (2x10^5 cells/well) were plated onto 12-well culture plates and incubated for 24 h. Then, cells were scraped with a plastic pipette tip and washed with PBS twice, and the medium was replaced with serum-free medium (RPMI-1640). Cells were photographed at 0, 24 and 48 h after scraping.

Quantitative cell migration assays were performed with fibronectin or collagen type I coated Boyden chambers according to the manufacturer's instructions (Chemicon Europe, Hampshire, UK). Briefly, subconfluent DB, Neo or C6 cells were cultured 24 h in serum-free medium (RPMI). Cells (2.5x10^5) in 300 μl were placed into fibronectin- or collagen type I coated Boyden chambers. After incubation for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2, the migratory cells on the underside of the chambers were stained and extracted according to manufacturer's instructions. Migrated cells were read by absorbance at 595 nm. All measurements were assessed in triplicates.

Invasion assay. The invasion assay was performed by using 24-well BD Biocoat Matrigel invasion chambers with 8-μm polycarbonated filters, coated with a three-dimensional matrix (Becton Dickinson, Heidelberg, Germany), as described by Albini *et al* (37). DB, Neo or C6 cells (25,000) were seeded on Matrigel invasion chamber plates, and cultured in routine medium. Cells were incubated for 22 h at 37˚C in a humidified atmosphere with 5% CO_2. Non-invaded cells at the upper surface of the filter were removed by wiping with a cotton swab. Invaded cells were stained with Cellstain solution (Chemicon Europe) after fixation with methanol. The cell number was counted under microscope vision, in four different areas, and the average cell number was determined. The assay was performed in triplicates.

Chicken chorioallantoic membrane (CAM) assay. A window was cut into the eggshell of 3-day-old chick embryos. The embryos were checked for normal development and the window was sealed with cellotape. Eggs were reincubated until day 10, when DB, Neo or C6 cells were inoculated onto the CAM. Cells were pipetted onto the CAM as described previously (38). Briefly, confluent cells were harvested by trypsinisation (0.125% trypsin/1% EDTA; Invitrogen), washed once with growth medium and resuspended in the same medium of 1x10^6 cells per 50 μl medium. Eggs were incubated at 37˚C and with 60% humidity. At day 17, tumors were first documented by photography, then the tumor volume were calculated by a mathematical formula: $[1/6\ \pi\ (R_{min})^2 \times (R_{max})^2]$ (39). Tumor sizes were measured by a silicon ruler. Tumors were then cut out and routinely embedded into paraffin. Tumor histology was evaluated on 5-μm thick sections stained with hematoxylin and eosin (H&E).

RNA isolation, reverse transcription and semi-quantitative RT-PCR for assessment of mmp-9 expression and expression of Ets family members. Total cellular RNA was extracted from DB, Neo or C6 cells by the RNeasy kit (Qiagen, Hilden, Germany). Generation of cDNAs by reverse transcriptase was performed in 20 μl reaction volume containing 1 μg of total cellular RNA, 2 μl of 10x first strand buffer (Invitrogen), 1 μl of 50 mM $MgCl_2$, 1 μl of 10 mM dNTP, 2 μl of 0,1 mM DTT and RNAse-free water. After incubation at 70˚C for 10 min 200 units of SuperscriptII reverse transcriptase (Invitrogen) were added and reverse transcription was performed at 42˚C for 50 min, followed by inactivation of the enzyme at 70˚C for 15 min. cDNA synthesis was controlled by PCR amplification of the constitutively expressed *rpL13* gene, encoding for a ribosomal protein. PCR reactions for Ets family members and rpL13 were performed during 25, and *mmp-9* and rpL13 during 30 cycles in a thermal cycler. Primer sequences and PCR-conditions for amplifications are shown in Table I.

Generation of subtracted libraries for both DB and Neo cells by subtractive suppression hybridization (SSH) and validation of differentially expressed genes. Analysis of differentially expressed genes between DB and Neo was performed by suppression subtractive hybridization (SSH) using the Clontech PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, USA). Briefly, 2 μg of both DB (tester) and Neo (driver) poly A$^+$ RNA were used for double stranded cDNA synthesis and the resulting cDNA was digested with Rsa I. The digested tester cDNA was split into 2 and ligated to either adaptor 1 or adaptor 2R. After second hybridization and selective amplification of hybrid molecules by first and second PCR, PCR products were analysed on a 2% agarose gel containing ethidium bromide. The PCR mixture, containing enriched differentially expressed transcripts, was cloned into the PCR®2.1-TOPO (Invitrogen, Groningen, The Netherlands). Plasmid DNA from subtracted clones were purified and sequenced. Blast search was used to analyse sequence homologies in the gene database. Sequences with no similarity to known rat sequences were compared to EST database. The ETS-binding sites in regulatory sequences of differentially expressed genes were determined using the MatInspector® transcription factor binding site search program (40). The subtraction efficiency was determined by the PCR analysis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in subtracted and unsubtracted cDNA libraries of both DB and Neo cell lines as described in the PCR-Select Subtraction protocol (Clontech). For validation of differential gene expression, quantitative-PCR analyses were performed with SYBR-Green by Roche LightCycler® system (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Specific primers for differentially expressed genes are listed in Table I. The gene encoding

Table I. Primers used for amplification of cDNAs for mmp-9, rpL13 and subtracted genes.

	Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
sq RT-PCR	*mmp-9*	ACAGCGAGACACTAAAGGC	GCCATACAGCTTATCCTGGTC
	rpL13	CCGCACAAGACCAAAAGA	GCACATAGAACCCTCCAGAAA
	ets-1	ACTATGTGCGTGACCGTGCT	TTCCTCTTTCCCCATCTCCT
	elf-1	CAATCACCCACGTGTCTGTC	GGTGCTGGTTATGGTTGCTT
	elk-1	ACGGGATGGTGGTGAGTT	AGGATGAAGGGGTGGCTGT
	fli-1	GGAGTGGGCCATAAAGGAAT	GAAGCTGGAGGAGGTGACAG
	etv-1	CAAGAAACATGGCTTGCTGA	ACTGGGTCGTGGTACTCCTG
q RT-PCR	*Sparc*	CAGAAACTGCGTGTGAAGAA	AGGTCTCAAAGAAGCGAGTG
	enpp1	CCCAAGTCATCCCAAAGAAGAG	GAAGTCCATGATCGGCACAA
	txn	GAAGCTGATCGAGAGCAAGGA	CACGTCTACTTCAAGGAACACCAC
	serpine2	GCCTTCACGTCTCTCACATCTT	CCTTCAGGGCTTGTTCACCT
	eefa1	CTGGTAAGAAGCTGGAAGATGG	AGCAAAACGACCAAGTGGAG
	nne	GACCCTCTTTCCTTCCTCC	CAATCTTCTCCTGCTCCACA
	eef β1	AGTCCCAAGTCTCCAAGCA	CAGCAAACCAAACAGGAAGG
	atp5b	ACCCATTGAAGAAGCTGTGG	AGGTCAGTGCAGAGTGCTGTT
	lrp	CTCTGTGGGAGCAACCTATC	TACTCGGTGTCTGGGTCAA
	grp58	GCCAATGATGTGCCTTCTC	GTCCTCTTGTGCCTTCTTCTTC
	ldha	GTGGTTGACAGTGCATACGAAG	GATACATGGGACGCTGAGGAA
	tpt1	GAGGTGGAGGGCAAGATGG	GGTGACTACTGTGCTTTCGGT
	rpL13	GGTGCCCTACAGTTAGATACCAC	GGATTTGTTTCGCCTCCTT
	mmp-9	CGTCTTCCCCTTCGTCTTC	ACCCCACTTCTTGTCAGCGT

sq, semi-quantitative; q, quantitative.

for ribosomal protein *(rpL13)* was used as internal control. All PCR reactions were performed during 45 cycles. Analyses were carried out in independent triplicates. Expression ratios of differentially expressed genes relative to *rpL13* were assessed automatically by the relative quantification software (Roche Diagnostics). The mean values of these quantifications were demonstrated as a ratio of DB and Neo.

Statistical analysis. Quantitative data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using analyses of variance (ANOVA) for multiple comparisons or Student's t-test for comparison of means of mRNA levels between DB and Neo with Prism version 5.0 (GraphPad Software, San Diego, CA). P-values ≤0.05 were considered significant for all analyses.

Results

Ets-DB expression in infected rat C6 glioma cells. MFG-DB or the MFG-Neo retroviral supernatant from GP+E86 packaging cells was used to infect rat C6 glioma cells and NIH3T3 cells. Viral titer was 10^7 cfu (colony forming units)/ml. Single C6 colonies were pooled and the expression of the Ets-DB protein in C6 glioma cells was determined by immunoprecipitation (Fig. 1a). In DB cells, the anti-Ets 1 antibody recognised a protein migrating at 13 kDa, the expected size for the amino acids 354-409. Fig. 1 shows the expression of Ets-DB in

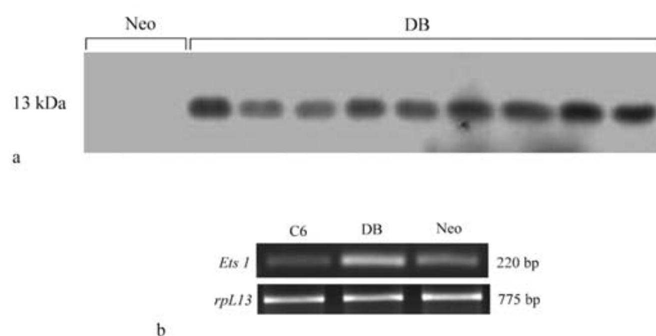

Figure 1. Expression of Ets-DB protein in rat C6 glioma cells. Expression of Ets-DB protein was analyzed in DB or Neo infected rat C6 glioma cells using immunoprecipitation. The bands show the presence of the Ets-DB protein (13 kDa, a). The relative expression of Ets 1 in DB, Neo and C6 cells has been analyzed through semi-quantitative RT-PCR using primers designed for amplification of Ets 1 DNA binding site cDNA. (b) Constitutive Ets 1 expression is seen in Neo and C6 cells. As expected a stronger signal is found in DB cells.

pooled DB cell clones. As expected pooled Neo control clones did not show any signal. After confirmation of Ets-DB expression in DB cells, randomly chosen pools for either Neo or DB cells were used in further experiments. We further determined the relative expression of Ets 1 in DB, Neo and C6 cells through semi-quantitative RT-PCR using primers designed for amplification of Ets 1 DNA binding site cDNA.

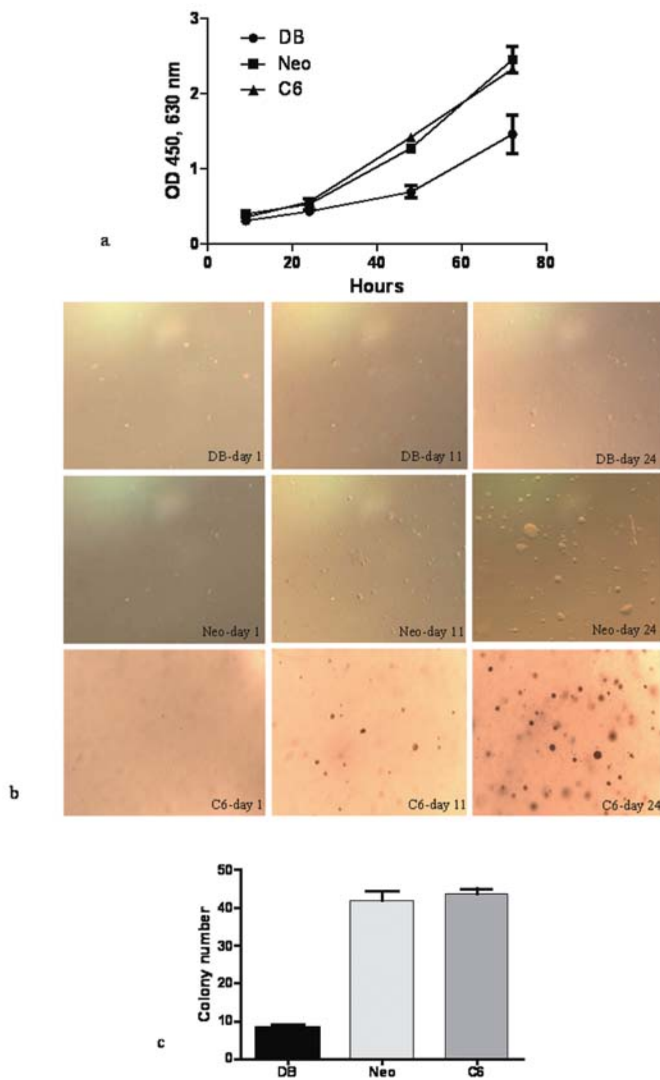

Figure 2. Assessment of cell proliferation and anchorage-independent growth of rat C6 glioma cells. Cell proliferation was assessed in 96-well plates using a modified MTT assay (a). DB, Neo or C6 cell proliferation was measured at 9, 24, 48 and 72 h. All measurements were performed in triplicates (P=0.0004, two-way ANOVA). The anchorage-independent growth was documented at several time-points likewise (b) and (c). Figures are presented from day 1, 11 and 24. Findings are documented at x35 magnification. Expression of Ets-DB strongly inhibits anchorage-independent growth of rat C6 glioma cells at day 24 compared to Neo and wild-type cells. Colony numbers according to triplicate experiments were counted for DB, Neo or C6 cells at day 24 and results presented in a graphic (c, P=0.0001, one-way ANOVA).

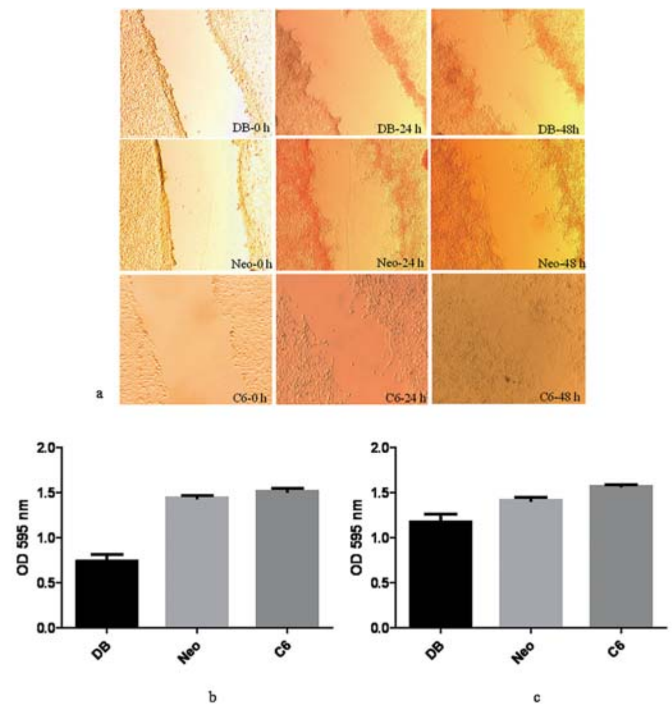

Figure 3. Ets-DB inhibits cells migration of C6 glioma cells. Confluent monolayers of DB, Neo or C6 cells (a) cultured on dishes were scraped by a plastic pipette tip and wound-induced cell migration documented after indicated time-points. All pictures were taken at magnification of x35. A slight inhibition of migration was evident for DB cells after 24 and 48 h compared to Neo and C6 cells. Cells were further placed into fibronectin (b, P<0.0001, one-way ANOVA) or collagen type I (c, P=0.0002, one-way ANOVA) coated Boyden chambers. Migrating cells were stained after 24 h and measured by absorbance at 595 nm. All measurements were performed in triplicates. The graphics show the mean value of all measurements. Ets-DB expression more or less inhibits cell migration.

We found constitutive Ets 1 expression in Neo and C6 control cells (Fig. 1b). As expected signals in DB cells were stronger than those in Neo and control cells.

Ets-DB inhibits C6 cell proliferation and anchorage-independent growth in soft-agar. We first assessed the effect of Ets-DB on cell proliferation using a modified MTT assay (Fig. 2a). Ets-DB inhibited cell proliferation at all time-points. A strong inhibition of proliferation was seen in DB cells at late time-points (48 and 72 h). Neo and C6 cells exhibited similar proliferation rates at all time-points (Fig. 2a), which showed that the retroviral infection had no effect on proliferation of these cells.

In anchorage-independent growth experiments (Fig. 2b and c) we found that Ets-DB strongly inhibited colony formation in soft-agar (beginning at day 11) compared to Neo and C6 controls. Colony numbers are quantified on day 24 in Fig. 2c. Neo and C6 cells showed similar colony formation properties.

Ets-DB reduces cell migration in wound assay as well as on fibronectin and on collagen type I coated Boyden chambers. We next investigated the effect of Ets-DB expression on cell migration using the wound assay (Fig. 3a). The migration of cells into the wound was slightly slowed down in DB cells at 48 h after scraping, compared to Neo and C6 controls.

For evaluation of the effect of Ets-DB on cell migration, we also used a quantitative migration assay with Boyden chambers coated with the extracellular matrix (ECM) proteins fibronectin and collagen type I, both known to play an important role in cell migration (36). We found a significantly stronger reduction of cell migration of DB cells on fibronectin coated Boyden chambers (49%), compared to collagen type I coated chambers (19%, Fig. 3b and c). Migration of Neo and C6 cells was comparable in both experiments (Fig. 3b and c).

Ets-DB inhibits invasion of C6 glioma cells on matrigel coated Boyden chambers. The biological roles of Ets 1 in tumors were

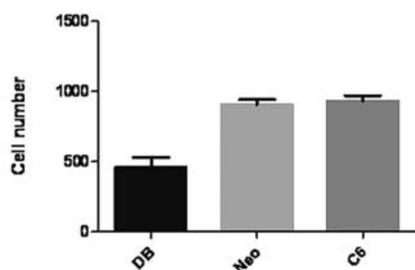

Figure 4. Inhibition of C6 glioma cell invasion by Ets-DB expression. Cells were placed into matrigel coated Boyden chambers. Invading cells were fixed, stained and counted in four different areas after 24 h. The graphic shows the mean value of cell numbers in 4 counted areas (P=0.0009, one-way ANOVA).

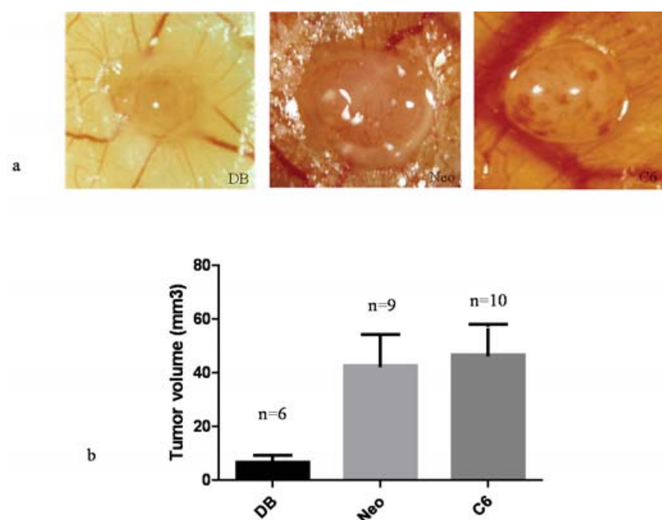

Figure 5. Ets-DB induced inhibition of tumor growth of C6 glioma cells on the chicken chorioallantoic membrane (CAM). DB, Neo or C6 cells were transplanted onto the CAM. Following incubation for 7 days, grown tumors were photographed (x25, a, representative images of each group) and tumor volumes were calculated by a mathematical formula (see Materials and methods, b, P=0.0031, one-way ANOVA). A significant reduction of tumor growth is evident for DB cells.

first linked to tumor angiogenesis and to invasion (9,41). It is known that Ets 1 is expressed in several invasive human tumors (11,42), where it participates to transcriptional regulation of matrix degrading proteases (9,16,17). We therefore, investigated the effect of Ets-DB expression on invasion of rat C6 glioma cells through a three-dimensional matrix using matrigel (an *in vitro* ECM) coated Boyden chambers. Fig. 4 demonstrates the inhibition of invasion of DB cells, compared to Neo and C6 cells. The observed inhibition was about 51%. The invasive behaviour of Neo and C6 cells did not differ significantly.

Ets-DB inhibits in vivo tumor growth on the chicken chorioallantoic membrane (CAM). We next assessed the effect of Ets-DB on tumor growth of rat C6 glioma cells *in vivo*. Therefore, we transplanted DB, Neo or C6 cells onto the chicken chorioallantoic membrane (CAM, Fig. 5a). We

Table II. Number of grown and non-developed rat C6 glioma tumors on the CAM.

Sample	No. of grown tumors	No. of non-developed tumors	Total no. of samples
DB	6	4	10
Neo	9	1	10
C6	10	-	10

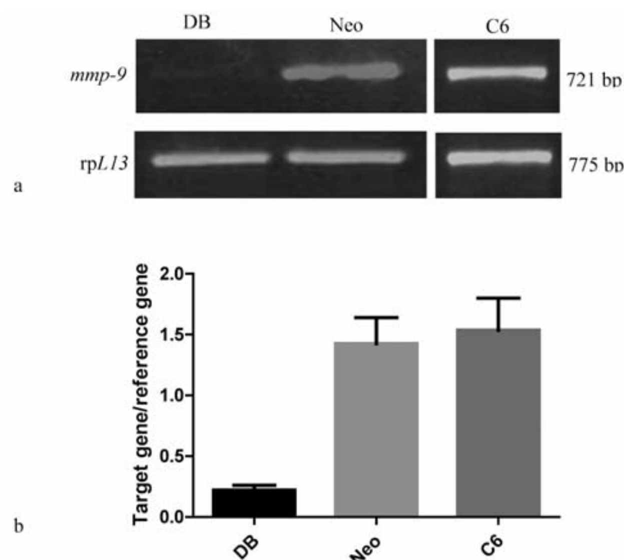

Figure 6. Ets-DB significantly reduces *mmp-9* expression in rat C6 glioma cells. *Mmp-9* gene expression relative to the housekeeping gene *rpL13* was evaluated by semi-quantitative (a) and quantitative RT-PCR (b, P=0.0057, one-way ANOVA). Quantification of RT-PCRs was done automatically by the relative quantification software (Roche Diagnostics) in which the ratio of the target gene (*mmp-9*) and the reference gene (*rpL13*) was assessed. Amplification was performed in triplicate. (b) Shows the mean value of these quantifications.

found that 4 of 10 transplantations of DB cells led to tumor formation on the CAM (Table II).

In contrast only one of 10 transplantations (10%) of Neo cells was not followed by tumor formation. Transplanted C6 cells formed tumors in each case. It was evident by both semiquantitative macroscopical evaluation (Fig. 5a) and mathematical calculation of tumor volumes (Fig. 5b) that DB tumors were significantly smaller (84.5%) than Neo and C6 control tumors. Histological analysis of the tumors showed no significant differences between DB, Neo and C6-tumors (data not shown).

Ets-DB inhibits mmp-9 expression in rat C6 glioma cells. To further clarify the role of Ets blockade in glioma cell invasion we evaluated the effect of Ets-DB on *mmp-9* gene expression using semi-quantitative (Fig. 6a) and quantitative RT-PCR (Fig. 6b). Both analyses showed that expression of Ets-DB caused a strong inhibition (quantitatively up to 87%) of *mmp-9* gene expression. Expression of *mmp-9* gene was comparable in Neo and C6-cells.

Table III. Identification of subtracted sequences after SSH by sequence analysis.

Function	Gene name	Accession no.	Incidence	cDNA insert size	Overexpression confirmed in DB cells
Translational elongation	Translation elongation factor-1 α (eef1a1)	NM_175838	7	1400	+
Translational elongation	Translation elongations factor-1 γ (eef-1b γ)	XM_233713	1	800	+
Collagen binding	Osteonectin (sparc)	NM_012656	4	300	+
Calcium binding	Translationally controlled tumor protein (tctp, tpt1)	NM_053867	1	590	+
Degradation of ECM and neurogenesis	Serine protease inhibitor (protease nexin I, PN-1, serpine 2)	XM_343604	2	920	+
Glycolysis	Lactate dehydrogenase-A (ldha)	NM_017025	2	1600	+
Glycolysis	Non-neural enolase (α enolase, enolase 1, nne)	XM_214956	1	900	+
Electron transport	Thioredoxin (txn)	NM_053867	1	400	+
Electron transport	Glucose regulated protein, 58 kDa (grp58)	NM_017319	1	900	+
Proton transport	ATP synthase ß (atp5B)	NM_134364	1	900	+
Protein tyrosine phosphatase activity	Protein tyrosine phosphatase α, receptor type (Ptpra, lrp)	Q03348	1	400	+
Nucleotid metabolism	Ectonucleotid pyrophosphatase/ phosphodiesterase I (enpp1)	NM_053535	1	400	+
Kinase activity	Rho-associated kinase 2 (rock2)	NM_013022	1	440	-
Metal ion binding	Cytochrome P450, subfamily 51 (cyp51)	NM_012941	1	600	-
Ubiquitin-protein ligase activity	Ubiquitin conjugated enzyme E2D3 (ube2d3)	NM_031237.1	1	1000	-
Actin filament polymerization	ß-actin	NM_031144	1	800	n.d.
DNA binding	H3 histon	NM_053985	1	280	n.d.
Structural constituent of ribosome	Ribosomal protein S6 (rps 6)	NM_017160	2	800	n.d.
Unknown	Similar to UPF0315 protein	XP_215167	1	810	n.d.
Unknown	Rattus norvegicus similar to RIKEN cDNA 0610038D11 (predicted) (RGD1309710_predicted), mRNA	XM_001072116	1	810	n.d.
Unknown	LRRGT00174	AAS66265	1		
Unknown	AGENCOURT_66933310 NIH_MGC_367 Rattus norvegicus cDNA clone, IMAGE: 8372424 5', mRNA sequence	DY310845	1	420	n.d.
Unknown	Liver regeneration after partial hepatectomy Rattus norvegicus cDNA, mRNA sequence	DW395162	2	800	n.d.
Unknown	AMGNNUC: NRHY7-00049-D3-A nrhy7 (10850) Rattus norvegicus cDNA, clone nrhy7-00049-d3 5' mRNA sequence	CB780399	1	800	n.d.
Unknown	Rattus norvegicus insulinoma RINm5F Rattus norvegicus cDNA clone RI01907 3', mRNA sequence,	BP502874	2	1100	n.d.
-	Not previously identified	-	1	570	n.d.

n.d., not defined.

Table IV. The Ets binding sites in the promoter regions of differentially expressed genes in DB and Neo rat c6 glioma cells.

Differentially expressed genes	Ets binding sites	Position	Stream	Sequence
eef1a1	Elk-1	119-135	(-)	cttgccGGAAtctacgt
	Pu.1 (Pu120)	573-589	(+)	atacgaGGAAatcgtta
	c-Ets-1 binding site	585-601	(+)	cgttaAGGAagtcagca
	ETS family member FLI	647-663	(-)	ccaaCCAGaaattggca
	c-Ets-1 (p54)	822-838	(+)	cctccAGGAtgtctata
serpine2	Pu.1 (Pu120)	195-211	(+)	actcgaGGAActaggct
	Elk-1	477-493	(+)	taaagtGGAAgtgcctt
	Elk-1	537-553	(-)	ggtcctGGAAgttcaca
	c-Ets-2 binding site	1236-1252	(-)	agaacAGGAaaggcctg
	Elk-1	1513-1529	(+)	ctgtacGGAAgaacgaa
	Elk-1	1654-1670	(-)	tctcccGGAAcgtcaca
	Pu.1 (Pu120)	1753-1769	(-)	agaaaaGGAAttaaaaa
	GABP:GA binding protein	1768-1784	(-)	tcgggtGGAAgtgagag
	Elk-1	1843-1859	(-)	agcaacGGAAacaacac
atp5b	GABP:GA binding protein	294-310	(+)	tgccttGGAAgtgcaag
	Pu.1 (Pu120)	436-452	(-)	ccaacaGGAAttttgat
	c-Ets-1 binding site	576-592	(+)	tgaacAGGAaattctgg
	Ets-family member ELF-2 (NERF1a)	924-940	(+)	agaccaGGAAggccaag
	Nuclear respiratory factor 2 (NRF 2)	960-976	(-)	tgaagcGGAAgatgttg
txn	c-Ets-1 binding site	268-284	(+)	ctgccAGGAAgttgctg
	Nuclear respiratory factor 2 (NRF 2)	307-323	(-)	agaactGGAAggtcggc
tpt1	Elk-1	218-234	(-)	ttcagcGGAAgcatttc
	ETS family member FLI	250-266	(+)	ggtaCCGAaagcacagt
ldha	c-Ets-1 (p54)	840-856	(-)	ggcccAGGAtgtgtaac
eef-1b	Nuclear respiratory factor 2 (NRF 2)	618-634	(-)	ggcctcGGAAgacccaa
	ETS family member FLI	677-693	(+)	gtcaCCCGaagcttctg
	GABP:GA binding protein	766-782	(-)	gcgcccGGAAgtgcgtc
	ETS family member FLI	839-855	(-)	gaaaCTGGaaatacctg
	Elk-1	980-996	(+)	cagaaaGGAAggtcagc
	c-Ets-1 (p54)	1028-1044	(-)	cgggcAGGAagccctgc
	c-Ets-1 binding site	1091-1107	(-)	caaacAGGAaggcaggg
	c-Ets-1 binding site	1145-1161	(+)	cccacAGGAagttttgg
	c-Ets-1 (p54)	1160-1176	(+)	ggcccAGGAagcaaatt
	c-Ets-1 binding site	1180-1196	(+)	ggcccAGGAagtagcaa
	GABP:GA binding protein	1352-1368	(+)	tgcgttGGAAgtgccgt
sparc	Ets-family member ELF-2 (NERF1a)	401-417	(-)	gtggcaGGAAgagtcga
	Pu.1 (Pu120)	1005-1021	(-)	ggtagtGGAAgtgggtg
	Pu.1 (Pu120)	1272-1288	(-)	gcttgaGGAAggcaagc
	c-Ets-1 (p54)	1369-1385	(-)	accccAGGAagcccaca
	Pu.1 (Pu120)	1746-1762	(+)	tcaagaGGAAgacagag
	Elk-1	1868-1884	(+)	gccaacGGAAggaatgg
nne	Pu.1 (Pu120)	69-85	(-)	tgcggaGGAAggaaaga
	Nuclear respiratory factor 2 (NRF 2)	194-210	(-)	cagcacGGAAgagacct
	GABP:GA binding protein	478-494	(+)	ccgtggGGAAggggtg
	Elk-1	628-644	(-)	gaaagaGGATgccccca

Table IV. Continued.

Differentially expressed genes	Ets binding sites	Position	Stream	Sequence
nne	c-Ets-1 binding site	752-768	(-)	tctccAGGAtgttaggt
	Pu.1 (Pu120)	1349-1365	(+)	cgaggaGGAActgggca
	ETS family member FLI	1420-1436	(+)	tggaCCGGagatccctg
lrp	Elk-1	273-289	(+)	gaaaccGGAAggccaga
	c-Ets-1 binding site	648-664	(-)	tccgcAGGAagtgggga
	Nuclear respiratory factor 2 (NRF 2)	696-712	(-)	tgcactGGAAggtagca
	Elk-1	879-895	(+)	tcgtacGGAAggaggtg
grp58	Pu.1 (Pu120)	476-492	(-)	ctgagaGGAActgaagc
	c-Ets-2 binding site	778-794	(+)	tattcAGGAaagcattt
	ETS family member FLI	1161-1177	(+)	gataCCTGaagtctgaa
enpp1	Ets-family member ELF-2 (NERF1a)	634-650	(-)	atgacaGGAAgaagtcc
	Elk-1	1324-1340	(-)	gtctccGGAAcctcggt
	Elk-1	1398-1414	(-)	aaggccGGAAgtgctgg
	GABP:GA binding protein	1531-1547	(+)	tattgtGGAAgtggatt
	c-Ets-1 (p54)	1767-1783	(-)	gggacAGGAagctctct
	Elk-1	1915-1931	(+)	cccaacGGAAggccccg
	Elk-1	1925-1941	(+)	ggccccGGAAtctgcag
	Pu.1 (Pu120)	1502-1518	(-)	gcaagaGGAAggctgaa

(-), up; (+), down.

Differentially expressed genes in DB and Neo cells selected by SSH (suppression subtractive hybridization). In addition to biological effects of Ets-DB expression on rat C6 glioma cells we wanted to assess effects on gene expression in order to find novel Ets target genes. We therefore identified differentially expressed genes in DB and Neo cells, using suppression subtractive hybridization (SSH). Differential gene expression was confirmed by quantitative RT-PCR. Database analyses revealed that 30 out of 40 analysed clones (75%) represented known rat genes with previously known functions. Three clones (7.5%) showed homology to known genes with unknown functions (Table III). Seven clones (17.5%) showed no homology to known rat sequences (Table III). Six of these clones showed similarity to EST sequences. One clone was identified as novel and showed no similarity to ESTs at all. Validation of differential gene expression was performed by quantitative RT-PCR (Lightcycler®, Roche Diagnostics, Fig. 7). Twelve of 15 verified genes (80%) showed a clear up-regulation in DB cells compared to Neo control cells (ß-actin, H3 histone and ribosomal protein S6 genes were not included into validation). These genes were translation elongation factor-1α (*eef1a1*) and gamma (*eef1b*), serine protease inhibitor *(serpine2*), osteonectin (*sparc*), thioredoxin (*txn*), ATP synthetase ß (*atp5b*), glucose regulated protein, 58kDa (*grp58*), translationally controlled tumor protein 1 (*tpt1*), ectonucleotid pyrophosphatase/phosphodiesterase 1 *(enpp1*), lactate dehydrogenase-A (*ldha*), non-neuronal enolase (*nne*) and protein tyrosine phosphatase α (*lrp*). For 3 of the 15 verified genes (20%) expression differences could not be confirmed by quantitative RT-PCR.

Ets binding sites in promotor regions of differentially expressed genes and identification of Ets family members in addition to Ets 1 in rat C6 glioma cells. Following identification of differentially expressed genes, we performed database searches for identification of Ets binding sites in the promoter regions of these genes. These analyses were performed with Transfac-MatInspector program. The list of binding sites for Ets transcription factors in the promoter regions of differentially expressed genes is shown in Table IV. The list shows that the subtracted genes all contain Ets binding sites in their promoter regions.

We next investigated if Ets family members other than Ets 1 are expressed in rat C6 glioma cells. For this puropose, we performed semi-quantitative RT-PCR analyses for Elk-1, Elf-1, Etv-1 and Fli-1 in DB, Neo and C6 cells. We found all these Ets members to be expressed in rat C6 glioma cells (Fig. 8). No differences in mRNA expression levels of the Ets members were seen between DB, Neo or C6 cells.

Discussion

In the present work, we demonstrated various suppressive effects of an Ets 1 dominant negative mutant on proliferation,

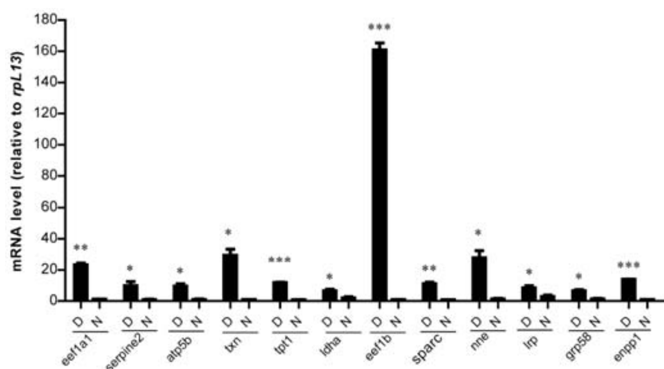

Figure 7. Verification of differential gene expression (identified through SSH) by quantitative RT-PCR. Specific primers for the actual genes were used for amplification of mRNA transcripts (cDNA) (see Table I). Normalization was performed by the housekeeping gene *rpL13* and was calculated automatically by the relative quantification software. The graphic shows mean values of mRNA (cDNA) levels of actual genes expressed in DB or Neo cells (*P<0.05, **P<0.01, ***P<0.0001, Student's t-test, DB versus Neo).

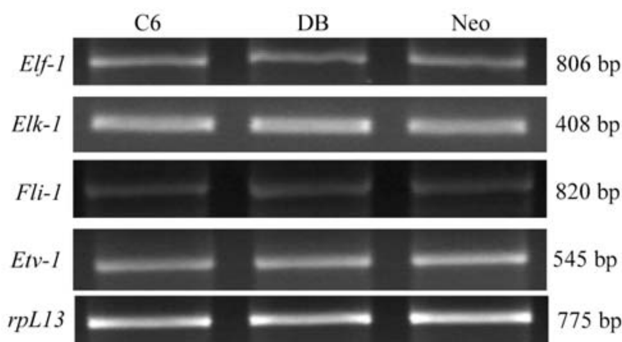

Figure 8. Identification of Ets family members other than Ets-1 in rat C6 glioma cells Specific primers for different Ets family members were used for amplification of mRNA transcripts (cDNA) by semi-quantitative RT-PCR (see Table I). The figure shows the relative expression of Ets family genes relative to the housekeeping gene *rpL13*.

anchorage-independent growth, migration, invasion, and *in vivo* tumor growth of rat C6 glioma cells. Expression of Ets-DB led to a strong reduction of proliferation. Analogous to our results, the stable transfection of a transdominant negative mutant of Ets 1 into the murine endothelial cell line MSS31 resulted in inhibition of several angiogenic properties including proliferation, migration, invasion, and tube formation in type-1 collagen gel (43). The mechanisms underlying reduced proliferation in these conditions are not yet well defined. Ets family members are important transcriptional effectors of the Ras-MAP-kinase signal transduction pathway (44,45). Some of them affect cell proliferation by regulating immediate early response genes such as *c-fos*, *c-jun* and *c-myc* and growth-associated genes encoding cell type specific growth factors, growth factor receptors, and integrins (26,46-48).

Anchorage-independent growth is a characteristic hallmark of cancer and associated with *in vivo* tumor growth. Accordingly we found an 80.2% inhibition of anchorage-independent growth of C6 DB-cells in soft agar (starting at day 11) compared to Neo and C6 control cells. The inhibition of colony formation was sustained at late time-points (day 24). Thus, the retroviral expression of Ets-DB is efficient over a long period. However, in the *in vivo* angiogenesis mouse ear model reported by Pourtier-Manzanedo and co-workers the retroviral expression of Ets-DB was less efficient after 3 weeks (33).

Our results revealed that Ets-DB expression inhibited further tumorigenic properties such as tumor cell migration and invasion. Reduced migration was more efective in fibronectin coated Boyden chambers than in collagen type I coated chambers or in uncoated plastic dishes. α5ß1-integrin is known to play an important role in adhesion of U251 glioma cells to fibronectin (49) and this role may have been affected by the present experiments (this has not been investigated). However, Kita *et al* (36) already showed that an expression of a dominant negative mutant of Ets 1 actually led to a down regulation of α5-integrin expression in U251 glioma cells and a binding site for Ets transcription factors has been identified in the promoter region of the α5-integrin gene near an AP1 binding site (50). Integrin mediated cell adhesion to components of the ECM (51) [resulting in an activation of the Ras-ERK cascade (52)] is not only important for migration but also for cell proliferation.

In our experiments, expression of Ets-DB further led to a 51% inhibition of invasion of rat C6 glioma cells through a porous membrane coated with matrigel. According to Delannoy-Courdent *et al*, invasion of murine breast carcinoma cells (MMT) was likewise inhibited by Ets-DB expressed through the same viral construct (31). In addition to invasion, we examined *mmp-9* expression in rat C6 glioma cells and found strong inhibition (87%) in DB cells compared to control cells. Matrix metalloproteinases (MMPs) not only participate in invasion but also directly or indirectly regulate other cellular events important for cancer progression, such as proliferation, differentiation, apoptosis and angiogenesis (39). Ets 1 is known to be involved in transcriptional regulation of many genes playing a role in these processes (53,54, reviewed in refs. 55-57). Besides the gene of MMP-9, those encoding MMP-3, MMP-1, and the urokinase type of plasminogen activator (uPA) are transactivated by Ets 1 (12,13, reviewed in ref. 58). Overexpression of MMP-9, in particular, correlates with aggressiveness and malignancy of gliomas (18,59,60). *In vitro* transfection of the human glioblastoma cell line SNB19 with an MMP-9 antisense construct has been shown to reduce invasion in matrigel and in spheroids (60). In the present experiments, diminished invasion of DB cells compared to control cells could therefore be linked to the strong reduction of *mmp-9* expression found in DB cells. Ets 1 is known to be involved in the regulation of MMP-9 in other cell types as well. Ets 1 antisense oligonucleotides downregulate Ets 1, MMP-9, and tenascin in bronchial fibroblasts (61) and suppression of endogenous Ets 1 via RNA interference leads to a reduction of *mmp-1* as well as *mmp-9* mRNA levels in the human breast cancer cell line MDA-MB-231 (62). MMP-9 expression is regulated via the PI 3-kinase (phosphatidylinositol 3-kinase) signaling pathway (63) and Ets transcription factors might be targets of this pathway. PI 3-kinase has already been shown to play a key role in glioma development by contributing to glioma cell survival (64). In addition to MMP-9, further

proteases regulated by Ets 1 could be involved in inhibiting invasion of Ets-DB expressing C6 cells. Among them is MMP-2, which is frequently expressed in gliomas (18) and which is transactivated by Ets 1, according to own unpublished data.

In *in vivo* experiments, we found that 4 of 10 samples of DB cells were unable to grow and form tumors on the chicken chorioallantoic membrane (CAM). In contrast, only 1 out of 10 samples of Neo control cells failed to form a tumor and 100% of C6 cells successfully formed tumors. Diminished adhesion, involving reduced integrin expression, could be responsible for decreased tumor formation of DB cells on the CAM (36). Calculation of tumor volumes of the three groups revealed an 84.5% reduced tumor volume of DB cells compared to Neo and C6 control cells. These results correlated well with those obtained for *in vitro* cell proliferation.

Using suppression subtractive hybridization (SSH), we identified a number of genes, which are differentially expressed between DB cells and Neo control cells. Differential gene expression was verified by quantitative RT-PCR. DB cells had upregulated several genes encoding products involved in cell metabolism. TXN and GRP58 are mitochondrial proteins implicated in electron transport (65,66). ATP5B is a proton-transporter playing a role in ATP-biosynthesis (67). ATP5B, as well as ENPP1, have nucleotide binding activities (68, reviewed in ref. 69). LDHA and NNE have functions in glycolysis (70,71) and eEF1A1, as well as eEF1B, are translation elongation factors (72). Besides its role in translation, eEF1A1 is up-regulated in cell death and is thought to be involved in ubiquitin-mediated protein degradation and a pro-apoptotic role in erythroleukemic (73,74), cardiomyocyte (75) and 3T3 cell lines (76). We found *eef1b* to be expressed 166.7-fold higher in DB cells compared to Neo control cells. eEF1B is part of eukaryotic translation elongation factor-1 complex and functions as the guanine nucleotide exchange factor (GEF) for eEF1A (72).

Other differentially expressed genes encode proteins that remodel the ECM. Among them are the serine protease inhibitor SERPINE 2 (77) and SPARC (78,79). SERPINE 2 also plays an important role in neurogenesis (80). We found *sparc* to be expressed 13.61-fold higher in DB cells compared to Neo controls. *In vitro* analyses showed that SPARC has a growth suppressive effect on U87 glioma cells under normal culture conditions and that it modulates migration of U87 glioma cells on brain matrix proteins (78,79). SPARC also significantly reduced glioma growth as assessed by MIB-1 proliferation index and determination of tumor volume (22). These findings are well in line with reduced migration and proliferation of DB cells compared to Neo controls in our experiments.

TPT1 (translationally controlled tumor protein, TCTP) is a calcium binding protein involved in the regulation of apoptosis and the stabilization of microtubules (81). It plays a role in cell cycle progression and malignant transformation (reviewed in ref. 82). In mammalian cells overexpression of TCTP is correlated with a reduced growth rate and a delay in cell cycle progression (83). This is in accordance with reduced proliferation of DB cells compared to Neo control cells in our experiments.

LRP has a protein tyrosine phosphatase activity and is implicated in a variety of biological processes including cell activation, differentiation, and neoplastic transformation (84). The extracellular domain of LRP inhibits adhesion of C6 glioma cells to tenascin (85). It has been hypothesized that LRP not only plays a role in the modulation of neural and glial adhesion, but also for neurit growth and signal transduction (86).

We searched for Ets transcription factor binding sites in the promoter regions of differentially expressed genes using data banks specific for transcription factor binding sites. We actually found binding sites not only for Ets 1, but also for other Ets family members, such as Elk-1 and Fli-1. In this context, it seems important that we demonstrated expression of these two Ets-family members and both Elf-1 and Etv-1 in rat C6 glioma cells. Two other studies have already shown that rat C6 glioma cells express Elk-1 (87,88). Thus the effects demonstrated in this study are probably due to a blockade of several Ets-family members including the prototype of this family, Ets 1. The fact that Ets-DB expression in C6 glioma cells led to an increased expression of many genes would imply that Ets transcription factors blocked by this strategy have trans-repressive effects. Thus, further investigations will have to define the precise roles of different Ets members for the biological effects obtained in rat C6 glioma cells by Ets DB-expression. However, in a previous study (8) we found that specific inhibition of Ets 1 by double stranded decoy oligonucleotides is sufficient to reduce proliferation and *mmp-9* expression of rat C6 glioma cells.

Our results encourage the use of Ets 1 and/or other Ets-family members as future targets in experimental glioma therapy.

Acknowledgements

DFG (Deutsche Forschungsgemeinschaft; grant no. WE 1104/8-1 and WE 1104/8-2).

References

1. Maher EA, Furnari FB, Bachoo RM, *et al*: Malignant glioma: genetics and biology of a grave matter. Genes Dev 15: 1311-1333, 2001.
2. Kondo Y, Hollingsworth EF and Kondo S: Molecular targeting for malignant gliomas (Review). Int J Oncol 24: 1101-1109, 2004.
3. Kanzawa T, Ito H, Kondo Y and Kondo S: Current and future gene therapy for malignant gliomas. J Biomed Biotechnol: 25-34, 2003.
4. Valter MM, Hugel A, Huang HJ, *et al*: Expression of the Ets-1 transcription factor in human astrocytomas is associated with Fms-like tyrosine kinase-1 (Flt-1)/vascular endothelial growth factor receptor-1 synthesis and neoangiogenesis. Cancer Res 59: 5608-5614, 1999.
5. Kitange G, Kishikawa M, Nakayama T, Naito S, Iseki M and Shibata S: Expression of the Ets-1 proto-oncogene correlates with malignant potential in human astrocytic tumors. Mod Pathol 12: 618-626, 1999.
6. Kitange G, Shibata S, Tokunaga Y, *et al*: Ets-1 transcription factor-mediated urokinase-type plasminogen activator expression and invasion in glioma cells stimulated by serum and basic fibroblast growth factors. Lab Invest 79: 407-416, 1999.
7. Fleischman LF, Holtzclaw L, Russell JT, Mavrothalassitis G and Fisher RJ: Ets-1 in astrocytes: expression and transmitter-evoked phosphorylation. Mol Cell Biol 15: 925-931, 1995.

8. Sahin A, Velten M, Pietsch T, *et al*: Inactivation of Ets 1 transcription factor by a specific decoy strategy reduces rat C6 glioma cell proliferation and mmp-9 expression. Int J Mol Med 15: 771-776, 2005.
9. Wernert N, Gilles F, Fafeur V, *et al*: Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. Cancer Res 54: 5683-5688, 1994.
10. Behrens P, Rothe M, Florin A, Wellmann A and Wernert N: Invasive properties of serous human epithelial ovarian tumors are related to Ets-1, MMP-1 and MMP-9 expression. Int J Mol Med 8: 149-154, 2001.
11. Behrens P, Rothe M, Wellmann A, Krischler J and Wernert N: The Ets-1 transcription factor is up-regulated together with MMP-1 and MMP-9 in the stroma of pre-invasive breast cancer. J Pathol 194: 43-50, 2001.
12. Iwasaka C, Tanaka K, Abe M and Sato Y: Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. J Cell Physiol 169: 522-531, 1996.
13. Wasylyk C, Gutman A, Nicholson R and Wasylyk B: The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J 10: 1127-1134, 1991.
14. Gouon V, Tucker GC, Kraus-Berthier L, Atassi G and Kieffer N: Up-regulated expression of the beta3 integrin and the 92-kDa gelatinase in human HT-144 melanoma cell tumors grown in nude mice. Int J Cancer 68: 650-662, 1996.
15. Hahne JC, Fuchs T, El Mustapha H, Okuducu AF, Bories JC and Wernert N: Expression pattern of matrix metalloproteinase and TIMP genes in fibroblasts derived from Ets-1 knock-out mice compared to wild-type mouse fibroblasts. Int J Mol Med 18: 153-159, 2006.
16. Rothhammer T, Hahne JC, Florin A, *et al*: The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. Cell Mol Life Sci 61: 118-128, 2004.
17. Hahne JC, Okuducu AF, Kaminski A, Florin A, Soncin F and Wernert N: Ets-1 expression promotes epithelial cell transformation by inducing migration, invasion and anchorage-independent growth. Oncogene 6, 2005.
18. Forsyth PA and Roa WH: Primary central nervous system tumors in adults. Curr Treat Options Neurol 1: 377-394, 1999.
19. Choe G, Park JK, Jouben-Steele L, *et al*: Active matrix metalloproteinase 9 expression is associated with primary glioblastoma subtype. Clin Cancer Res 8: 2894-2901, 2002.
20. Raithatha SA, Muzik H, Rewcastle NB, Johnston RN, Edwards DR and Forsyth PA: Localization of gelatinase-A and gelatinase-B mRNA and protein in human gliomas. Neurooncology 2: 145-150, 2000.
21. Rooprai HK, van Meter T, Rucklidge GJ, Hudson L, Everall IP and Pilkington GJ: Comparative analysis of matrix metalloproteinases by immunocytochemistry, immunohistochemistry and zymography in human primary brain tumours. Int J Oncol 13: 1153-1157, 1998.
22. Schultz C, Lemke N, Ge S, Golembieski WA and Rempel SA: Secreted protein acidic and rich in cysteine promotes glioma invasion and delays tumor growth in vivo. Cancer Res 62: 6270-6277, 2002.
23. Ohnishi T, Hiraga S, Izumoto S, Arita N, *et al*: Role of fibronectin-stimulated tumor cell migration in glioma invasion *in vivo*: clinical significance of fibronectin and fibronectin receptor expressed in human glioma tissues. Clin Exp Metastasis 16: 729-741, 1998.
24. Span PN, Manders P, Heuvel JJ, *et al*: Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer. Oncogene 21: 8506-8509, 2002.
25. Fujimoto J, Aoki I, Toyoki H, Khatun S and Tamaya T: Clinical implications of expression of ETS-1 related to angiogenesis in uterine endometrial cancers. Ann Oncol 13: 1605-1611, 2002.
26. Davidson B, Goldberg I, Gotlieb WH, Kopolovic J, Ben-Baruch G and Reich R: PEA3 is the second Ets family transcription factor involved in tumor progression in ovarian carcinoma. Clin Cancer Res 9: 1412-1419, 2003.
27. Takai N, Miyazaki T, Nishida M, Nasu K and Miyakawa I: c-Ets1 is a promising marker in epithelial ovarian cancer. Int J Mol Med 9: 287-292, 2002.
28. Sasaki H, Yukiue H, Moiriyama S, *et al*: Clinical significance of matrix metalloproteinase-7 and Ets-1 gene expression in patients with lung cancer. J Surg Res 101: 242-247, 2001.
29. Pande P, Mathur M, Shukla NK and Ralhan R: Ets-1: a plausible marker of invasive potential and lymph node metastasis in human oral squamous cell carcinomas. J Pathol 189: 40-45, 1999.
30. Tokuhara K, Ogata Y, Nakagawa M and Shirouzu K: Ets-1 expression in vascular endothelial cells as an angiogenic and prognostic factor in colorectal carcinoma. Int Surg 88: 25-33, 2003.
31. Delannoy-Courdent A, Mattot V, Fafeur V, *et al*: The expression of an Ets1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. J Cell Sci 111: 1521-1534, 1998.
32. Mattot V, Vercamer C, Soncin F, *et al*: Constitutive expression of the DNA-binding domain of Ets1 increases endothelial cell adhesion and stimulates their organization into capillary-like structures. Oncogene 19: 762-772, 2000.
33. Pourtier-Manzanedo A, Vercamer C, Van Belle E, Mattot V, Mouquet F and Vandenbunder B: Expression of an Ets-1 dominant-negative mutant perturbs normal and tumor angiogenesis in a mouse ear model. Oncogene 22: 1795-1806, 2003.
34. Markowitz D, Hesdorffer C, Ward M, Goff S and Bank A: Retroviral gene transfer using safe and efficient packaging cell lines. Ann NY Acad Sci 612: 407-414, 1990.
35. Gilles F, Raes MB, Stehelin D, Vandenbunder B and Fafeur V: The c-ets-1 proto-oncogene is a new early-response gene differentially regulated by cytokines and growth factors in human fibroblasts. Exp Cell Res 222: 370-378, 1996.
36. Kita D, Takino T, Nakada M, Takahashi T, Yamashita J and Sato H: Expression of dominant-negative form of Ets-1 suppresses fibronectin-stimulated cell adhesion and migration through down-regulation of integrin alpha5 expression in U251 glioma cell line. Cancer Res 61: 7985-7991, 2001.
37. Albini A, Iwamoto Y, Kleinman HK, *et al*: A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res 47: 3239-3245, 1987.
38. Ribatti D, Vacca A, Roncali L and Dammacco F: The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis. Int J Dev Biol 40: 1189-1197, 1996.
39. Lakka SS, Gondi CS, Yanamandra N, *et al*: Synergistic down-regulation of urokinase plasminogen activator receptor and matrix metalloproteinase-9 in SNB19 glioblastoma cells efficiently inhibits glioma cell invasion, angiogenesis, and tumor growth. Cancer Res 63: 2454-2461, 2003.
40. Quandt K, Grote K and Werner T: GenomeInspector: a new approach to detect correlation patterns of elements on genomic sequences. Comput Appl Biosci 12: 405-413, 1996.
41. Wernert N, Raes MB, Lassalle P, *et al*: c-ets1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. Am J Pathol 140: 119-127, 1992.
42. Behrens P, Mathiak M, Mangold E, *et al*: Stromal expression of invasion-promoting, matrix-degrading proteases MMP-1 and -9 and the Ets 1 transcription factor in HNPCC carcinomas and sporadic colorectal cancers. Int J Cancer 107: 183-188, 2003.
43. Nakano T, Abe M, Tanaka K, Shineha R, Satomi S and Sato Y: Angiogenesis inhibition by transdominant mutant Ets-1. J Cell Physiol 184: 255-262, 2000.
44. Sawka-Verhelle D, Escoubet-Lozach L, Fong AL, *et al*: PE-1/METS, an antiproliferative Ets repressor factor, is induced by CREB-1/CREM-1 during macrophage differentiation. J Biol Chem 279: 17772-17784, 2004.
45. Foulds CE, Nelson ML, Blaszczak AG and Graves BJ: Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. Mol Cell Biol 24: 10954-10964, 2004.
46. Albanese C, Johnson J, Watanabe G, *et al*: Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 270: 23589-23597, 1995.
47. Bassuk AG and Leiden JM: The role of Ets transcription factors in the development and function of the mammalian immune system. Adv Immunol 64: 65-104, 1997.
48. Kopp JL, Wilder PJ, Desler M, *et al*: Unique and selective effects of five Ets family members, Elf3, Ets1, Ets2, PEA3, and PU.1, on the promoter of the type II transforming growth factor-beta receptor gene. J Biol Chem 279: 19407-19420, 2004.
49. Pijuan-Thompson V and Gladson CL: Ligation of integrin alpha5beta1 is required for internalization of vitronectin by integrin alphavbeta3. J Biol Chem 272: 2736-2743, 1997.

50. Birkenmeier TM, McQuillan JJ, Boedeker ED, Argraves WS, Ruoslahti E and Dean DC: The alpha 5 beta 1 fibronectin receptor. Characterization of the alpha 5 gene promoter. J Biol Chem 266: 20544-20549, 1991.
51. Aplin AE, Short SM and Juliano RL: Anchorage-dependent regulation of the mitogen-activated protein kinase cascade by growth factors is supported by a variety of integrin alpha chains. J Biol Chem 274: 31223-31228, 1999.
52. Renshaw MW, Ren XD and Schwartz MA: Growth factor activation of MAP kinase requires cell adhesion. EMBO J 16: 5592-5599, 1997.
53. Lelievre E, Lionneton F, Soncin F and Vandenbunder B: The Ets family contains transcriptional activators and repressors involved in angiogenesis. Int J Biochem Cell Biol 33: 391-407, 2001.
54. Takai N, Miyazaki T, Fujisawa K, Nasu K and Miyakawa I: Expression of c-Ets1 is associated with malignant potential in endometrial carcinoma. Cancer 89: 2059-2067, 2000.
55. Oikawa T and Yamada T: Molecular biology of the Ets family of transcription factors. Gene 303: 11-34, 2003.
56. Sato Y: Transcription factor ETS-1 as a molecular target for angiogenesis inhibition. Hum Cell 11: 207-214, 1998.
57. Chakraborti S, Mandal M, Das S, Mandal A and Chakraborti T: Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253: 269-285, 2003.
58. Sementchenko VI and Watson DK: Ets target genes: past, present and future. Oncogene 19: 6533-6548, 2000.
59. Rao RK, Shantaram M, Aroor AR, Raja A, Davis TP and Rao A: Molecular variants of epidermal growth factor in malignant astrocytoma. Peptides 17: 179-181, 1996.
60. Kondraganti S, Mohanam S, Chintala SK, *et al*: Selective suppression of matrix metalloproteinase-9 in human glioblastoma cells by antisense gene transfer impairs glioblastoma cell invasion. Cancer Res 60: 6851-6855, 2000.
61. Nakamura Y, Esnault S, Maeda T, Kelly EA, Malter JS and Jarjour NN: Ets-1 regulates TNF-alpha-induced matrix metalloproteinase-9 and tenascin expression in primary bronchial fibroblasts. J Immunol 172: 1945-1952, 2004.
62. Dittmer J, Vetter M, Blumenthal SG, Lindemann RK and Kolbl H: Importance of ets1 proto-oncogene for breast cancer progression. Zentralbl Gynakol 126: 269-271, 2004.
63. Esteve PO, Robledo O, Potworowski EF and St-Pierre Y: Induced expression of MMP-9 in C6 glioma cells is inhibited by PDGF via a PI 3-kinase-dependent pathway. Biochem Biophys Res Commun 296: 864-869, 2002.
64. Bowers DC, Fan S, Walter KA, *et al*: Scatter factor/hepatocyte growth factor protects against cytotoxic death in human glioblastoma via phosphatidylinositol 3-kinase- and AKT-dependent pathways. Cancer Res 60: 4277-4283, 2000.
65. Murthy MS and Pande SV: A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme. Biochem J 304: 31-34, 1994.
66. Sahlin L, Holmgren A and Eriksson H: Thioredoxin messenger ribonucleic acid is regulated by estradiol in the rat uterus. Biol Reprod 57: 1056-1059, 1997.
67. Cross RL and Muller V: The evolution of A-, F-, and V-type ATP synthases and ATPases: reversals in function and changes in the H+/ATP coupling ratio. FEBS Lett 576: 1-4, 2004.
68. Antes I, Chandler D, Wang H and Oster G: The unbinding of ATP from F1-ATPase. Biophys J 85: 695-706, 2003.
69. Goding JW, Grobben B and Slegers H: Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. Biochim Biophys Acta 1638: 1-19, 2003.
70. Dutta SK and Verma M: Isolation and characterization of non-neuronal enolase (NNE) from Neurospora crassa and comparison with neuron specific enolase isolated from neuroblastoma cell line NG108. Biochem Int 15: 223-234, 1987.
71. Miyajima H, Takahashi Y and Kaneko E: Characterization of the glycolysis in lactate dehydrogenase-A deficiency. Muscle Nerve 18: 874-878, 1995.
72. Bohnsack MT, Regener K, Schwappach B, *et al*: Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. EMBO J 21: 6205-6215, 2002.
73. Kato MV, Sato H, Nagayoshi M and Ikawa Y: Upregulation of the elongation factor-1alpha gene by p53 in association with death of an erythroleukemic cell line. Blood 90: 1373-1378, 1997.
74. Kato MV: The mechanisms of death of an erythroleukemic cell line by p53: involvement of the microtubule and mitochondria. Leuk Lymphoma 33: 181-186, 1999.
75. Chen E, Proestou G, Bourbeau D and Wang E: Rapid up-regulation of peptide elongation factor EF-1alpha protein levels is an immediate early event during oxidative stress-induced apoptosis. Exp Cell Res 259: 140-148, 2000.
76. Duttaroy A, Bourbeau D, Wang XL and Wang E: Apoptosis rate can be accelerated or decelerated by overexpression or reduction of the level of elongation factor-1 alpha. Exp Cell Res 238: 168-176, 1998.
77. Bergman BL, Scott RW, Bajpai A, Watts S and Baker JB: Inhibition of tumor-cell-mediated extracellular matrix destruction by a fibroblast proteinase inhibitor, protease nexin I. Proc Natl Acad Sci USA 83: 996-1000, 1986.
78. Vadlamuri SV, Media J, Sankey SS, Nakeff A, Divine G and Rempel SA: SPARC affects glioma cell growth differently when grown on brain ECM proteins in vitro under standard versus reduced-serum stress conditions. Neurooncology 5: 244-254, 2003.
79. Rempel SA: Molecular biology of nervous system tumors. Hematol Oncol Clin North Am 15: 979-1006, 2001.
80. Meier R, Spreyer P, Ortmann R, Harel A and Monard D: Induction of glia-derived nexin after lesion of a peripheral nerve. Nature 342: 548-550, 1989.
81. Arcuri F, Papa S, Carducci A, *et al*: Translationally controlled tumor protein (TCTP) in the human prostate and prostate cancer cells: expression, distribution, and calcium binding activity. Prostate 60: 130-140, 2004.
82. Bommer UA and Thiele BJ: The translationally controlled tumour protein (TCTP). Int J Biochem Cell Biol 36: 379-385, 2004.
83. Gachet Y, Tournier S, Lee M, Lazaris-Karatzas A, Poulton T and Bommer UA: The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. J Cell Sci 112: 1257-1271, 1999.
84. Saadat M, Nakamura K, Mizuno Y, Kikuchi K and Yoshida MC: Regional localization of rat and mouse protein-tyrosine phosphatase PTP alpha/LRP gene (Ptpra) by fluorescence *in situ* hybridization. Jpn J Genet 70: 669-764, 1995.
85. Grumet M, Milev P, Sakurai T, *et al*: Interactions with tenascin and differential effects on cell adhesion of neurocan and phosphacan, two major chondroitin sulfate proteoglycans of nervous tissue. J Biol Chem 269: 12142-12146, 1994.
86. Milev P, Friedlander DR, Sakurai T, *et al*: Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. J Cell Biol 127: 1703-1715, 1994.
87. Storm SM and Khawaja XZ: Probing for drug-induced multiplex signal transduction pathways using high resolution two-dimensional gel electrophoresis: application to beta-adreno-ceptor stimulation in the rat C6 glioma cell. Brain Res Mol Brain Res 71: 50-60, 1999.
88. Chung EY, Shin SY and Lee YH: Amitriptyline induces early growth response-1 gene expression via ERK and JNK mitogen-activated protein kinase pathways in rat C6 glial cells. Neurosci Lett 422: 43-48, 2007.