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ARTICLE

Herpes Simplex Virus 1 Amplicon Vector-Mediated siRNA Targeting Epidermal Growth Factor Receptor Inhibits Growth of Human Glioma Cells *in Vivo*

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In primary glioblastomas and other tumor types, the epidermal growth factor receptor (EGFR) is frequently observed with alterations, such as amplification, structural rearrangements, or overexpression of the gene, suggesting an important role in glial tumorigenesis and progression. In this study, we investigated whether posttranscriptional gene silencing by vector-mediated RNAi to inhibit EGFR expression can reduce the growth of cultured human gli36 glioma cells. To "knock down" EGFR expression, we have created HSV-1-based amplicons that contain the RNA polymerase III-dependent H1 promoter to express double-stranded hairpin RNA directed against EGFR at two different locations (pHSVsiEGFR I and pHSVsiEGFR II). We demonstrate that both pHSVsiEGFR I and pHSVsiEGFR II mediated knock-down of transiently transfected full-length EGFR or endogenous EGFR in a dose-dependent manner. The knock-down of EGFR resulted in the growth inhibition of human glioblastoma (gli36-luc) cells both in culture and in athymic mice *in vivo*. Cell cycle analysis and annexin V staining revealed that siRNA-mediated suppression of EGFR induced apoptosis. Overall HSV-1 amplicons can mediate efficient and specific posttranscriptional gene silencing.

Key Words: EGFR, HSV-1 amplicons, tumor growth, posttranscriptional gene silencing

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and most aggressive brain tumor. With an incidence of 3–4/100,000, GBM constitutes approximately 20–25% of all primary intracranial neoplasms [1,2]. Overall effectiveness of standard treatment (surgery, radiation, chemotherapy) is limited as these tumors invariably recur. The mean life expectancy is only 1–2 years after diagnosis [3]. Together with all brain tumors, GBM constitutes the second most common cause of death from a neurological disorder after stroke [3,4].

A complex series of molecular changes occurs in the development of GBM including loss, mutation, or hypermethylation of tumor suppressor and cell cycle-regulating genes (e.g. TP53, CDKN2A/p16, p14ARF, PTEN), as well as activation or amplification of oncogenes and growth factors and/or their receptors (e.g., MDM2, CDK4, cyclins D1 and D3, EGFR, PDGFR, and TGF-β) [5,6]. During progression from low-grade astrocytoma (WHO grade II) to anaplastic astrocytoma (WHO grade III) and to GBM (WHO grade IV), a step-wise accumulation of genetic alterations may occur [7]. These so-called secondary glioblastomas can be distinguished from primary *de novo* glioblastomas on the basis of molecular genetic findings. In primary GBM, overexpression or amplification of the epidermal growth factor receptor (EGFR) plays a central role in glial tumorigenesis and is found in up to 50% of all cases. These EGFR alterations have also been described in a number of further premalignant and malignant diseases such as mammary and hepatic carcinoma [8].

EGFR is a transmembrane glycoprotein of 170 kDa and is composed of an extracellular ligand-binding domain, a single hydrophobic membrane-spanning domain, and a cytoplasmic tyrosine kinase domain [9]. In the EGFR-Ras signaling pathway, the binding of

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ligands to EGFR activates Ras, which in turn activates Raf. The activated Raf kinase can initiate the mitogen-activated protein (MAP) kinase cascade. The activated MAP kinase then migrates into the nucleus and induces phosphorylation of transcription factors (e.g., c-myc, c-jun, and c-ets), which affects gene expression and ultimately cell growth [9].

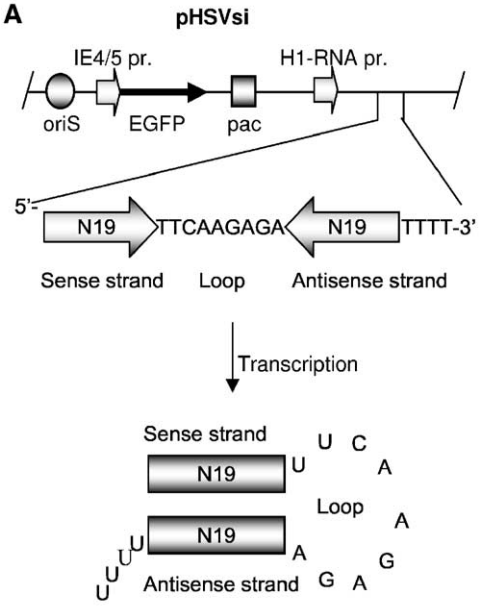
Several studies have exploited EGFR as a target for cancer-therapeutic procedures, including the use of (i) anti-EGFR antibodies [10–12], (ii) toxin-conjugated ligands [13], and (iii) tyrosine kinase-specific inhibitors [14]. A promising, new approach is the selective degradation of mRNA by RNA interference (RNAi). RNAi is a sequence-specific, posttranscriptional gene silencing mechanism, which is triggered by small interfering, double-stranded RNA (siRNA) and causes degradation of mRNA homologous in sequence to the siRNA. Here, we used helper-virus-free HSV-1 amplicon vectors [15] expressing siRNAs to (i) mediate posttranscriptional silencing of EGFR and (ii) analyze the effect of EGFR suppression on the proliferation of human glioblastoma cells in culture and *in vivo*.

RESULTS

HSV-1 Amplicon-Encoded siRNAs Inhibit the Synthesis of EGFR and EGFP

To "knock down" EGFR expression, we have created HSV-1 amplicon vectors that contain the HSV-1 immediate-early 4/5 promoter to express enhanced green fluorescent protein (EGFP) and the RNA polymerase III dependent H1 promoter to express double-stranded hairpin RNA directed against either EGFR at two different locations (pHSVsiEGFR I and pHSVsiEGFR II) or EGFP (Fig. 1). To test the functionality of these vectors, we infected Vero 2-2 cells and human glioma gli36-luc cells at a multiplicity of infection (m.o.i.) of 10 transducing units (TU) per cell (Fig. 2A). In gli36-luc cells, pHSVsiEGFP mediated the complete inhibition of EGFP expression by day 1 after infection; in Vero 2-2 cells, inhibition of EGFP was initially inefficient, but almost complete after 7 days. EGFP fluorescence in the pHSVsiEGFR I- and II-infected cultures was present in virtually 100% of the cells and was stable over at least 7 days.

To test the functionality of the EGFR-specific siRNAs, we first transfected Vero 2-2 cells with a vesicular stomatitis virus G protein (VSVG)-tagged EGFR expression vector (pMVEGFR-VSV) and, 4 h later, mock infected or infected them with pHSVsiEGFP or pHSVsiEGFR I or II amplicons. Four days later, we fixed the cells and stained them with an anti VSVG antibody and a Cy3-conjugated secondary antibody. We examined the cells under the fluorescence microscope using a rhodamine filter, which allows codetection of both EGFR-VSVG (red, transfected cells) and EGFP (green, amplicon-transduced cells). The results are shown in Fig. 2B and can be summarized as



B

siRNA	Sequence	Target nt
EGFR I	CACAGTGGAGCGAATTCCT	526-544, Exon 3
EGFR II	GAGTCGGGCTCTGGAGGAA	307-325, Exon 1
EGFP	GATCCCCGCTGACCCTGAA	125-143

FIG. 1. Schematic drawing of the pHSVsi amplicon vector. (A) Sequences encoding siRNA with 19 nt of homology (N19) to the target sequences are synthesized as 64-bp double-stranded DNA oligonucleotides and inserted downstream of the H1 RNA promoter. The amplicon contains two HSV-1 elements, an origin of DNA replication (oriS) and a DNA packaging/cleavage signal (pac), and EGFP coding sequences under the control of the HSV-1 IE 4/5 promoter. (B) The siRNA target sequences within EGFP and exons 1 and 3 of EGFR (I and II) are shown.

follows: Transfection of the cells with pMVEGFR-VSV resulted in approximately 10% EGFR-VSVG-positive cells. Superinfection of these cultures with pHSVsiEGFR I or pHSVsiEGFR II reduced the number of EGFR-VSVG-positive cells by about 40%. By increasing the m.o.i. from 1 to 5 TU of pHSVsiEGFR II per cell, the number of green (vector transduced) cells increased threefold, while the number of EGFR-VSVG-positive cells (red) was further reduced by 60–70%. Very few cells were positive for both EGFP- and EGFR-VSVG in the pHSVsiEGFR I- and II-infected cultures, indicating that, once a cell is transduced by amplicon, suppression of EGFR-VSVG synthesis was efficient. By contrast, infection of the cells with the control amplicon (pHSVsiEGFP, m.o.i. of 5) did not reduce the number of EGFR-VSVG-positive cells, compared to mock-infected cells. Moreover, although the number of EGFP-fluorescent cells was small (due to posttranscriptional silencing of EGFP), EGFR-VSVG and EGFP were codetected in a high proportion of the cells,

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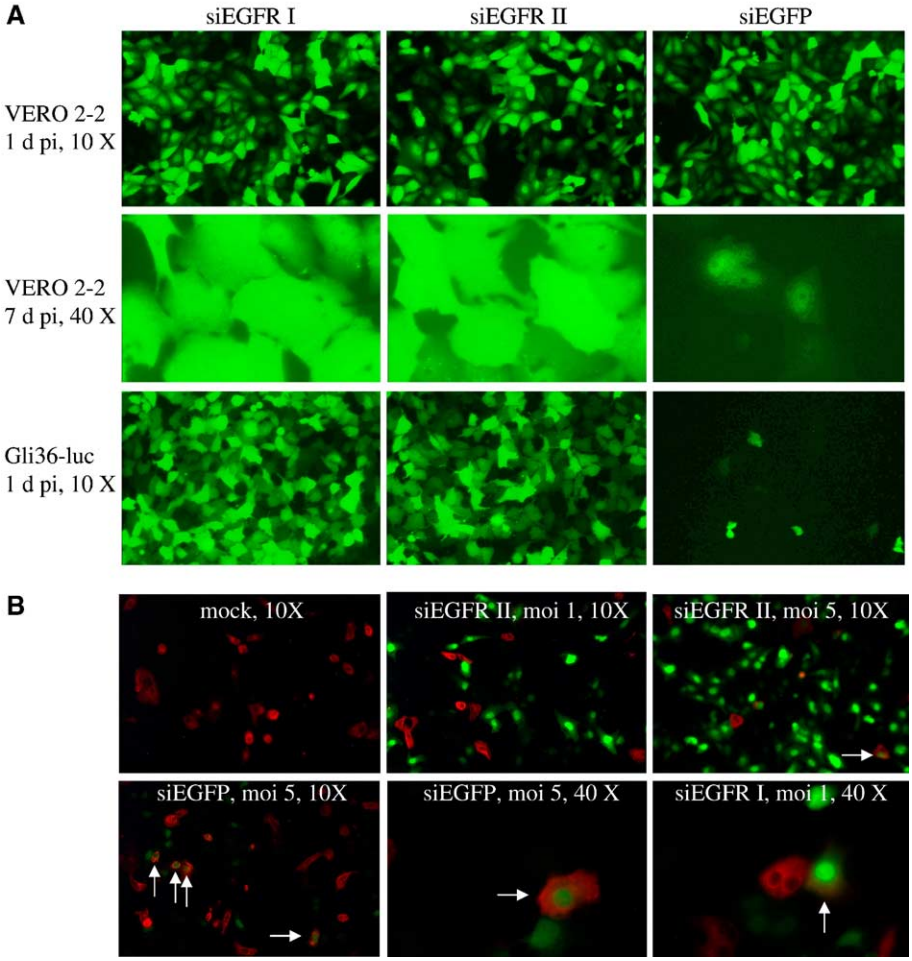

FIG. 2. HSV-1 amplicon-mediated posttranscriptional silencing of EGFR and EGFP. (A) Vero 2-2 cells or gli36-luc cells were infected with pHSVsiEGFR I, pHSVsiEGFR II, or pHSVsiEGFP amplicon vectors at an m.o.i. of 5. EGFP, which is expressed from all three vectors, was monitored at the indicated days postinfection (pi) and magnification (10×, 40×) under the fluorescence microscope. (B) 2-2 cells were first transfected with the EGFR-VSVG expression plasmid (pMVEGFR-VSV) and, 4 h later, mock infected or infected with pHSVsiEGFR I, pHSVsiEGFR II, or pHSVsiEGFP at the indicated m.o.i. Four days after infection, cells were fixed and stained with an anti-VSVG antibody and examined under the fluorescence microscope using a red filter (rhodamine), which allows codetection of both EGFR-VSVG (red) and EGFP (green). Arrows show cells that are positive for both colors.

indicating that transduction of the cells with the control amplicon did not affect EGFR-VSVG expression. These results demonstrate the efficiency and specificity of the vector-encoded siRNAs for their respective targets, EGFR and EGFP.

HSV-1 Amplicon-Mediated EGFR-Specific siRNA Expression Reduces the Levels of Endogenous EGFR Transcript in gli36-luc Cells

We evaluated the effects of HSV-1 amplicon-mediated siRNA synthesis on the expression of EGFR by reverse transcription and PCR (RT-PCR) analysis. We mock infected Gli36-luc cells or infected them with pHSVsiEGFR I, pHSVsiEGFR II, or pHSVsiEGFP (m.o.i. of 5). We harvested the cells and isolated total RNA at 4 days after infection. RT-PCR revealed that the EGFR transcripts were significantly reduced in both pHSVsiEGFR I- and II-infected cells (Fig. 3A, lanes 3 and 4), but not in control amplicon- (Fig. 3A, lane 2) or mock- (lane 1) infected cells. The level of the endogenous human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was not affected (Fig. 3A). Quantification of the RT-PCR products obtained from the gli36-luc cells revealed a reduction of 50–75% in EGFR mRNA levels following infection with pHSVsiEGFR I or pHSVsiEGFR II, compared to mock-infected or pHSVsiEGFP-infected cells (Fig. 3B). We confirmed the functionality and specificity of the vector-encoded siRNAs also by Western analysis. While endogenous EGFR protein was almost undetectable in pHSVsiEGFR I- or pHSVsiEGFR II-infected gli36-luc cells, it was readily detected in mock-infected or pHSVsiEGFP-infected cells (Fig. 3C). As a control, the levels of endogenous β-tubulin were not affected by any of the vectors tested (Fig. 3C).

Vector-Mediated siRNA Targeting EGFR Inhibits Growth of Human Glioma Cells in Culture and *in Vivo*

We determined different proliferation parameters to assess the growth capability of gli36-luc cells in culture and, following subcutaneous implantation, in nude mice. For all assays, gli36-luc cells were either mock infected or infected with HSV-1 amplicons at an m.o.i. of 5. As a first

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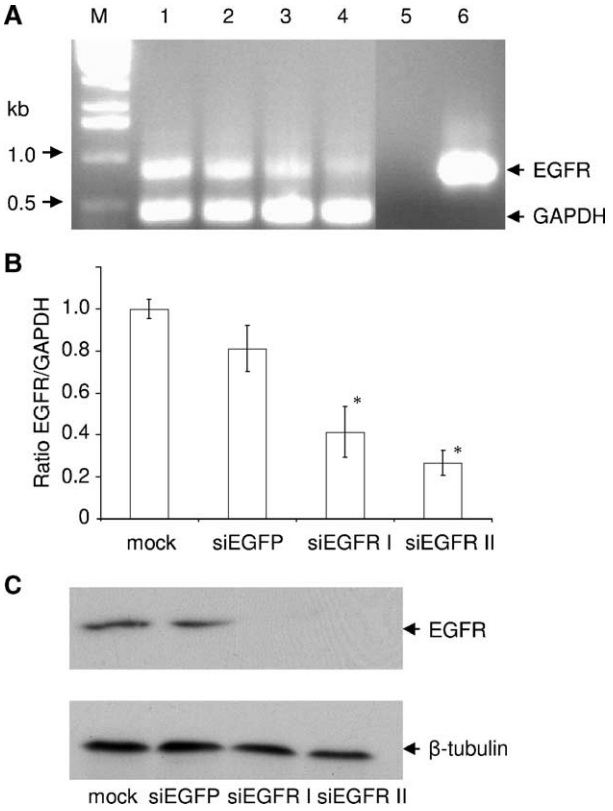

FIG. 3. HSV-1 amplicon-mediated EGFR-specific siRNA expression reduces the levels of endogenous EGFR in gli36-luc cells. (A) Gli36-luc cells were mock infected or infected with HSV-1 amplicons expressing EGFP-, EGFR I-, or EGFR II-specific siRNAs (m.o.i. of 5). Four days later, total RNA was isolated and reverse transcribed, and conventional PCR was performed. A representative agarose gel from three independent RT-PCRs is shown. Lane 1, mock; lane 2, pHSVsiEGFP; lane 3, pHSVsiEGFR I; lane 4, pHSVsiEGFR II; lane 5, H_2O; lane 6, pMVEGFR-VSV plasmid DNA; lane M, 1 kb molecular weight marker. The arrows indicate EGFR- and GAPDH-specific RT-PCR products. (B) After scanning and analyzing the RT-PCR product bands by using Image Quant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA), we calculated relative mRNA levels of EGFR by normalization with GAPDH mRNA levels. The results were obtained from three independent experiments. *Significant reduction ($P < 0.05$). (C) Gli36-luc cells were infected as in (A), and proteins were resolved by SDS–polyacrylamide gel electrophoresis and analyzed using monoclonal antibodies that recognize EGFR or β-tubulin.

parameter, we determined the cell number at days 5 and 10 after infection. There was a significant difference in the growth rate between pHSVsiEGFR I- or II- and control amplicon- or mock-infected cells, with a 40–50% reduction in growth following expression of the EGFR-specific siRNAs (Fig. 4A). Infection with pHSVsiEGFR I or II, but not with control amplicon, also significantly reduced the growth of a different human glioma cell line (U87; not shown). Because the gli36-luc cells constitutively express the firefly luciferase gene, we determined luciferase activity as a second proliferation parameter. We observed a pronounced reduction of luciferase activity in cells infected with pHSVsiEGFR I and II (not shown). We used a BrdU incorporation ELISA as a third parameter for cell proliferation. BrdU is a thymidine analogue that is incorporated into replicating DNA and can be quantified using antibodies [16]. We found that pHSVsiEGFR I or II, but not pHSVsiEGFP, significantly decreased BrdU incorporation in gli36-luc cells, compared to mock-infected cells (Fig. 4B). We performed soft agarose assays to test whether targeting of EGFR by siRNA can reduce the ability of gli36-luc cells to form colonies. At 24 h after infection, we placed the cells into medium containing soft agarose and evaluated colony formation after 11 days. Cells infected with pHSVsiEGFR I and II formed fewer (40–50%) and smaller colonies than mock-infected cells or cells infected with the control amplicon (Figs. 5A and 5B). We performed two experiments to investigate whether the observed reduction in the growth capability of the glioma

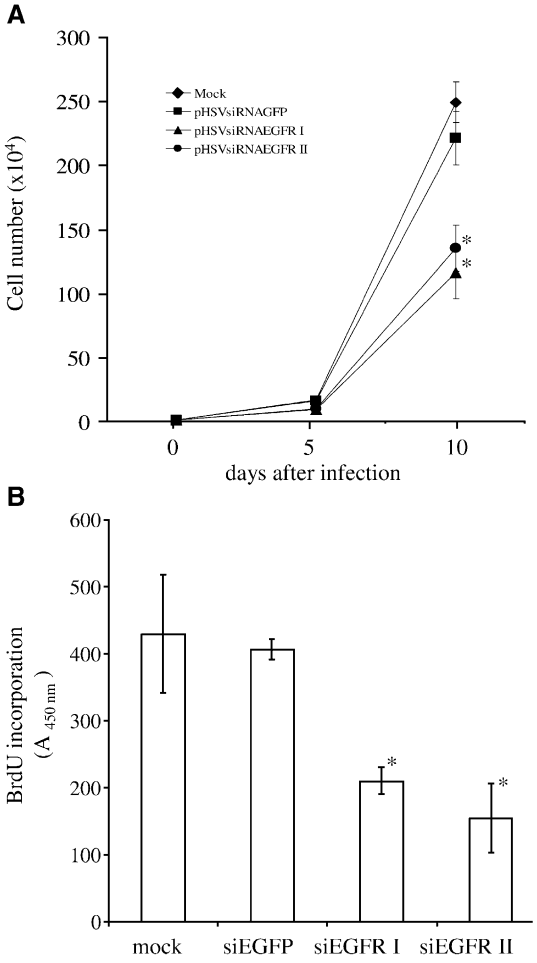

FIG. 4. Effects of posttranscriptional silencing of EGFR on cellular proliferation of human glioblastoma cells in culture. Cells were infected as described under Materials and Methods and proliferation was assayed by (A) cell number and (B) BrdU incorporation. All values are presented as the mean ± SD of three independent experiments. *Significant reduction ($P < 0.05$).

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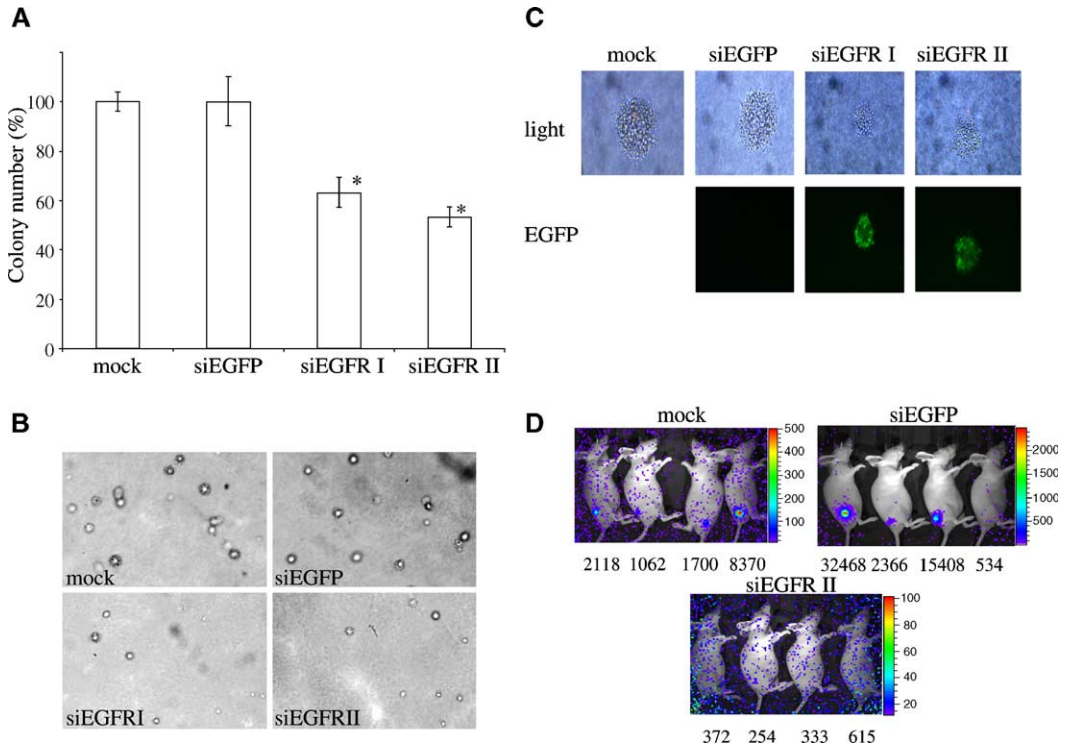
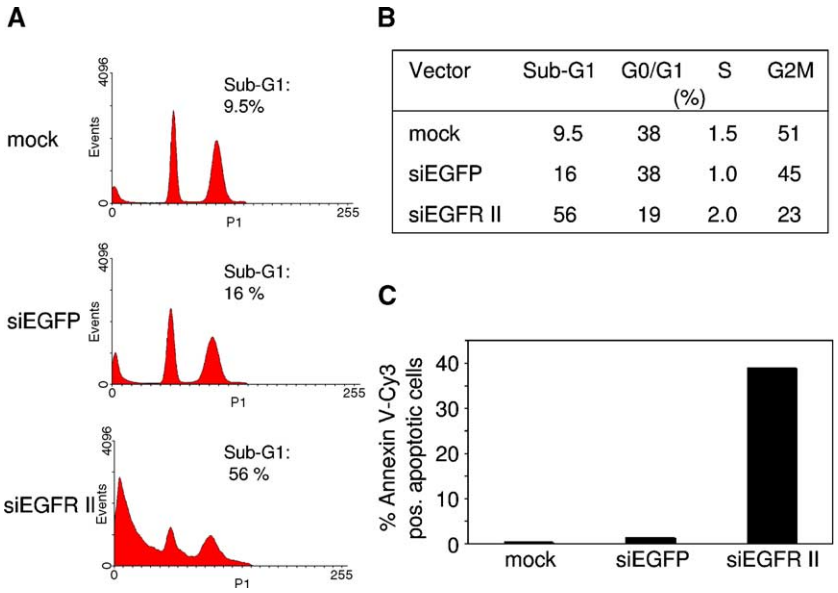


FIG. 5. The effects of EGFR inhibition on tumor growth of gli36-luc cells *in vitro* and *in vivo*. (A) Colony soft agar assay was evaluated as an *in vitro* tumor growth assay. The colony numbers in mock-infected plates were taken as a value of 100% colony formation. (B and C) Representative colonies were photographed at 11 days after infection using a light microscope or a fluorescence microscope equipped with a filter specific for EGFP. Original magnification: (B) ×2.5, (C) ×40. The values in (A) are presented as the mean ± SD of three independent experiments. *Significant reduction ($P < 0.05$). (D) To assess the growth capacity *in vivo*, gli36-luc cells were infected and, 24 h later, infused sc into athymic mice (1×10^6 cells per animal; $n = 4$). Luciferase expression was visualized 2 weeks later using the Xenogen IVIS system. The numbers indicate photons per 10 s.

cells resulted from inhibition of the cell cycle and/or the induction of apoptosis. We mock infected Gli36-luc cells or infected them with HSV-1 amplicons, either pHSVsiEGFR II or pHSVsiEGFP (m.o.i. of 5) and, 4 days later, analyzed them for cell cycle distribution and apoptosis. FACS analyses showed that approximately



Vector	Sub-G1	G0/G1	S	G2M
		(%)		
mock	9.5	38	1.5	51
siEGFP	16	38	1.0	45
siEGFR II	56	19	2.0	23

FIG. 6. Cell cycle analysis and apoptosis. Gli36-luc cells were mock infected or infected with HSV-1 amplicons expressing EGFP- or EGFR II-specific siRNAs (m.o.i. of 5). After 4 days, the cells were stained with (A and B) propidium iodide or (C) annexin V-Cy3.

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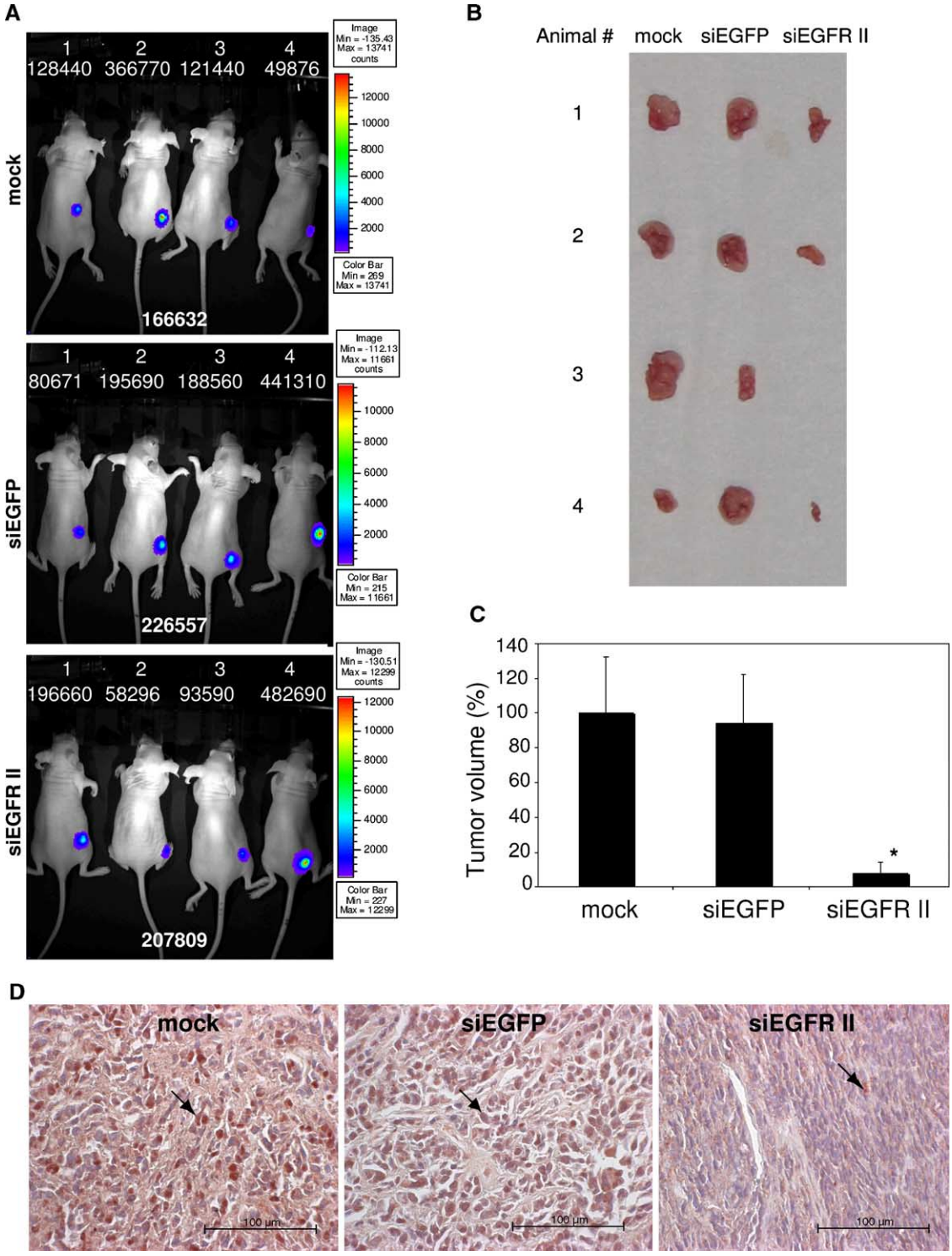

FIG. 7. Effects of HSV-1 amplicon-mediated siRNA expression on the growth of preestablished tumors in athymic mice. Gli36-luc cells were subcutaneously injected into the right flanks of athymic mice (nu/nu, 5-week-old males). (A) Two weeks after implantation, tumors were monitored by IVIS and the animals were divided into groups of four. The number and photon counts of each animal (top), as well as the average photon counts of the group (bottom), are shown. Vectors (1×10^6 TU in 20 μl) were injected into tumors three times per week for 2 weeks. Then, the tumors were removed (B and C) to determine the volumes and (D) for immunohistochemical detection of EGFR. The arrows in (D) point to EGFR-positive cells (brown cytoplasmic staining). *Significant reduction ($P < 0.05$).

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56% of the pHSVsiEGFR II-infected cells were in the sub-G1 fraction (representing apoptotic cells). In mock-infected cultures or cultures infected with the control vector pHSVsiEGFP the amounts of cells in the sub-G1 fraction were 9.5 and 16%, respectively (Figs. 6A and 6B). We obtained similar results with U87 cells (sub-G1 fractions: mock, 19.4%; siEGFP, 26.7%; siEGFRII, 57.5%). We performed annexin staining to confirm that siRNA-mediated inhibition of EGFR can induce apoptosis. We found a population of 40% apoptotic cells in the presence of EGFR gene silencing. We observed no relevant apoptosis in mock-infected cells or in cells infected with the control amplicon pHSVsiEGFP (Fig. 6C).

Next we tested the growth capacity of gli36-luc cells following implantation in mice. For this we chose pHSVsiEGFR II, as this vector was consistently more efficient in all *in vitro* assays. We infected the cells as above and, 24 h later, trypsinized, washed, and subcutaneously infused them into nude mice (10^6 cells per animal). We placed one portion of the cells (10^5) back into the tissue culture dish and further cultivated them; these cells were viable and the pHSVsiEGFR II-infected cells continued to express EGFP for at least 7 days (data not shown). Bioluminescence imaging performed 14 days after implantation showed that all animals infused with mock-infected gli36-luc cells and three of four animals infused with control amplicon pHSVsiEGFP-infected cells emitted light from the infusion site. However, none of the animals infused with pHSVsiEGFR II-infected gli36-luc cells showed light emission markedly above background level at this time point (Fig. 5C); luciferase activity (5704 photons per 10 s) was detectable in one of these animals for the first time at 3 weeks after infusion. We also examined the effects of HSV-1 amplicon-mediated inhibition of EGFR on the growth of preestablished tumors. For this experiment, we infused 3.5×10^6 gli36-luc cells subcutaneously into nude mice. Two weeks after implantation, we scored tumors by IVIS and divided the animals into three groups with comparable photon counts (Fig. 7A). Then, we injected the tumors three times per week with vector (pHSVsiEGFP or pHSVsiEGFR II; 1×10^6 TU in 20 μl phosphate-buffered saline (PBS)) or PBS (20 μl). After 2 weeks of treatment, the pHSVsiEGFR II-treated tumors were significantly smaller and expressed markedly lower EGFR levels than grafts treated with pHSVEGFP or PBS (Figs. 7B–7D).

Discussion

The genetic abnormalities of EGFR in glioblastomas have led to extensive studies of its relationship to cell proliferation, differentiation, and patient survival as well as its potential as a target of a number of alternative therapeutic interventions [17,18]. One therapeutic intervention, the specific downregulation of EGFR by virus vector-mediated delivery of siRNAs to inhibit glioma cell proliferation, has not yet been assessed. HSV-1-based vectors are well suited to treat diseases affecting the central nervous system, including the selective killing of brain tumor cells with replication-conditional HSV-1 vectors, an approach that has moved already toward clinical application [19,20]. In addition to replication-conditional HSV-1 mutants, the "gutless" HSV-1 amplicon vector has several properties that make it a promising candidate gene transfer vehicle. HSV-1 amplicons are bacterial plasmids that contain two noncoding genetic elements from HSV-1, an origin of DNA replication and a DNA packaging/cleavage signal, which allow their replication and packaging as a concatemer of 150 kb into HSV-1 particles. Depending on the size of the seed amplicon, numerous copies of the transgene sequences are packaged in one vector particle (e.g., approx 22 copies in a pHSVsiEGFR vector particle), supporting high expression levels. As no virus proteins are encoded, amplicons are nearly nontoxic when packaged without contaminating helper virus. Moreover, amplicon vectors can infect most mammalian cell types, including human glioma cells [21–26].

This study establishes the basis for the viral vector-mediated, posttranscriptional suppression of epidermal growth factor receptor as a potential therapeutic intervention in neuro-oncology. Using helper-virus-free HSV-1 amplicon vectors to express siRNAs, we achieved a significant and specific inhibition of either transfected or endogenous EGFR expression in Vero 2-2 cells or human gli36-luc cells, respectively (Figs. 2 and 3); GAPDH and β-tubulin expression was not affected (Fig. 3). The pHSVsiEGFP amplicon vector, which expresses EGFP from the HSV-1 immediate-early 4/5 promoter and EGFP-specific siRNA from the H1 promoter mediated the efficient posttranscriptional silencing of EGFP, but not that of EGFR or GAPDH, further demonstrating the specificity of the assay (Figs. 2A and 3). The downregulation of EGFR had a significant effect on the growth capability of the glioma cells both in culture and *in vivo*, which was expected, as the transfection of EGFR-specific, synthetic siRNAs into cultured glioblastoma cell lines was recently reported to induce a blockade at the G_2M phase of the cell cycle followed by apoptosis [27]. An antiproliferative effect following transfection of EGFR-specific siRNAs was observed in other cultured tumor cells as well [28,29]. In this study we show that the reduced cell proliferation following vector-delivered siRNA inhibition of EGFR was due to apoptosis (Fig. 6).

The advantage of the HSV-1 amplicon approach presented in our study is that it is safe, because the vector contains no viral genes and remains episomally in the cell nucleus, and can target cells and tissues not accessible by transfection. Moreover, the large transgene

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capacity of HSV-1 amplicons allows the inclusion of additional genes that have therapeutic, regulatory, and/or imaging functions. The incorporation of the EGFP marker gene in our vectors allowed the titration of the vector stocks and the visualization of cell transduction over time. For example, in the cell proliferation assays, we noted that a large fraction of the cells was still EGFP positive at 11 days after infection with pHSVsiEGFR I or II vectors, while no EGFP-positive cells were observed at 11 days after infection with the pHSVsiEGFP vector, indicating that RNA interference was still effective at this time point.

In conclusion, the approach presented here provides a novel tool to study the function of EGFR in tumor development and may find applications in gene therapy for gliomas. HSV-1 amplicons are easy to construct and, therefore, allow the rapid evaluation of numerous siRNA target sequences, tailored to the physical state of the EGFR gene or other target genes in a specific tumor. It will be interesting to test the effect of vector delivery into preestablished tumors, perhaps in combination with replication-conditional HSV-1 helper viruses to increase the intratumoral spread of the vectors. Finally, the strategy described here is not limited to neuro-oncological conditions, but may be applicable to neurodegenerative disorders as well, such as the recently reported RNAi-mediated suppression of polyglutamine-induced neurodegeneration [30].

MATERIALS AND METHODS

Cells

Human glioma cells (gli36) were kindly provided by Dr. David N. Louis (Harvard Medical School, Massachusetts General Hospital, Charlestown, MA, USA). These cells are negative for both GFAP and S-100 on immunohistochemistry and they carry a p16-CDKN2A homozygous deletion; moreover, gli36 cells do not have the EGFR gene amplification or the deletion of exons 2–7 (ΔEGFR), as these genetic abnormalities are not stably maintained in most cultured glioma cells (D.N. Louis, personal communication). To generate glioma cells stably expressing luciferase (gli36-luc), gli36 cells were cotransfected with plasmids pSV2Neo and pGL3, which expresses firefly luciferase from the SV40 early promoter (Promega, Wallisellen, Switzerland). Gli36-luc-positive clones were selected using 2000 μg/ml G418 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and checked for luciferase expression by a luminometer. The clone with the highest luciferase level was used for further studies. The selected gli36-luc cell clone and Vero 2-2 cells [31] were grown in DMEM supplemented with 10% FBS, 500 μg/ml G418, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2/95% O_2 atmosphere.

Plasmids

pMVEGFR-VSV (kindly provided by Dr. Mark I. Greene, University of Pennsylvania School of Medicine, Philadelphia, PA, USA) expresses a full-length EGFR protein fused to VSVG under the control of the CMV immediate-early (IE) 1 promoter/enhancer [32]. Plasmid pSUPER, which contains the RNA polymerase III-dependent H1 promoter and a well-defined start of transcription and a termination signal consisting of four consecutive thymidines, was described previously [33]. To create the basic pHSVsi amplicon, the 3.6-kb *Bam*HI/*Sal*I fragment of ploxP-A-H6 (kindly provided by Dr. Yoshinaga Saeki, Ohio State University Medical Center, Columbus, OH, USA), which contains an HSV-1 origin of DNA replication (OriS), the HSV-1 DNA packaging/cleavage signal (Pac), and an EGFP under control of the HSV-1 IE 4/5 promoter, was first inserted between the *Bam*HI/*Sal*I sites of pUC19. The resulting plasmid was designated pUC19-OriS-Pac-EGFP. The *Bam*HI/*Pst*I fragment of pUC19-OriS-Pac-EGFP was then inserted between the *Bam*HI and the *Pst*I sites of pSUPER. The resulting amplicon, pHSVsi, expresses EGFP to facilitate titration (Fig. 1A). DNA oligonucleotides targeting EGFR at two different locations (Fig. 1) were synthesized (Microsynth, Balgach, Switzerland) as 64-mer sense and antisense oligonucleotide templates (19 × 2 nucleotides specific to the targeted genes and 26 nucleotides for restriction enzyme recognition sites and hairpin structure) and inserted into pHSVsi between the *Bgl*II and the *Hin*dIII sites. An amplicon that contains DNA oligonucleotides targeting EGFP (Fig. 1) was used as a control. These amplicons are designated here as pHSVsiEGFR I, pHSVsiEGFR II, and pHSVsiEGFP. Amplicon packaging was performed as described [23] and titered (TU/ml) by counting the number of green cells 24 h following infection of Vero 2-2 cells using a fluorescence microscope.

Western Analysis

Gli36-luc cells were seeded into 6-cm-diameter tissue culture plates (1.2×10^6 cells per plate) and, the following day, either mock infected or infected with HSV-1 amplicon vectors (m.o.i. of 5). After 4 days, cells were harvested, and total protein was separated on a SDS–12% polyacrylamide gel and blotted onto nitrocellulose. The membrane was blocked with 5% nonfat dry milk in PBS-T (PBS containing 0.05% Tween 20) for 2 h at 37°C and then rinsed once with PBS-T and washed twice for 15 min and twice for 5 min at room temperature with TBS-T (50 mM Tris–HCl, pH 7.9, 150 mM NaCl, 0.01% Tween 20). The primary antibodies were diluted 1:50 (anti-EGFR antibody; NeoMarkers, Fremont, CA, USA) or 1:1000 (anti-β-tubulin antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TBS-T containing 2% nonfat dry milk and applied to the membrane. After incubation overnight at 4°C, the membrane was washed twice for 15 min and twice for 5 min at room temperature with TBS-T and then incubated for 1 h at room temperature with a horseradish-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology) diluted 1:2500 in TBS-T containing 2% nonfat dry milk. Bands were visualized using the ECL system according to instructions provided by the supplier (Amersham, Buckinghamshire, UK).

RT-PCR Analysis

Total RNA from 1×10^5 gli36-luc cells either infected with amplicons (m.o.i. of 5) or mock infected was isolated using the Micro-to-Midi total RNA isolation kit (Invitrogen, Basel, Switzerland). Total RNA was reverse transcribed with random primers using the RT-PCR kit from Promega. PCR for EGFR was performed by using the following primers: forward primer, 5′-CACCTGCGTGAAGAAGTGTCC-3′; reverse primer, 5′-CCGTCCTGTGCAGGTGATGTT-3′. PCR amplification of the housekeeping gene GAPDH was performed as control of sample loading and to allow normalization between samples. The following primers were used for the GAPDH PCR: forward primer, 5′-GGGGAGCCAAAAGGGTCATCATCT-3′; reverse primer, 5′-ACGCCTGCTTCACCACCTTCTTGA-3′. PCR was performed under the following conditions: EGFR, 95°C for 2 min; 35 cycles at 94°C for 1 min, 60.5°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min; GAPDH, 95°C for 2 min; 35 cycles at 94°C for 1 min, 63°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min.

Indirect Immunofluorescence

Vero 2-2 cells were seeded in 24-well plates at 5×10^4 cells/well and, the following day, transfected with pMVEGFR-VSV (0.2 μg/well) using Lipofectamine 2000 (Invitrogen). After 4 days of incubation, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After incubation in 0.1 M glycine (in PBS) for 15 min, the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min and then incubated for 1 h at room temperature with monoclonal mouse anti-VSVG antibody (Sigma; 1:5000 in 3% BSA, 0.2% Triton X-100 in PBS). As a fluorescent secondary antibody, rabbit anti-mouse IgG–Cy3 was used (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cells were examined under a Zeiss Axiovert 100 fluorescence microscope equipped with a Hamamatsu CCD digital camera and a filter specific for

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rhodamine, which allows covisualization of both Cy3 (EGFR-VSVG) and EGFP.

Cellular Proliferation Assays

Growth rate. Gli36-luc cells (5×10^3) were seeded in each well of a six-well plate and infected on the next day with HSV-1 amplicons (m.o.i. of 5). Cells were counted at 5 and 10 days after infection using a hemocytometer.

Luciferase activity. Cells were infected with HSV-1 amplicons as described above and, 5 or 10 days later, lysed by three cycles of freezing and thawing. After centrifugation (14,000 rpm, 5 min), 10 μl of supernatant was mixed with 100 μl of luciferase assay solution (Promega) and analyzed in a luminometer.

BrdU index. Gli36-luc cells (1×10^3) were infected with HSV-1 amplicons (m.o.i. of 5). Cells were fixed at 5 days after infection and DNA was denatured using reagents supplied with the cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA). Cells were incubated with anti-BrdU peroxidase conjugate, washed, and incubated with color development substrate. The absorbance of the samples was measured at 450-nm wavelength (A_{450}).

Statistical analysis. All measurements for cell proliferation were performed in triplicate and the values are expressed as means ± SD; P values were calculated by using the Student t test, and values of $P < 0.05$ were regarded as significant.

Colony Soft Agarose Assay

Colony soft agarose assays were performed as described in detail elsewhere [34]. Briefly, each well of a six-well plate was coated with 2 ml of bottom agarose mixture (DMEM/10% FBS/0.6% agarose). After the bottom layer had solidified, 2 ml of top agarose mixture (DMEM/10% FBS/0.4% agarose) containing 5×10^3 cells, infected at an m.o.i. of 5 TU per cell with either pHSVsiEGFP or pHSVsiEGFR I or II amplicons, was added to each well. Triplicate cultures were incubated in 5% CO_2/95% O_2 atmosphere at 37°C. Colonies were counted and photographed 11 days after the cells were plated. The number of colonies formed in the mock-infected cultures was set as the value of 100% colony formation.

FACS Analysis and Apoptosis Assays

Gli36-luc cells were seeded into 12-well plates (1×10^5 cells per well) and, on the next day, mock infected or infected with HSV-1 amplicon vectors (m.o.i. of 5). After 4 days, the cells were fixed with 70% ethanol on ice for 30 min, washed with PBS, treated with RNase A (100 μg/ml) at 37°C for 30 min, and then, 20 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) was added. A total of 64,000 stained nuclei were analyzed in a FACSCalibur flow cytometer (Becton–Dickinson, Basel, Switzerland) using the BD-CellQuest software (Becton–Dickinson). DNA histograms were analyzed using WinMDI 2.8 software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA). Apoptotic cell death was determined using the Annexin V–Cy3 Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. Briefly, infection was performed as above, and cells were harvested at 4 days after infection, washed with PBS, and then suspended in annexin V binding buffer. The cells were stained for 15 min at room temperature in the dark and analyzed on a FACSAria flow cytometer using BD-FACSDiVa software (Becton–Dickinson).

Glioma Cell Implantation and Bioluminescence Imaging

All animal procedures were approved and performed according to guidelines issued by the Committee of Animal Care of the Canton of Zurich. Human glioblastoma gli36-luc cells were either mock infected or infected with pHSVsiEGFR II or pHSVsiEGFP at an m.o.i. of 5 TU per cell. After 24 h, the cells were trypsinized, washed, and subcutaneously implanted (1×10^6 cells in 100 μl per animal) into the flanks of athymic mice (nu/nu, 5-week-old males, four animals per group; Charles River Laboratories, Germany). After 14 days, the animals were first injected intraperitoneally with D-luciferin (150 μg/g body wt; Xenogen) and then anesthetized with isoflurane. Ten minutes after the injection of D-luciferin, images were acquired for 10 s using the Xenogen IVIS system and quantified by Living Image analysis software (Xenogen). A surface image of the animals was acquired, onto which the pseudocolor image representing the distribution of the photon counts was projected to allow correlation of bioluminescence and anatomy. For the treatment of preestablished tumors, 3.5×10^6 gli36-luc cells in 100 μl PBS were subcutaneously injected into the right flanks of athymic mice (nu/nu, 5-week-old males). Two weeks after implantation, tumors were monitored by IVIS as described above, and the animals were divided into groups of four. Vectors (1×10^6 TU in 20 μl) were injected into tumors three times per week for 2 weeks. Then, the tumors were removed and photographed, and the tumor volumes were determined using AxionVision 2.05 software (Zeiss, Feldback, Switzerland). For immunohistochemical detection of EGFR, tumor sections were incubated overnight at room temperature with a goat anti-EGFR antibody (1:15; Santa Cruz Biotechnology). LSAB+ (DAKO Cytomation, Zug, Switzerland) was used as the secondary antibody and aminoethylcarbazole (Zymed, San Francisco, CA, USA) as the chromagen according to instructions provided by the manufacturers.

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