Effective silencing of EGFR with RNAi demonstrates non-EGFR dependent proliferation of glioma cells

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Abstract. The epidermal growth factor receptor (EGFR, ErbB1) is frequently dysregulated in a variety of solid human tumors, including malignant glioma. EGFR expression has been associated with disease progression, resistance to standard therapies and poor survival. The application of small interfering RNAs (siRNAs) has become an effective and highly specific tool to modulate gene expression, and a wide range of oncogenes have been silenced successfully. Here we show the siRNA-mediated down-regulation of EGFR in two established glioma cell lines with different EGFR expression levels (U373 MG, LN18). The expression of EGFR mRNA and protein was down-regulated by 70-90%. However, siRNA treatment had no inhibitory effect on cell proliferation, migration and activation status of EGFR-coupled signaling cascades. In accordance with these results, gene expression analysis with microarrays revealed only small, albeit specific changes in expression patterns. In conclusion, these data indicate that the specific down-regulation of EGFR might not be sufficient for a single agent therapeutic approach in malignant glioma.

Introduction

Malignant glioma represent the most common primary brain tumors in the adult. Their anatomical localization, the infiltration of the surrounding normal brain parenchyma and the suppression of a tumor-directed immune response contribute to their highly aggressive phenotype. Despite extensive efforts to improve surgery, radiotherapy and chemotherapy, the median survival for patients with glioblastoma multiforme (GBM) averages only 14 months (1). One of the most frequently altered genes in glioblastoma is the epidermal growth factor receptor (EGFR, ErbB1, HER-1). EGFR gene amplification occurs in 40-50% of GBM, and tumors without amplification might also display an overexpression of this receptor (2-4). A common mutation (EGFRvIII), occurring in about half of the glioma with receptor amplification, confers enhanced tumorigenicity by rendering the receptor constitutively active (5). EGFR represents the prototype of class I receptor tyrosine kinases (RTKs). Besides EGFR, the ErbB family of receptors comprises three more members, ErbB2 (HER-2/NEU), ErbB3 (HER-3) and ErbB4 (HER-4). Receptor activation takes place after binding of specific ligands, e.g., epidermal growth factor (EGF), transforming growth factor α (TGFα), Amphiregulin, heparin-binding EGF (6). Ligand binding induces receptor homo- or heterodimerization and activation of the tyrosine kinase domain, which in turn phosphorylates both the receptor itself and downstream effector molecules. This results in signaling through multiple pathways, including the activation of extracellular regulated kinases 1/2 (ERK1/2), protein kinase B (PKB) and members of the signal transducer and activator of transcription (STAT) family. Ultimately, cells respond with enhanced proliferation, migration and transcriptional activity, as well as decreased apoptosis (7-9). Several new strategies have been developed to target EGFR, including monoclonal antibodies (mAbs) (10), small molecule tyrosine kinase inhibitors (TKIs) (11), ribozymes (12) and antisense oligonucleotides (AS-ODNs) (13). Whereas mAbs and TKIs function by blocking EGFR activity, ribozymes and AS-ODNs inhibit its protein biosynthesis. An alternative approach to the latter methods is the use of small interfering RNAs (siRNAs), which show superior specificity and an up to 1000-fold higher efficiency in down-regulating target gene expression compared to AS-ODNs (14). Since the initial report of siRNA-mediated gene silencing in mammalian cells (15), siRNA technology has been established as a powerful tool to regulate gene expression or determine gene function. The therapeutic potential of this new class of

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Abbreviations: EGFR, epidermal growth factor receptor; siRNA, small interfering RNA; GBM, glioblastoma multiforme

Key words: AG1478, epidermal growth factor receptor, gene expression analysis, glioma, siRNA

molecules is highlighted by several *in vitro* studies showing marked down-regulation of a variety of oncogenes and *in vivo* by initial studies demonstrating successful application of siRNAs at least in some organs (16,17).

The aim of this study was to examine the effect of siRNA-mediated 'knockdown' of EGFR on established glioma cell lines. The specific down-regulation of protein and mRNA expression was shown, and the effect on cellular proliferation, anchorage-independent growth, migration and activation status of EGFR-coupled signaling cascades (ERK1/2, PKB) was examined. In addition, microarray analyses were performed to investigate the influence of EGFR down-regulation on global gene expression.

Materials and methods

Materials. Tyrphostin AG1478 [4(3-chloranilino)-6,7-dimethoxyquinazoline] was purchased from Calbiochem (Bad Soden, Germany). Epidermal growth factor (EGF) and the anti-EGFR antibody for immunoblotting (1 μg/ml) were purchased from R&D Systems (Minneapolis, MN, USA). Biotinylated anti-EGFR for flow cytometry (1:50) was purchased from Cymbus Biotechnology (Hants, UK). Anti-ß-actin antibody (1:5000) was obtained from Sigma (Steinheim, Germany). All other primary antibodies (1:1000) were purchased from Cell Signaling Technology (Frankfurt, Germany). Secondary antibodies for immunoblotting (1:5000) were purchased from Chemicon (Hofheim, Germany). Streptavidin-Cyan5 conjugate for flow cytometry (1:50) was from Jackson Immuno Research (West Grove, USA).

Cells and cell culture. U373 MG cells were obtained from the brain tumor bank of the Department of Neurology, University of Regensburg. LN18 cells were purchased from the American Type Culture Collection (ATCC No. CRL-2610). The cells were maintained in Dulbecco's minimal essential medium (DMEM), supplemented with 10% fetal calf serum (FCS), 5% non-essential amino acids and 5% vitamin solution (all from Biochrom AG, Berlin, Germany) without antibiotics in a humidified atmosphere of 5% CO_2 at 37˚C. For ligand stimulation, cells were serum-starved for 24 h before addition of 100 ng/ml EGF.

siRNA transfection and inhibitor treatment. siRNAs were designed and synthesized by Alnylam Europe AG (Kulmbach, Germany) as duplexes of 21 nucleotides plus an asymmetrical overhang of two uridines at the 3'-end of the antisense strand. The sequence of the EGFR-specific siRNA (EGR10) corresponds to GenBank accession number NM_005228, positions 2456-2478. The control siRNA (EGR12) was identical in sequence to EGR10, but contained four mismatch point mutations at positions 4, 8, 12 and 16 of the sense strand. Transient transfections with siRNAs were performed using oligofectamine and serum-reduced optimem (both Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions and the guidelines published by Harborth *et al* (18). Unless indicated otherwise, a final siRNA concentration of 10 nM was used. To obtain maximum transfection efficiency, cells were seeded 24 h prior to transfection such that 20-30% confluency was reached on the next day. Six hours post-transfection, the medium was replaced with standard culture medium. For inhibitor treatment, AG1478 was administered in parallel to siRNA transfections, i.e., concomitantly with the replacement of transfection medium.

Immunoblotting and flow cytometry. For immunoblots, cells were washed with ice-cold PBS followed by the addition of lysis buffer [1% SDS, 50 mM Tris-Cl pH 7.4, 5 mM EDTA, 10% protease inhibitor cocktail solution (Sigma)]. The protein concentration was determined by BC Assay (Uptima, Montlucon Cedex, France). Equal amounts of protein were separated on 8% SDS gels and transferred to nitrocellulose (NC) membranes. NC membranes were blocked for 1 h in 5% non-fat dry milk in phosphate-buffered saline (PBS) with 0.1% Tween and incubated overnight with primary antibodies at 4˚C. HRP-conjugated secondary antibodies were used for labeling (1 h at room temperature). Proteins were visualized by standard chemiluminescence methods.

For flow cytometry, cells were trypsinized and washed three times with PBS. Prior to the experiments it was shown that trypsinization did not affect EGFR surface expression as compared to non-enzymatic cell detachment by cell scrapers. Cells were then incubated on ice with biotinylated primary antibody for 1 h, washed with PBS and incubated 45 min with Cyan-5-coupled streptavidin on ice. After washing with PBS, cells were resuspended in PBS and antigen detection was performed using the FACS Calibur System with CellQuest Software (BD Biosciences, San Jose, CA, USA).

Real-time PCR analysis. For quantification of specific mRNA transcripts, cell pellets were lysed and total RNA was extracted (Nucleospin RNAII, Macherey-Nagel, Düren, Germany). Reverse transcription was performed with SuperScript First-Strand Synthesis System (Invitrogen) with amounts of total RNA ranging from 800 pg to 2000 ng per reaction and random hexamer primers (25 μM). Amplification was performed using the 5'-nuclease method with pre-designed gene expression assays (TaqMan) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Proliferation assay. Cell number was determined using a crystal-violet staining assay (19). Briefly, cells grown in 96-well culture dishes were fixed with 1.25% glutardialdehyde for 20 min, washed with PBS and stained with 0.02% crystal violet solution (N-hexamethylpararosanilin x HCl in H_2O) for 30 min. The staining solution was removed by repeated washing of dishes with deionized H_2O. Nucleoprotein-bound crystal violet was solubilized by adding 70% ethanol and vigorous shaking for 2 h. Staining intensity was measured with a microplate reader (Emax precision microplate reader, Molecular Devices, Munich, Germany) at an emission wavelength of 578 nm.

Colony formation assay. Six-well plates were coated with DMEM containing 1% FCS and 1% low melting agar (Difco, Heidelberg, Germany). The next day, 10^4 cells were re-suspended in 2 ml of DMEM with 1% FCS and 0.5% low melting agar, seeded in the prepared wells and overlaid with DMEM (1% FCS). The medium was changed weekly. After

Table I. Scoring system for evaluating transcripts as significantly regulated.[a]

Parameters	Score
Paired t-test MAS 5.0 <0.1	2
Paired t-test RMA <0.1	2
No. of MAS 5.0 'change calls' (probe set is rated as decreased or increased)	1 per 'change call' (maximum 3)
Average change of signal log ratio MAS 5.0 <-0.4 or >0.4	2
Average change of signal log ratio RMA <-0.4 or >0.4	2
Total	11

[a]See Materials and methods for details.

five weeks, colonies (ε 20 μm diameter) per field (0.25 cm^2) were counted (12 fields/well; magnification x25). Each assay was done in triplicate.

Migration assay. Migration studies were performed using a modified Boyden-Chamber assay (20). Briefly, after a serum-starvation of 24 h, $2x10^5$ cells were seeded in the upper compartment of the chamber (Blind Well Chamber, Costar, Corning, USA). Fibroblast-conditioned medium was used as chemoattractant and filled in the lower compartment. Both compartments were separated by an uncoated, 8 μm Nucleopore filter (Nucleopore Track Etch Membrane, Whatman, Clifton, USA) and a layer of Matrigel (Matrigel Membrane Matrix, Becton-Dickinson, Bedford, CA, USA) on top of the filter. Cells were allowed to migrate for 24 h in a humidified atmosphere of 5% CO_2 at 37˚C. Filters with migrated cells were stained with hemalaun/eosin (Hemacolor, Merck, Darmstadt, Germany) and quantified by counting 'cells/field' (magnification x125) with five fields per filter. Each assay was done in triplicate.

Microarray analysis. Total RNA was extracted (Nucleospin RNAII) and labeled cRNA was prepared according to the standard Affymetrix protocol using 15 μg of total RNA as starting material. The labeled cRNA was hybridized to Human Genome HG-U133A chips (Affymetrix, Inc., Santa Clara, CA, USA) containing 18,400 transcripts of 14,500 genes. The array analysis was performed three times, each time comparing RNA of cells transfected with EGFR-specific siRNA with RNA of control-siRNA-transfected cells (EGR10 vs. EGR12). For each of the three array analyses, independently generated RNAs were used and the results of the three independent experiments were compared. Microarray data were analyzed by using in parallel Affymetrix Microarray Suite 5.0 (MAS 5.0) software and RMAExpress (Robust Multichip Average, www.stat.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html). Because of the relatively small changes found in the array analysis, a set of criteria was defined to classify genes as significantly regulated (Table I). Paired t-tests were performed for each probe set using logged MAS 5.0 or logged RMA signal values, respectively. 'Change call' is a MAS 5.0-defined output indicating whether a significant expression change is detectable for a given probe set in a pairwise comparison setting (MAS 5.0 baseline comparison analysis). This resulted in a maximum of three points for probe sets displaying significant change calls in all three replicate analyses. Additional points were assigned after calculating average fold change values for both the MAS 5.0 and the RMA expression measures. Since overall changes in expression patterns were relatively small, a threshold of ±0.4 was chosen for average signal log ratios (corresponding to a roughly 1.3-fold change). The maximum score added up to 11 points and transcripts with a score of 8 or above were selected for further analysis.

Results

Inhibition of EGFR protein and mRNA expression by transfection of siRNA. U373 MG and LN18 cells were transiently transfected with different concentrations (0.01-100 nM) of siRNA EGR10 and siRNA EGR12, respectively. The reduction of EGFR protein expression was determined by immunoblot (Fig. 1) and flow cytometric analysis (Fig. 2). With both methods, a specific reduction of EGFR of up to 90% in U373 MG cells and 70% in LN18 cells could be demonstrated. Protein down-regulation could be already observed at siRNA concentrations as low as 0.1 nM, concentrations above 10 nM did not enhance the effect any further. In time course experiments, down-regulation of protein was shown to last for at least five days (Fig. 2). This could also be demonstrated for EGFR mRNA, which was readily reduced to 20% after a few hours in U373 MG cells. No attenuation of this effect was observed up to 120-h post-transfection (Fig. 3).

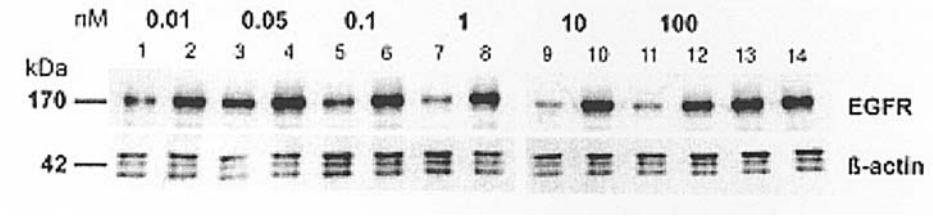

Figure 1. Down-regulation of EGFR protein expression using different siRNA concentrations in U373 MG glioma cells. U373 MG cells were transfected with different concentrations of EGFR-specific siRNA EGR10 (odd numbers) and control siRNA EGR12 (even numbers), respectively: 0.01 nM (lanes 1 and 2), 0.05 nM (lanes 3 and 4), 0.1 nM (lanes 5 and 6), 1 nM (lanes 7 and 8), 10 nM (lanes 9 and 10), 100 nM (lanes 11 and 12), oligofectamine only (lane 13), untreated control (lane 14).

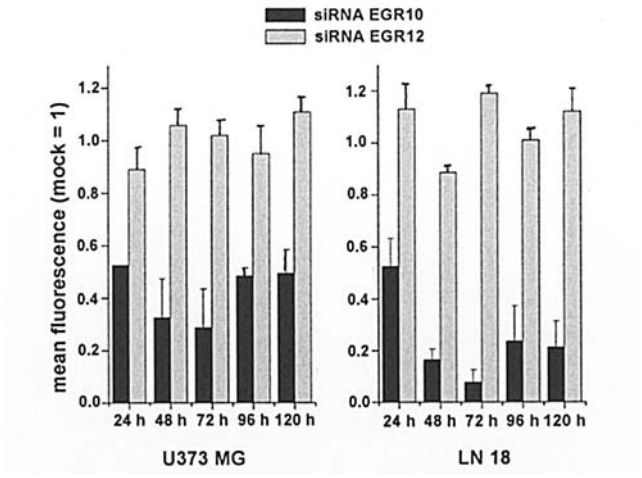

Figure 2. EGFR protein expression at different time points after transfection with 10 nM siRNA. U373 MG and LN18 cells were transfected with either 10 nM of EGFR-specific siRNA EGR10 or control siRNA EGR12, respectively. EGFR expression was determined by flow cytometry. Expression data were normalized to mock-treated controls. Bars represent mean values of four independent experiments, error bars indicate standard deviation.

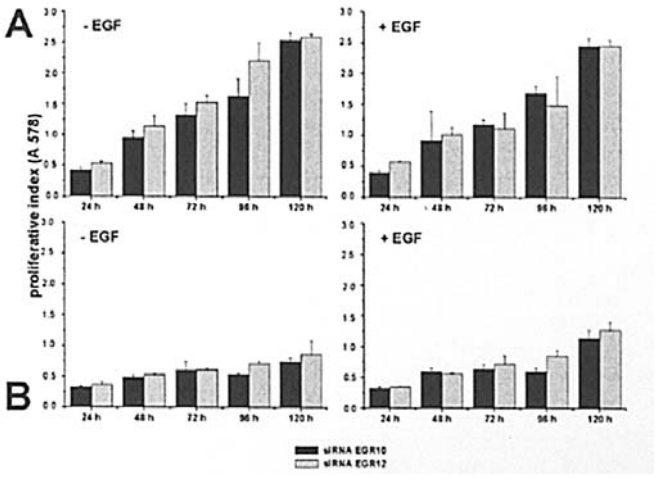

Figure 4. Proliferation of U373 MG cells after transfection with 10 nM siRNA. U373 MG cells were either transfected with 10 nM siRNA EGR10 or siRNA EGR12. (A), Proliferation in the presence of 10% FCS, with or without addition of EGF (20 ng/ml). (B), Proliferation in the absence of serum, with or without addition of EGF (20 ng/ml). Proliferative activity was determined by crystal violet staining assay (absorbance at 578 nm). Error bars indicate standard deviation.

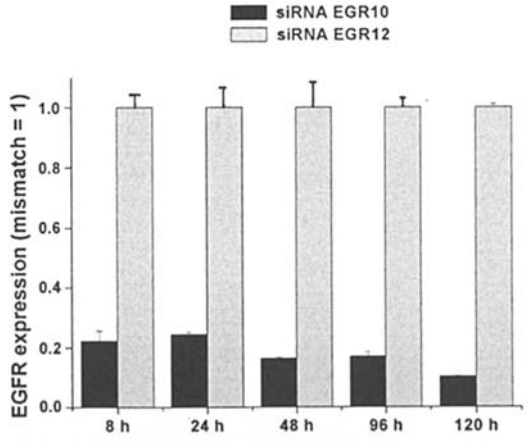

Figure 3. EGFR mRNA expression at different time points after transfection with 10 nM siRNA. U373 MG cells were transfected with either 10 nM of EGFR-specific siRNA EGR10 or control siRNA EGR12, respectively. EGFR mRNA expression was quantified by real-time PCR. Values for EGR10-transfected cells were normalized to EGR12-treated controls (EGR12=1). Bars represent mean values of three independent experiments, error bars indicate standard deviation.

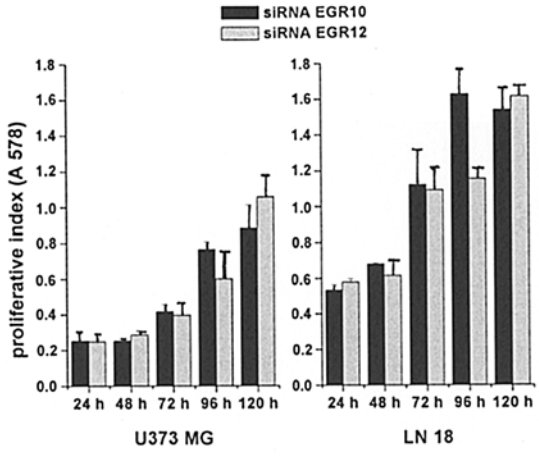

Figure 5. Proliferation of U373 MG and LN18 cells after transfection with increased concentrations of siRNA. Cells were transfected with 100 nM siRNA under serum-free conditions with addition of 20 ng/ml EGF. Proliferative activity was determined by staining with crystal violet. Bars represent mean values of three independent experiments, error bars indicate standard deviation.

Effect of EGFR down-regulation on glioma cell proliferation. To evaluate siRNA-mediated effects, the EGFR-specific tyrosine kinase inhibitor (TKI) AG1478 was used. Initial experiments had demonstrated the inhibition of cell proliferation at a concentration of 20 μM AG1478 in both cell lines under investigation (data not shown).

To analyze the effect of siRNA mediated EGFR down-regulation on U373 MG and LN18 cells, first their proliferative activity after transfection of siRNAs was tested under multiple culture conditions. The addition of epidermal growth factor (EGF) had been shown to enhance proliferation under serum-free conditions in both cell lines, indicating functional signaling through EGFR (data not shown). Cell proliferation after siRNA transfection was measured in DMEM with and without serum, with or without addition of EGF (20 ng/ml) and with different concentrations of siRNA (1-100 nM). Surprisingly, in none of the experiments an inhibiting effect on cell proliferation could be detected (Figs. 4 and 5).

To elucidate if simultaneous treatment of glioma cells with siRNAs and TKI would result in an enhanced inhibition of cell proliferation by AG1478, the TKI was added 6- and 72-h post-transfection, respectively. No additive effect was detected, inhibition of U373 MG and LN18 proliferation was observed at AG1478 concentrations identical to those in experiments without siRNA transfection (20 μM; Fig. 6).

Effect of siRNA transfection on anchorage-independent growth. To determine if the siRNA-mediated down-regulation of EGFR

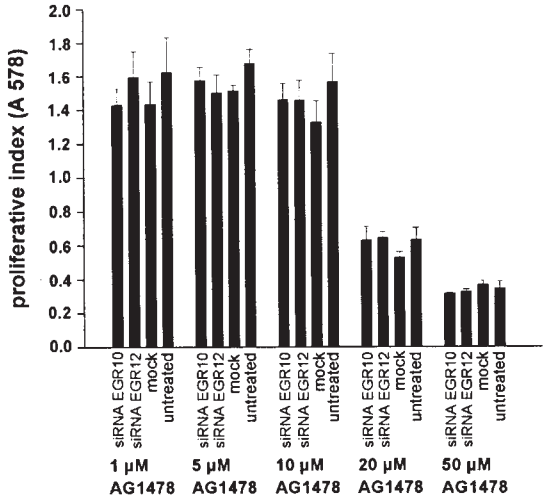

Figure 6. Proliferation of U373 MG cells after transfection of 10 nM siRNA EGR10 and simultaneous treatment with AG1478. U373 cells were transfected with 10 nM siRNA EGR10 and siRNA EGR12 or treated with oligofectamine only (mock). 6-h post-transfection, the transfection medium was replaced with DMEM (10% FCS) and increasing amounts of AG1478 were added (1-50 μM). Proliferation was measured over a period of 120 h; the chart exemplifies cell mass at 96 h. Error bars indicate standard deviation.

would have an inhibiting effect in three-dimensional, rather than in two-dimensional proliferation assays, the capacity of U373 MG cells to form colonies in soft agar was investigated. The cells were routinely transfected with 10 nM of siRNA or pretreated with 20 μM of AG1478. 72-h post-transfection, cells were seeded in wells coated with soft agar. AG1478 was added to the supernatant only once at the day of seeding. Colony formation was measured 5 weeks later by microscopically counting colonies with a diameter of ε 20 μm.

No inhibition of colony formation could be observed in samples treated with EGFR-specific siRNA EGR10 compared to EGR12-transfected or oligofectamine-treated controls. In contrast, AG1478 significantly impaired cell growth (Fig. 7).

Effect of siRNA transfection on migration of U373 MG glioma cells. Although no inhibiting effect of siRNA-mediated EGFR depletion on U373 MG or LN18 cells was detectable to this point, it remained possible that EGFR exerted its effect mainly on migratory or invasive properties rather than on proliferative activity. This was tested using a Boyden-Chamber assay where cells migrate through an artificial basement membrane containing different extracellular matrix proteins. Again, transfection with siRNA EGR10 did not cause an inhibitory effect on migration, whereas treatment with AG1478 resulted in a marked decrease in the number of migrated cells (Fig. 8).

Effect of siRNA transfection on EGFR-coupled PKB and ERK1/2 signaling pathways. Since EGFR stimulation by EGF results in activation of two main signaling pathways, by phosphorylation of extracellular regulated kinases (ERK1/2) and protein kinase B (PKB), the activation status of these molecules after treatment with EGF-specific siRNA or AG1478 was investigated. Whole cell lysates were subjected to immunoblot analysis and the amount of phosphorylated PKB and ERK1/2 was determined. Additionally, the expression of EGFR protein was shown to verify the specific down-regulation by siRNA EGR10. Stimulation with EGF resulted in further reduction of receptor by endocytosis and degradation after 15 min (Fig. 9A). Immunoblot analysis revealed that in both cell lines PKB is constitutively activated (Fig. 9C). Accordingly, treatment with EGF did not result in an increase in phosphorylated PKB, whereas activation of ERK1/2 was

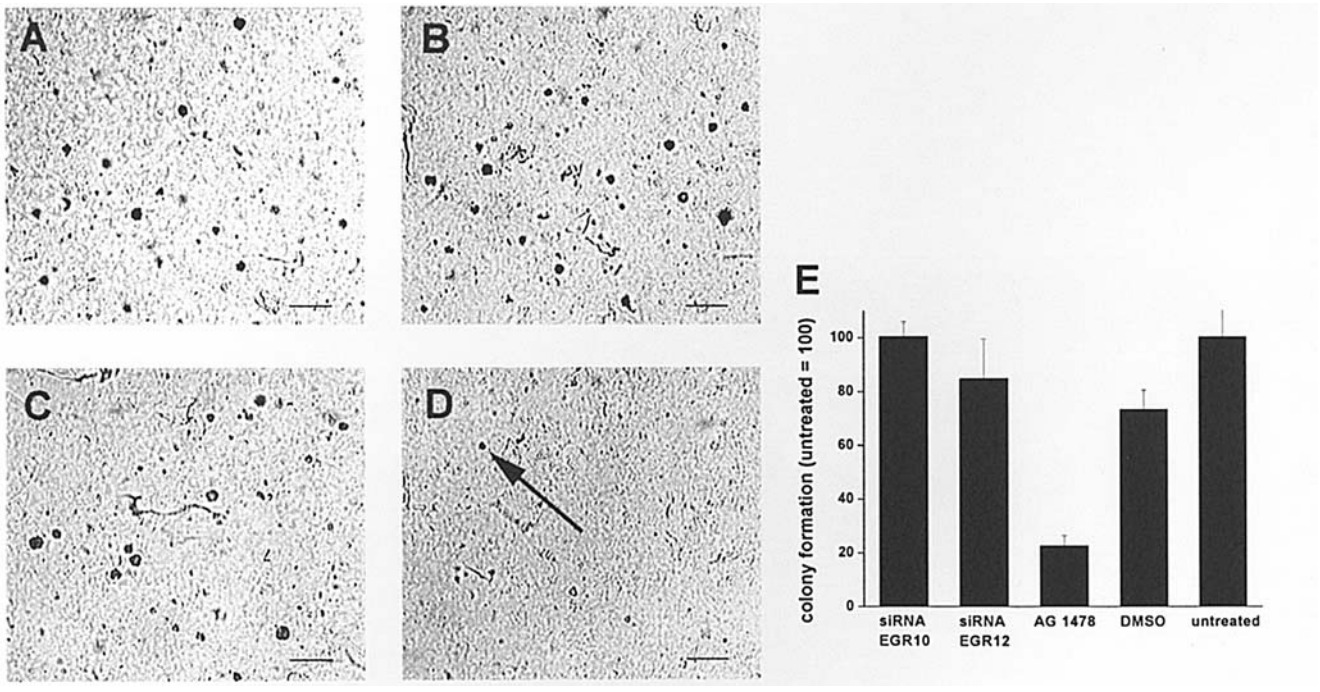

Figure 7. Colony formation of U373 MG cells. U373 cells were either transfected with 10 nM of control siRNA EGR12 (A) or siRNA EGR10 (B) or treated with DMSO (C) and 20 μM AG1478 (D), respectively. The arrow in (D) indicates a single small colony; scale bar, 250 μm. In (E), colony number is depicted relative to untreated controls. Bars represent mean values of three independent experiments, error bars indicate standard deviation.

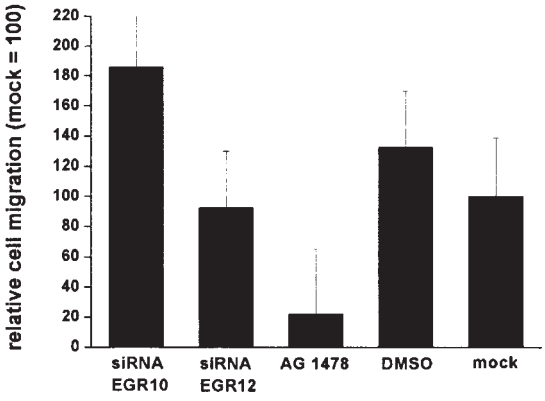

Figure 8. Migration of U373 MG cells. U373 MG cells were transfected with siRNAs EGR10, EGR12 or treated with DMSO or AG1478 (20 μM), respectively. 72-h post-transfection, migration was determined by using a modified Boyden-Chamber assay. Each assay was performed in triplicate and the experiment was reproduced twice. Error bars represent standard deviations of the measurements from nine independent samples.

markedly enhanced in both cell lines. Similar to the results obtained with proliferation and migration assays, no inhibitory influence on phosphorylation of ERK1/2 and PKB could be observed with siRNA EGR10 (Fig. 9C and G). In contrast, AG1478 treatment caused a significant reduction in phospho-ERK and phospho-PKB (Fig. 9E and I).

Effect of siRNA transfection on gene expression in U373 MG cells. In order to get a more comprehensive understanding of the siRNA treatment effects on the molecular level, we performed gene expression analysis on Affymetrix U133A microarrays. U373 MG cells were transfected with 10 nM of siRNA EGR10 or EGR12, respectively. 72-h post-transfection, cells were serum-starved for 24 h and then stimulated with 100 ng/ml EGF for 6 h. The experiment was independently performed three times for a total of six arrays (triplicates for siRNA EGR10 and siRNA EGR12 transfection, respectively). Since experiments from different time points formed a tighter cluster than experimental groups (data not shown), a pairwise

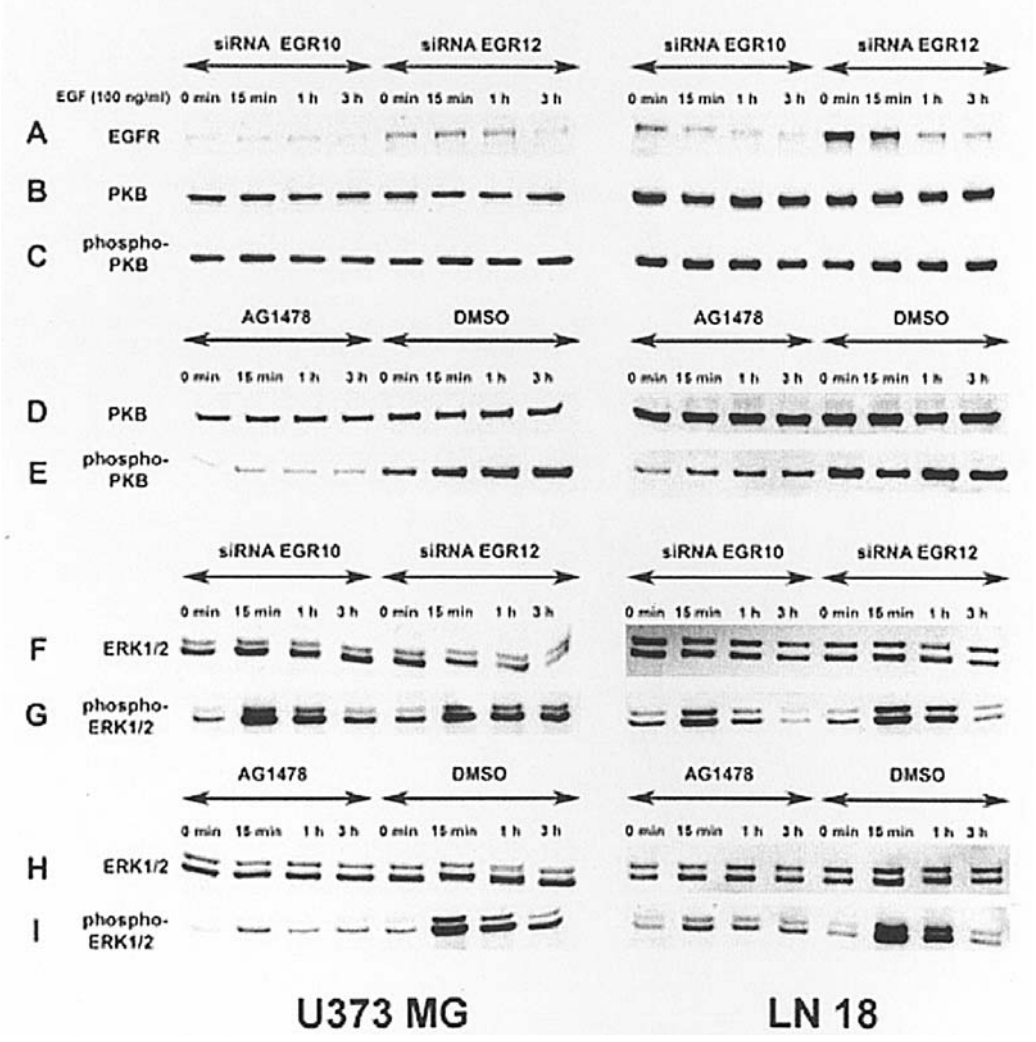

Figure 9. Activation status of protein kinase B (PKB/AKT) and mitogen-activated protein kinases (ERK1/2) in U373 MG and LN18 glioma cells. U373 MG and LN18 cells were transfected with siRNA EGR10 or siRNA EGR12 (10 nM f.c.) or treated with DMSO or AG1478 (20 μM f.c.). 72-h post-transfection, cells were serum starved for 24 h and then EGF (100 ng/ml) was added. At the indicated time points, whole cell lysates were prepared and samples were subjected to immunoblot analysis. Activation of PKB and ERK1/2 was detected by phospho-specific antibodies, total PKB and ERK1/2 expression were used as controls. (A), EGFR-expression after transfection with siRNA EGR10 or EGR12. (B), Unphosphorylated PKB after transfection with siRNAs EGR10, EGR12. (C), Phospho-PKB after transfection with siRNAs EGR10, EGR12. (D), Unphosphorylated PKB after treatment with AG1478 or DMSO. (E), Phospho-PKB after treatment with AG1478, DMSO. (F), Unphosphorylated ERK1/2 after transfection with siRNAs EGR10, EGR12. (G), Phospho-ERK1/2 after transfection with siRNAs EGR10, EGR12. (H), Unphosphorylated ERK1/2 after treatment with AG1478, DMSO. (I), Phospho-ERK1/2 after treatment with AG1478, DMSO.

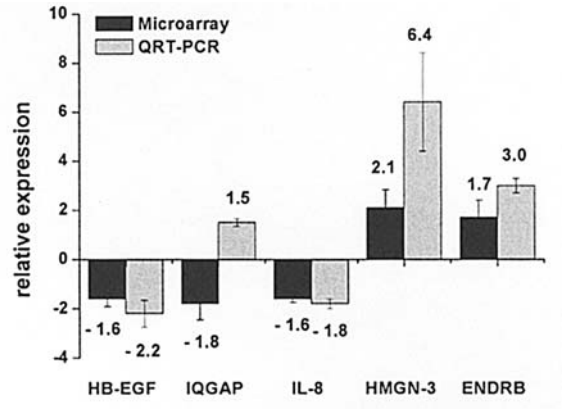

Figure 10. Validation of microarray data by quantitative real-time PCR. Three down-regulated and two up-regulated genes were randomly chosen for confirmation of microarray results by TaqMan real-time PCR as described in Materials and methods. Values for each gene were normalized to expression levels of GAPDH and ratios comparing expression in siRNA EGR10 and control siRNA EGR12 transfected cells were calculated. Bars represent mean values of three PCR experiments and three independent microarray analyses, respectively. Error bars indicate standard deviation.

comparison was conducted. Transcripts which were undetectable in all six arrays (i.e., which had 'Absent' calls assigned to them by the MAS 5.0 software) were eliminated from data analysis; all other transcripts were analyzed according to the criteria defined in Table I. Using this set of parameters, a total of 103 transcripts representing 95 genes were found to be reproducibly regulated, with up-regulation of 44 genes and down-regulation of 51 genes (Table II).

The overall difference in expression patterns between siRNA EGR10-transfected and control-transfected cells was only diminutive. EGFR itself represented the most prominently altered transcript with a 5-fold change (RMA value), equivalent to a down-regulation of 80%. This was in very good accordance with the results obtained in the real-time PCR experiments, indicating the general validity of the microarray analyses. That is further supported by the identification of genes whose transcription is coupled to EGFR-signaling, e.g., the EGFR-specific ligand heparin-binding EGF (HB-EGF, DTR) (21), or urokinase plasminogen activator receptor (UPAR, PLAUR) (22), which is able to transactivate EGFR (23) and is up-regulated in highly malignant glioma (24). Some of the down-regulated transcripts play a role in cellular migration and invasion (DDEF1, ESDN, FN, HMGA1, IQGAP, ITGA7, PTHLH, TIAM1, TNC, UPAR), others are proliferation-promoting (FOSL2, FOXM1B, MAP2K3, TCF7L2) or anti-apoptotic (BCL2L, MCL1). The induced transcripts form a highly heterogeneous group. In addition to a few proapoptotic genes (TNFRSF10B, PCD2), DNA- and RNA-binding transcripts were up-regulated (ANKT, SCA1, SMARCA1 and PAIP1, SSB, THOC2, SFRS7, respectively), as well as some genes coding for proteins associated with the extracellular matrix (COL XI 1A, ALCAM, IGSF4, EFEMP1).

Validation of microarray data by quantitative real-time PCR. To corroborate the results obtained in the microarray experiments, the expression levels of five exemplary genes (DTR, IQGAP, IL-8, HMGN3, EDNRB) were determined by quantitative real-time PCR (QRT-PCR) with newly generated RNA samples, i.e., a different cDNA from those used in the three microarray experiments was used for the QRT-PCR. For 4 of the 5 genes, the observed regulations were confirmed both qualitatively and quantitatively; only the down-regulation of IQGAP was irreproducible (Fig. 10). This remarkable degree of congruence between the two methods validates our scoring approach and suggests that the majority of the 95 genes listed in Table I are indeed affected by the EGFR siRNA treatment.

Discussion

In this report we have examined the effect of siRNA-mediated, specific down-regulation of EGFR on human malignant glioma cell lines U373 MG and LN18. The substantial reduction of about 80% of EGFR protein and mRNA expression was demonstrated (Figs. 1-3 and 9A). However, no inhibitory effect on cell proliferation could be observed (Figs. 4-7). Likewise, cell cycle analysis by flow cytometry showed no reduction in S-phase fraction or increase in the G1- or G2-arrested cell fraction (data not shown). Since it has been repeatedly demonstrated that EGF might enhance cellular responses like migration and invasion rather than stimulating proliferative activity (25,26), migration of U373 MG cells after siRNA transfection was investigated (Fig. 9). Again, no inhibitory effect was detectable. Similar results were obtained by determining the phosphorylation status of members of downstream signaling cascades (PKB, ERK1/2, Fig. 9). In contrast to these findings, treatment of U373 MG and LN18 cells with the tyrphostin AG1478 caused inhibition of proliferation and migration and reduced activation of effector molecules. AG1478 is known as a selective inhibitor of EGFR, with up to 10^4-fold higher IC_{50} values for other tyrosine kinases (27; Calbiochem product data sheet) and it is widely used in glioma *in vitro* studies (28-30). Nevertheless, Lipson *et al* reported unspecific inhibition of platelet-derived growth factor receptor (PDGFR) at concentrations above 10 μM AG1478 (31). AG1478 belongs to the same class of synthetic tyrosine kinase inhibitors as Gefitinib ('Iressa', ZD1839), the first FDA-approved EGFR-targeting drug (32). As for AG1478, no specifications exist concerning the influence of Gefitinib on receptor tyrosine kinases (RTK's) besides EGFR. However, investigations with fibroblasts and endothelial cells showed blocking of insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) induced proliferation at concentrations similar to those at which EGF-induced proliferation was inhibited (32). Since these values are within the range of clinically relevant plasma concentrations of Gefitinib (32), the observed cytotoxic effects after treatment might result from concomitant inhibition of EGFR and other receptor tyrosine kinases like IGF1R and PDGFR. Since AG1478 and Gefitinib belong to the same class of substances and a high concentration (20 μM) of AG1478 is needed to induce inhibitory effects in U373 MG and LN18 glioma cells, unspecific effects similar to those seen with Gefitinib can not be excluded.

In accordance with our previous results, transfection of U373 MG cells with siRNA EGR10 resulted in marked down-regulation of EGFR transcript, but only a marginal effect

Table II. Expression of 95 genes regulated in U373 MG cells after transfection with EGFR-specific siRNA EGR10.[a]

Gene symbol	Title	Average fold change (RMA/MAS)	p-value (RMA/MAS)	Score
EGFR	Epidermal growth factor receptor	-4.98/-4.29	0.012/0.008	11
ARHI	Ras homolog gene family member 1	-1.97/-1.95	0.037/0.076	10
ALDH1A3	Aldehyd dehydrogenase family member A3	-1.79/-1.91	0.038/0.039	11
IQGAP1	IQ motif containing GTPase activating protein 1	-1.77/-2.0	0.005/0.001	10
DUSP5	Dual specifity phosphatase 5	-1.75/-1.74	0.048/0.004	10
ESDN	Endothelial and smooth muscle cell-derived neuropilin-like protein	-1.68/-2.19	0.196/0.067	8
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	-1.66/-1.41	0.095/0.101	8
PTPN12	Protein tyrosine phosphatase, non-receptor type 12	-1.65/-1.55	0.08/0.066	10
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	-1.6/-1.41	0.175/0.1	9
IL8	Interleukin 8	-1.59/-1.41	0.036/0.09	10
P37NB	37 kDa leucine rich repeat protein	-1.58/-1.7	0.023/0.194	8
PLXNA1	Plexin A1	-1.58/-1.87	0.033/0.001	11
HSMPP8	M-phase phosphoprotein mpp8	-1.56/-1.48	0.007/0.024	10
RIS1	Ras-induced senescence 1	-1.55/-2.05	0.039/0.101	8
DTR	Diphteria toxin receptor (heparin-binding epidermal growth factor-like growth factor, HB-EGF)	-1.55/-1.82	0.21/0.01	11
LIF	Leukemia-inhibitory factor	-1.49/-1.59	0.027/0.062	9
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	-1.49/-1.38	0.147/0.042	8
SYBL1	Synaptobrevin-like 1	-1.49/-1.48	0.113/0.091	9
PHLDA1	Pleckstrin homology-like domain, family A member 1	-1.48/-2.52	0.101/0.099	8
DDEF1	Development and differentiation enhancing factor 1	-1.48/-1.52	0.015/0.074	11
FOSL2	FOS-like antigen 2	-1.47/-1.35	0.096/0.052	10
PTHLH	Parathyroid hormone-like hormone	-1.45/-1.35	0.074/0.045	10
MAP2K3	Mitogen-activated protein kinase kinase 3	-1.43/-1.52	0.047/0.121	9
PLAUR	Plasminogen activator, urokinase receptor	-1.43/-1.32	0.012/0.047	10
FN1	Fibronectin 1	-1.42/-1.35	0.045/0.019	10
TCF7L2	Transcription factor 7-like 2/TCF 4	-1.42/-1.26	0.058/0.067	8
SPRY4	Sprouty homolog 4 (*Drosophila*)	-1.41/-1.52	0.051/0.072	10
HMGA1	High mobility group AT-hook 1	-1.41/-1.7	0.009/0.206	9
SFRP1	Secreted frizzled-related protein 1	-1.41/-1.38	0.05/0.045	10
FOXM1	Forkhead box M1	-1.4/-1.38	0.255/0.088	8
RBPMS	RNA binding protein with multiple splicing	-1.38/-1.41	0.016/0.065	8
ITGA7	Integrin alpha 7	-1.38/-1.59	0.183/0.094	8
CPA4	Carboxypeptidase A4	-1.37/-1.45	0.199/0.036	8
SLC5A3	Solute carrier family 5 (inositol transporters) member 3	-1.36/-1.29	0.003/0.03	8
SHC1	SHC (Src homology 2 domain containing transforming protein)	-1.35/-1.29	0.026/0.029	8
DCTN1	Dynactin 1	-1.35/-1.55	0.169/0.079	8
SAC2	Sac domain-containing inositol phosphatase 2	-1.35/-1.35	0.216/0.089	8
BCL2L1	BCL2-like 1	-1.35/-1.48	0.053/0.076	9
TIAM1	T-cell lymphoma invasion and metastasis 1	-1.34/-1.32	0.025/0.075	10
PITPN	Phosphatidylinositol transfer protein	-1.34/-1.23	0.014/0.07	8
DUSP6	Dual specifity phosphatase 6	-1.34/-2.09	0.006/0.068	11
p66alpha	p66alpha	-1.29/-1.35	0.018/0.086	8
TNC	Tenascin C	-1.27/-1.32	0.04/0.026	8
FLJ12750		-1.27/-1.41	0.006/0.048	8
RAB31	RAB31 member RAS oncogene family	-1.25/-1.32	0.05/0.044	8
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	-1.24/-1.32	0.053/0.01	8
MET	Met proto-oncogene (hepatocyte growth factor receptor)	-1.24/-1.35	0.051/0.079	8

Table II. Continued.

Gene symbol	Title	Average fold change (RMA/MAS)	p-value (RMA/MAS)	Score
INPP5A	Inositol phosphatase-5-phosphatase, 40 kDa	-1.23/-1.32	0.001/0.047	8
NR1D1	Nuclear receptor subfamily 1, group D, member 1	-1.22/-2.09	0.011/0.035	8
P4HA2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase	1.25/1.35	0.058/0.072	8
NUDT9	Nudix (nucleoside diphosphate linked moiety X) type motif 9	1.32/1.41	0.221/0.063	8
ANXA1	Annexin A1	1.34/1.38	0.011/0.004	10
AKR1C1	Aldo-keto reductase family 1 member C1	1.34/1.45	0.081/0.153	8
SFRS7	Splicing factor, arginine/serine-rich 7, 35 kDa	1.34/1.35	0.107/0.038	8
SMARCA1	SW1/SNF related, matrix-associated, actin-dependent regulator of chromatin, subfamily A member 1	1.35/2.7	0.014/0.046	8
DUT	DUTP pyrophosphatase	1.35/1.45	0.052/0.015	10
ANKT	Nucleolar protein 1	1.35/1.38	0.002/0.056	10
MYLK	Myosin light polypeptide kinase	1.36/1.45	0.074/0.032	10
NUP88	Nucleoporin 88 kDa	1.38/1.35	0.099/0.089	9
HEBP2	Heme binding protein 2	1.41/1.35	0.05/0.026	9
CREG	Cellular repressor of E1A-stimulated genes	1.41/1.32	0.07/0.096	10
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1.41/1.38	0.004/0.01	11
FLJ12671		1.41/1.48	0.014/0.028	9
ALCAM	Activated leukocyte cell adhesion molecule	1.41/1.41	0.002/0.056	9
SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A, member 3	1.42/1.32	0.388/0.012	8
PDCD2	Programmed cell death 2	1.42/1.41	0.074/0.218	8
CSPG6	Chondroitin sulfate proteoglycan 6 (Bamacan)	1.42/1.48	0.219/0.096	8
RARG-1	Retinoic acid repressible protein	1.44/1.32	0.021/0.044	8
OAZ1	Ornithine decarboxylase antizyme1	1.44/1.38	0.026/0.02	10
HBLD2	HESB-like domain containing 2	1.45/1.45	0.082/0.105	8
C6ORF111	Chromosome 6 open reading frame 111	1.45/1.48	0.025/0.156	9
ARL5	ADP-ribosylation factor-like 5	1.46/1.45	0.036/0.115	8
DMN	Desmuslin	1.46/1.38	0.074/0.141	8
GPC4	Glypican 4	1.46/1.35	0.068/0.07	10
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	1.47/1.45	0.017/0.069	11
SSB	Sjogren syndrome antigen B (autoantigen La)	1.48/1.52	0.086/0.039	11
SCRG1	Scrapie responsive protein 1	1.48/1.41	0.097/0.138	8
IGSF4	Immunoglobulin superfamily, member 4	1.49/1.45	0.018/0.023	9
THOC2	THO complex	1.53/1.35	0.016/0.026	10
TOP2A	Topoisomerase (DNA) II alpha 170 kDa	1.54/1.55	0.069/0.047	11
SCA1	Spinocerebellar ataxia 1	1.58/1.7	0.034/0.069	11
S164	S 164 protein	1.61/1.62	0.074/0.055	10
MYCBP	c-myc binding protein	1.64/1.66	0.019/0.161	9
AKR1B10	Aldo-keto-reductase family 1, member B10	1.65/1.59	0.1/0.056	8
EDNRB	Endothelin receptor type B	1.65/1.48	0.002/0.084	11
CUTL1	Cut-like 1, CCAAT displacement protein (*Drosophila*)	1.67/1.59	0.057/0.038	11
CDC10	CDC10 cell division cycle 10 homolog	1.69/1.59	0.011/0.022	11
COL11A1	Collagen type XI, alpha 1	1.69/1.74	0.088/0.121	8
FABP7	Fatty-acid binding protein 7, brain	1.74/1.66	0.035/0.104	9
ARL6IP	ADP-ribosylation factor-like 6 interacting protein	1.9/1.74	0.09/0.125	8
PAIP1	Polyadenylate binding protein interacting protein 1	1.93/2.0	0.068/0.049	11
HMGN3	High mobility group nucleosomal binding domain 3	2.05/2.0	0.081/0.142	9

[a]Genes are listed in ascending order from most down-regulated to strongest up-regulated transcript. See Materials and methods and Table I caption for details on the scoring system.

on overall gene expression was observed in microarray studies. However, the results strongly suggest that the detected differences between the EGR10 and the control group were indeed attributable to altered EGFR transcription, since several differentially regulated genes are coupled to EGFR-dependent signaling. For example, expression of DTR, PTHLH, UPAR and IL-8 has been shown to be induced by EGF (21,22,33,34). Additionally, DDEF1 is involved in EGFR recycling (35), the invasion-promoting TNC might function as membrane-bound EGFR ligand (36) and one of the main adaptor proteins for EGFR, SHC1, was also slightly repressed. The activation of transcription factor TCF4 is also coupled to EGF (37). FOXM1B, another repressed transcription factor has been shown to be up-regulated specifically in highly malignant forms of glioma (38,39). In agreement with reports postulating a tumor-promoting effect of EGFR through enhancement of migration rather than proliferation, quite a few of the 51 down-regulated transcripts play a role in cellular invasion and migration. However, these small, albeit reproducible, effects on the transcript level do not translate into changes of corresponding cellular parameters, like adhesion to matrix proteins (data not shown) or migration through an artificial basement membrane (Fig. 8). It is possible that the small changes in expression do not result in sufficiently reduced amounts of protein. Transcriptional up-regulation was found for 41 genes. As opposed to the group of repressed transcripts, no significant functional relation between the induced transcripts could be established. Some of them are related to the transcription factor myc, e.g., MYCBP, which enhances transcriptional activity of myc (40), the RNA-binding SCA1 (41), and ARL6IP, which belongs to the ARF-family of GTP-binding proteins (42). Of note, EDNRB, a G-protein-coupled receptor (GPCR), was among the most strongly up-regulated transcripts. EDNRB is known to be highly expressed in glioma cells, where binding of its ligand endothelin-1 (ET-1) mediates proangiogenetic and antiapoptotic stimuli (43). Since EDNRB is known to transactivate EGFR (44), one could speculate about a compensation mechanism for the down-regulation of EGFR by induction of EDNRB. The results obtained by the microarray analyses were confirmed by quantitative real-time PCR. For four of five randomly chosen genes, the results of the QRT-PCR were in agreement with the microarray results, although the expression differences were generally more pronounced in the PCR, an observation also made by other investigators (45). For one transcript (IQGAP), the down-regulation found in the microarray could not be confirmed. Since for the QRT-PCR analysis a new transfection experiment was conducted, the reproducibility of four genes with relatively low expression differences strongly argues for the general validity of the microarray results.

Although altered EGFR expression is a hallmark of glioma pathogenesis, only very limited information is available on the application of EGFR-targeting drugs like small tyrosine kinase inhibitors (e.g., Gefitinib, Erlotinib) and antibodies (e.g., Cetuximab) in these tumors. One study of Cetuximab in glioma cells showed that application of the substance caused cell cycle arrest and enhanced apoptosis only in cell lines with EGFR gene amplification (46). Halatsch *et al* (47) report that sensitivity to Erlotinib in glioma cells is independent of EGFR expression level and significantly higher concentrations of inhibitor were needed to block proliferation compared to other tumor cell lines (e.g., colon carcinoma, head and neck tumors). Little more literature is available on the use of Gefitinib in glioma. While apoptosis was enhanced at simultaneous Gefitinib treatment and radiation (48), no inhibitory effect on proliferation was seen with Gefitinib alone (49). In a phase II clinical study, no objective response to Gefitinib in patients with recurrent glioblastoma multiforme could be demonstrated (50). A hint to what might cause these weak effects could be deduced from the work of Li *et al* (51), who demonstrated effective inhibition of EGFR phosphorylation at low concentrations of Gefitinib, but no concomitant effect on EGFR-coupled signaling. Much higher concentrations were needed to inhibit PKB and ERK1/2, although this effect could not be explained and might perhaps be attributed to unspecific effects. The undiminished signaling through PKB and ERK1/2 might be due to their redundant activation by other receptor tyrosine kinases, for example through members of the ErbB family, e.g., ErbB2. By immunoblot analysis, U373 MG and LN18 cells were shown to express ErbB2 (data not shown), with especially high expression in the U373 MG line. However, upon stimulation with EGF, no phosphorylation of ErbB2 could be detected (data not shown). Likewise, no inhibition of proliferation could be achieved by blocking ErbB2 with the specific TKI AG825, and concomitant application of AG825 with AG1478 or with siRNA EGR10 did not result in enhanced inhibition of proliferation (data not shown). These results indicate that an assumed signaling through the ErbB2 portion of EGFR-ErbB2 heterodimers or through ErbB2 homodimers is not responsible for the observed resistance of the investigated glioma cells to EGFR inhibition. This is in accordance with results of Li *et al* who found that simultaneous inhibition of EGFR and ErbB2 had no additive effect in reducing PKB and ERK1/2 activation (51). U373 MG cells were shown to have no detectable expression of ErbB3 and ErbB4 (52), so it can be excluded that one of the ErbB-receptors is compensating for the decreased EGFR. Increased signaling through IFG1R has been implicated in mediating resistance to AG1478 in glioma cell lines (28). Since U373 MG cells express IGF1R (LN18 show only very weak expression; data not shown), this might represent a mechanism by which these cells make up for diminished EGFR signaling. Nevertheless, it can not be ruled out that the EGFR remaining after siRNA transfection is still sufficient to mediate signaling. However, this is contrary to assumptions made by two different research groups, who proposed a threshold for functional EGFR signaling, since they observed inhibition of proliferation despite a residual amount of EGFR protein of up to 30% after transfection with siRNAs (53,54). Taken together, the results presented in this study demonstrate that the specific, siRNA-mediated down-regulation of EGFR has no impact on the malignant phenotype of the investigated glioma cells.

First results of clinical studies on the application of EGFR-targeting therapeutics in human glioma are now being published (50). Further advanced trials in other types of tumors yielded rather disappointing results, which has been attributed to wrong dosage or combination of drugs and to a lack of patient selection in particular (55). This notion is further supported by recent findings showing that responsiveness to Gefitinib in non-small cell lung cancer depends on mutations

in the EGFR gene (56,57). The high specificity of siRNAs, as verified here by gene expression profiling, makes them a valuable tool to validate potential therapeutic targets. Regarding our *in vitro* results and taking into account recent observations on the lack of prognostic significance of EGFR status in glioma patients (58), the role of EGFR as a single target for therapeutic approaches should be reconsidered. By defining subsets of glioma susceptible to EGFR inhibition and through combination with other treatment options, the application of siRNA might yet prove useful as a highly specific tool to modulate the tumor-promoting effects mediated by this receptor.

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