

# Tumor Therapy Mediated by Lentiviral Expression of shBcl-2 and S-TRAIL<sup>1</sup>

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## Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) can selectively kill tumor cells and, in combination with other agents, could enhance tumor therapy. We explored the combined therapeutic effects of a secretable form of (S) TRAIL-induced apoptosis and the downregulation of Bcl-2 in human gliomas. We constructed a lentiviral delivery system: 1) for the expression of short hairpin (sh) RNA to downregulate Bcl-2 and for the expression of S-TRAIL to induce apoptosis in glioma cells; and 2) to follow delivery *in vitro* and the fate of tumors in real time *in vivo*. We demonstrate that lentiviral-mediated simultaneous downregulation of Bcl-2 and S-TRAIL–induced apoptosis leads to an increased expression of activated caspase-3 and caspase-7, thus resulting in accelerated S-TRAIL–mediated apoptosis in glioma cells *in vitro*. Using a highly malignant human glioma model expressing EGFRvIII and firefly luciferase, we show that the combined effect of Bcl-2 downregulation and S-TRAIL–induced apoptosis results in complete eradication of gliomas compared to S-TRAIL monotherapy. These results show that simultaneous triggering of TRAIL-mediated death receptor pathway and downregulation of Bcl-2 by shRNA leads to enhanced eradication of gliomas and serves as a template in developing and monitoring combination therapies for the treatment of drug-resistant cancers.

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## Introduction

Cancer cells are characterized by self-sufficiency in growth signals, insensitivity to antigrowth signals, sustained angiogenesis, tissue invasion and metastasis, limitless replicative potential, and evasion of apoptosis [1]. The molecular mechanism of drug-induced apoptosis is associated with mitochondrial dysfunction that is characterized by an increase in mitochondrial membrane permeability and is regulated by Bcl-2 [2,3]. The overexpression of Bcl-2 in tumor cells results in antiapoptotic effects triggered through the extrinsic death receptor–dependent pathway [4], but not

the death receptor–independent pathway [5]. Targeted repression of Bcl-2 has the potential to facilitate tumor cell apoptosis induced through a number of extrinsic apoptosis-inducing agents, such as tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) [6,7], which offers one of the most promising strategies in the field of death ligands and receptors.

Apoptosis initiated by TRAIL involves the binding of TRAIL to death receptors DR4 and DR5, causing the intracellular death domains of these receptors to trimerize [8–10]. Previously, we have engineered a secretable form of TRAIL (S-TRAIL) consisting of an N-terminal fusion of the extracellular domain of Flt3L, a ligand for Flt3 tyrosine kinase receptor, with extracellular domain of TRAIL [9]. We have shown that S-TRAIL has more potent apoptotic effects compared to TRAIL itself, in addition to having a bystander effect for tumor cells both in culture and in mouse models of glioma [10,11]. Recent studies revealed that inhibitors of apoptosis (Bcl-2, XIAP, FLIP, HDAC, or survivin) play an important role in mediating resistance to the apoptotic effects of cytokines TRAIL and TNF- $\alpha$  in melanoma cells [12,13]. Human gliomas overexpress Bcl-2, which has been shown to induce complex changes in glioma cell phenotype and protects glioma cells from various proapoptotic stimuli [14,15].

A vital aspect to successful glioma therapy is to achieve robust and prolonged transgene expression and to follow the fate of tumors overtime. Recently, viral vectors have emerged as an efficient method of integrating transgenes into the host genome. In particular, lentiviral vectors have been used in several studies to genetically modify cells *ex vivo* as these viruses allow stable integration of transgenes into the host genome irrespective of their state of division [16]. Several approaches to imaging gene delivery and monitoring tumor fate have been described [9,17,18]. Bioluminescent imaging

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offers high detection sensitivity, low background luminescence from normal tissues, and rapid turnover of luciferase substrates, making this method suited for temporal *in vivo* imaging of gene expression [19].

In this study, we have engineered S-TRAIL lentiviral vectors and have tested the combined potential of downregulating Bcl-2 by lentiviral-mediated expression of shRNA and by induction of apoptosis by S-TRAIL, released from transduced cells in a highly malignant EGFRvIII glioma model. Furthermore, we have employed noninvasive real-time imaging to follow changes in glioma burden *in vivo*. To our knowledge, this is the first study to show the effect of downregulating Bcl-2 by lentiviral-mediated shRNA in human gliomas and following the combined effect of S-TRAIL (released within tumors) and Bcl-2 downregulation in real time *in vivo*. The mode of combined cancer therapy described in this study has three important advantages: 1) the use of glioma cells stably expressing shRNA; 2) the use of an enhanced apoptosis-inducing recombinant S-TRAIL with a bystander effect; and 3) real-time imaging of glioma burden *in vivo*. This study should immensely help in developing clinically relevant combination therapies *in vivo*.

## Methods

### Generation of Lentiviral Vectors

Short hairpin (sh) RNA plasmid constructs for Bcl-2 and green fluorescent protein (GFP) were constructed in pSuper vector (Oligoengine, Seattle, WA) to include the specific 19-bp target sequence for the shBcl-2 construct (5'-TGTGGATGACTGAGTACCTGA-3') [14] and the shGFP construct (5'-GAACGGCATCAAGGTGAAC-3') [20] in front of the H1 RNA polymerase III promoter, resulting in pSuper-shBcl-2 and pSuper-shGFP. H1 promoter shBcl-2 or shGFP fragments were amplified from pSuper vectors using polymerase chain reaction and cloned into CSCRW [derived from CSCGW [21] lentiviral (LV) vector in which GFP sequences were replaced by DsRed2 cDNA sequences], resulting in LV-shBcl-2 and LV-shGFP lentiviral vectors. All constructs were verified by sequencing secreted form of TRAIL (S-TRAIL), which was created by fusing the coding sequences for the extracellular domain of Flt3L, and an isoleucine zipper with the N-terminal of TRAIL [9] was placed under a Cytomegalovirus (CMV) promoter in a vector coexpressing GFP through an internal ribosomal entry sequence (IRES), thus resulting in an LV-S-TRAIL construct. Similarly, cDNA sequences for firefly luciferase (Fluc) were placed under the CMV promoter in a vector coexpressing LacZ through IRES, resulting in the LV-Fluc construct. For a detailed description of lentivirus vector production, see Sena-Esteves et al. [21]. In brief, lentiviral genome (CMVR8.91) was transfected into 293T cells together with an envelope-coding plasmid (VSVG) and vector constructs. LVs were harvested 40 hours posttransfection and concentrated by ultracentrifugation. Titters were determined on 293T cells as transducing units using serial dilutions of vector stocks with 8  $\mu$ g/ml polybrene (Sigma Chemical, St. Louis, MO).

### Generation of shBcl-2-Expressing Glioma Cells

Human glioma lines U87, U-251, Gli36, and Gli36-EGFRvIII (stably expressing the mutant form of EGFR associated with a high malignancy of this tumor type) [19,20] and 293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Sigma) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). Different adherent glioma cell lines were transduced with LV-shBcl-2 or LV-shGFP at multiplicities of infection (MOI) ranging from 1 to 3, and the cells were visualized for DsRed2 expression by fluorescence microscopy. Transduction was carried out for 12 hours at 37°C in the presence of polybrene (8  $\mu$ g/ml; Sigma). Bcl-2 levels were measured by Western blot analysis 5 days after transduction, as described below. Gli36-EGFRvIII glioma cells were also transduced with LV-Fluc at an MOI of 3; 72 hours later, cells were incubated in a medium containing 150  $\mu$ g/ml D-luciferin (Biotium, Hayward, CA), and bioluminescence imaging was performed as described below.

### S-TRAIL Western Blot Analysis, Enzyme-Linked Immunosorbent Assay (ELISA), and Immunocytochemistry

Human Gli36-EGFRvIII glioma cells and 293T cells were transduced with LV-S-TRAIL vector at an MOI of 1; 36 hours later, a culture medium and the cells were harvested. Proteins were isolated from harvested cells, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with anti-TRAIL (ProScience, Poway, CA) and anti-cleaved PARP (Cell Signaling, Beverly, MA) antibodies. S-TRAIL concentration in the conditioned culture medium was measured by ELISA with the TRAIL Immunoassay Kit (Biosource International, Camarillo, CA) according to the manufacturer's protocol, using recombinant human TRAIL expressed in *Escherichia coli* as a standard. Different glioma lines were plated in 96-well plates with five replicate wells and incubated with varying concentrations (0–250 ng/ml) of S-TRAIL obtained from transduced 293T cells. Twenty-four hours later, glioma cell viability was determined as described below. Gli36-EGFRvIII glioma cells were incubated with 80 ng/ml S-TRAIL; 24 hours later, cells were fixed, permeabilized, and incubated with a cleaved caspase-3 antibody (1:100; Cell Signaling) for 1 hour at 37°C. Cells were then washed and incubated with goat anti-rabbit Alexa dye (540 nm)–conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 hour, washed again, mounted, and examined by fluorescence microscopy.

### Cytochrome c and BH3-Interacting Domain Death Agonist (BID) Immunoblotting

For assessing the combined effect of S-TRAIL and Bcl-2 downregulation, an S-TRAIL concentration of 40 ng/ml was used. Gli36-EGFRvIII-shGFP and Gli36-EGFRvIII-shBcl-2 cells were incubated with S-TRAIL (40 ng/ml) for 24 hours, and the total cytochrome *c* in mitochondrial fractions was analyzed by using cytochrome *c* assay kit (Calbiochem, San Diego, CA) according to the manufacturer's protocol. Briefly,  $5 \times 10^6$  of Gli36-EGFRvIII-shGFP and Gli36-EGFRvIII-shBcl-2 cells with or without S-TRAIL treatment

was harvested by centrifugation at 700g for 5 minutes. After washes with phosphate-buffered saline (PBS), the cells were resuspended in a 250- $\mu$ l extraction buffer containing a protease inhibitor mixture and dithiothreitol, and then incubated on ice for 10 minutes, homogenized, and centrifuged at 700g for 10 minutes at 4°C. Supernatant was collected and further centrifuged at 10,000g for 30 minutes at 4°C. Resulting supernatants were harvested and designated as cytosolic fractions, and pellets were resuspended in an appropriate buffer and designated as mitochondrial fractions. Cytochrome *c* was analyzed using Western blot analysis with cytochrome *c* monoclonal antibody (Calbiochem) or control antibody against tubulin. Mitochondrial fractions were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with rabbit anti-BID antibody (Cell Signaling, Cambridge, MA).

#### *Caspase-3/7 and Cell Viability Assay*

Different glioma lines either expressing shBcl-2 or shGFP were plated at  $1 \times 10^4$  cells/well in 96-well plates with five replicate wells for each condition. Cells were either left untreated or treated with 40 ng/ml S-TRAIL for 24 hours, and the metabolic activity of the cells was determined using a luminescent adenosine triphosphate (ATP)-based assay (CellTiter GLO; Promega, Madison, WI), according to the manufacturer's instructions. Results were read with a luminometer with a read time of 1 second/well. Glioma cells were also plated as described above, incubated with 40 ng/ml S-TRAIL for 24 hours, and analyzed using ApoONE Homogeneous Caspase 3/7 Assay (Promega), according to the manufacturer's instructions. Samples were read after 1 hour of incubation with the caspase substrate, as described above. S-TRAIL-treated cells were analyzed for viability by luminescent ATP-based assay (CellTiter GLO; Promega) 24 hours later, as described above.

#### *Tumor Models and Glioma Implantations*

Athymic nude mice (nu/nu, 6–7 weeks old; Charles River Laboratories, Wilmington, MA) were anesthetized by an intraperitoneal injection of a mix of ketamine (1.6 mg/mice) and xylazine (240  $\mu$ g/mice) in saline. For correlation studies, different concentrations of Gli36-EGFRvIII-FI cells (ranging from  $5 \times 10^4$  to  $5 \times 10^6$ ;  $n = 2$  tumors for each cell number) were implanted subcutaneously, and the mice were imaged for Fluc activity 24 hours later, as described below. For assessing the effect of Bcl-2 downregulation and S-TRAIL on glioma proliferation, we employed our previously developed model of assessing the bystander effect of S-TRAIL released within the tumor [10]. Gli36-EGFRvIII-shBcl-2-Fluc or Gli36-EGFRvIII-shGFP-Fluc glioma cells (Gli36-EGFRvIII-FI cells transduced with either LV-shBcl-2 or LV-shGFP) in midlog phase were harvested by trypsinization, and single-cell suspensions were transduced with LV-S-TRAIL at an MOI of 1 for 6 hours at 37°C. S-TRAIL-transduced cells were washed with PBS, and mice were implanted with: 1) Gli36-EGFRvIII-FI-shBcl-2; 2) Gli36-EGFRvIII-FI-shGFP; 3) a mix of  $1.5 \times 10^6$  of S-TRAIL-transduced cells and  $6.0 \times 10^6$  of Gli36-EGFRvIII-FI-shBcl-2 cells; and 4) a mix of  $1.5 \times$

$10^6$  of S-TRAIL-transduced cells and  $6.0 \times 10^6$  of Gli36-EGFRvIII-FI-shGFP cells, in the two opposite flanks of the mice ( $n = 10$  tumors in each case). Three days after glioma cell implantations, mice were imaged for Fluc activity, as described below. All animal protocols were approved by an institutional review board.

#### *Bioluminescence Imaging in Culture and In Vivo*

Fluc-bearing glioma cells were incubated with 150  $\mu$ g/ml D-luciferin and assayed for luciferase activity using a liquid nitrogen charged cooled device (CCD; Roper Scientific, Trenton, NJ). Mice were given an intraperitoneal injection of D-luciferin (4.5 mg/25 g body weight; prepared in 150  $\mu$ l of saline) and imaged for Fluc activity for 1 minute after D-luciferin administration using a cryogenically cooled high-efficiency CCD camera system (Roper Scientific). Mice were imaged for Fluc activity on the fourth, sixth, and eighth days after tumor implantation. Postprocessing and visualization were performed as described previously [22].

#### *Tissue Processing and Immunohistochemistry*

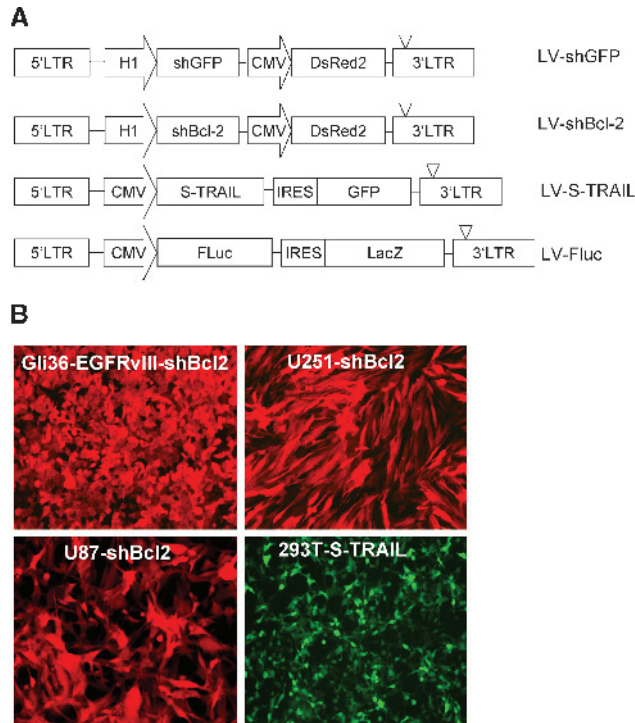
Two hours following the last luciferase imaging session, mice were sacrificed, tumor tissues were fixed in 10% buffered formalin and embedded in paraffin, and 6- $\mu$ m sections were cut on a cryostat (CM 3000; Leica Microsystems [Wetzlar, Germany]). The sections were dehydrated in xylene and ethanol then immersed in PBS, and caspase-3 staining was performed according to the manufacturer's protocol using caspase-3 immunostaining kit (Cell Signaling). Immunostained sections were also stained with hematoxylin and eosin.

#### *Statistical Analysis*

Data are expressed as mean  $\pm$  SEM and were analyzed by either Student's *t* test or analysis of variance (after Bartlett's test of homogeneity of variance), followed by Newman-Keuls correction for multiple comparisons. Differences were considered significant at  $P < .05$ .

## **Results**

The plasmid constructs used to generate lentivirus vectors (LVs) in this study are diagrammed in Figure 1A. To determine the transduction efficiency of these LVs, 293T and Gli36 glioma cells were transduced and visualized for DsRed2 and GFP fluorescence. The presence of 90% to 95% DsRed2-positive and GFP-positive glioma cells revealed the high transduction efficiency of LVs (Figure 1B). Gli36-EGFRvIII human glioma cells were transduced with LV-S-TRAIL; 24 hours after transduction, total protein was isolated from tumor cells, fractionated by denaturing SDS-PAGE, and immunoblotted using antiserum against TRAIL. Immunoreactive proteins corresponding to the size of S-TRAIL were present in the respective LV-S-TRAIL-transduced cells and not in control vector-transduced cells (Figure 2A). Quantitation of TRAIL in the cell supernatant confirmed the secretion of 230 ng/ $10^6$  cells per 24 hours by glioma cells transduced with LV-S-TRAIL, with no significant amounts

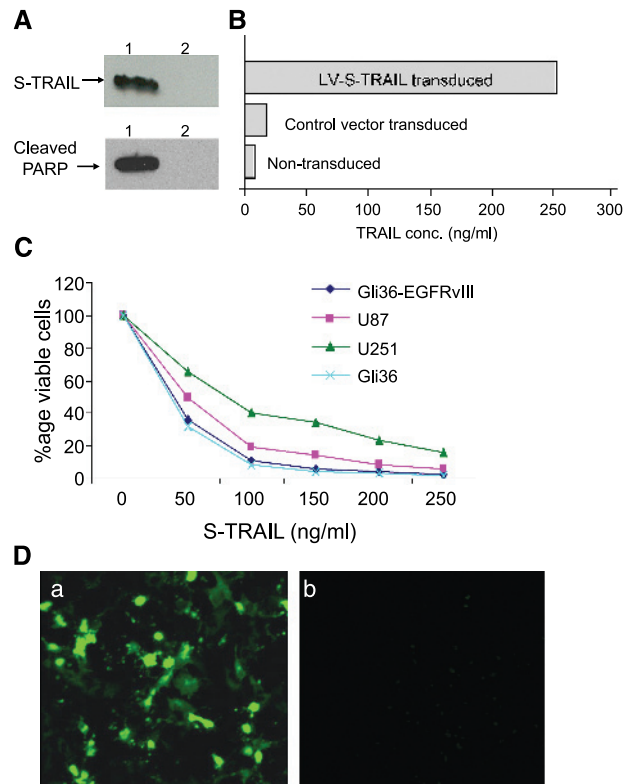


**Figure 1.** shRNA, S-TRAIL, and Fluc lentiviral vectors. (A) A self-inactivating lentiviral system based on HIV-1 (CS-CG) [36] was used to construct vectors (i) expressing shRNA against human Bcl-2 and GFP under H1 RNA polymerase III promoter and DsRed2 under the CMV promoter; and (ii) expressing S-TRAIL or Fluc under the CMV promoter with an IRES sequence followed by GFP and LacZ, respectively. (B) Human glioma, Gli36-EGFRvIII, U251, and U87 cells transduced in culture with LV-shBcl-2 and 293T cells transduced with LV-S-TRAIL at an MOI of 1 were visualized for DsRed2 or GFP fluorescence. Original magnification,  $\times 10$  (B).

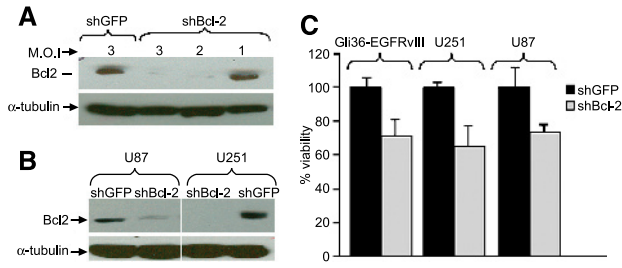
produced by nontransduced or control vector-transduced cells (Figure 2B). Cell viability assays on different glioma cell lines treated with varying concentrations of S-TRAIL revealed that both Gli36 and Gli36-EGFRvIII are more susceptible to S-TRAIL-mediated cell killing than are U251 and U87 glioma cells (Figure 2C). Immunocytochemistry and immunoblotting with antibodies detecting cleaved caspase-3 (Figure 2D) and cleaved PARP (Figure 2A) respectively revealed that lentivirally expressed S-TRAIL induced apoptosis in glioma cells. These results show that TRAIL secreted by lentiviral-transduced cells is active and can induce apoptosis in glioma cells in culture.

To test whether shBcl-2 could effectively knock down protein levels of Bcl-2 in glioma cells, Gli36-EGFRvIII glioma cells were transduced with LV-shBcl-2 and control LV-shGFP, with MOI ranging from 1 to 3, and the levels of Bcl-2 protein were then determined by immunoblotting after 5 days of transduction using antiserum against Bcl-2. Immunoreactive proteins corresponding to the size of Bcl-2 were present in the respective LV-shGFP-transduced cells and not in LV-shBcl-2-transduced cells (Figure 3A). The other well-established glioma lines U87 and U251 showed a similar downregulation of Bcl-2 when transduced with shBcl-2 lentiviral vectors (Figure 3B). Viability assays revealed a 25% to 30% reduction in cell viability over a 24-hour period in cells expressing shBcl-2 compared to glioma cells expressing shGFP (Fig-

ure 3C). As all the glioma lines tested showed a similar decrease in cell viability, we used Gli36-EGFRvIII glioma cells for further studies, as EGFRvIII expression in gliomas has been previously reported to enhance tumor growth [23]. Next, we investigated whether the combined effect of S-TRAIL and Bcl-2 downregulation would influence the viability of glioma cells in culture. Gli36-EGFRvIII glioma cells were transduced with LV-S-TRAIL with an MOI of 1; 24 hours later, the medium was collected and quantified by ELISA. Both shGFP and shBcl-2-expressing glioma cells were incubated with a medium from LV-S-TRAIL-transduced cells (40 ng/ml) and assessed for caspase-3 activity, cytochrome c release, BID cleavage, and cell viability. At 24 hours, a significantly higher ( $P < .02$ ) caspase-3/7 activity was assessed in shBcl-2-expressing glioma cells compared to shGFP-expressing cells (Figure 4A). A considerably reduced expression of the 22-kDa BID protein indicating its cleavage was observed in S-TRAIL-treated shBcl-2-expressing cells than in shGFP-expressing glioma cells (Figure 4B). Higher levels of cytochrome c were



**Figure 2.** S-TRAIL-induced apoptosis and bystander effect in human glioma cells. (A) Gli36-EGFRvIII glioma cells were transduced with LV-S-TRAIL vector or control vector bearing only IRES-GFP under the CMV promoter; 24 hours after transduction, the cells were harvested, and proteins were resolved by SDS-PAGE and immunoblotted with antibodies against TRAIL and anti-cleaved PARP. Lane 1: S-TRAIL-transduced cell lysates. Lane 2: Control vector-transduced cell lysates. (B) Immunoreactive S-TRAIL protein concentration in the medium of transduced cells, as determined by ELISA. (C) Different glioma lines were plated in 96-well plates with five replicate wells and incubated with varying concentrations (0–250 ng/ml) of S-TRAIL for 24 hours, and glioma cell viability was determined by using a luminescent ATP-based assay. (D) Gli36-EGFRvIII glioma cells were incubated with 80 ng/ml S-TRAIL; 24 hours later, immunohistochemistry was performed with anti-cleaved caspase-3 antibody: (a) S-TRAIL-incubated cells, and (b) control-incubated cells. Original magnification,  $\times 20$  (B).



**Figure 3.** Downregulation of Bcl-2 using lentiviral-delivered shRNA against Bcl-2. (A) Gli36-EGFRvIII glioma cells were transduced with LV-shBcl-2 or LV-shGFP with different MOI ranging from 1 to 3, and cells were harvested 5 days after transduction. Proteins were resolved by SDS-PAGE and immunoblotted with anti-Bcl-2 and anti- $\alpha$ -tubulin antibodies. (B) U87 and U251 glioma cells were transduced with LV-shBcl-2 or LV-shGFP at an MOI of 3 and harvested after 5 days. Proteins were resolved and blotted as in (A). (C) Different glioma lines were plated; 24 hours later, cell viability was determined using a luminescent ATP-based assay.

present in mitochondrial fractions of untreated shBcl-2 glioma cells compared to S-TRAIL-treated shBcl-2 glioma cells (Figure 4C). Furthermore, cell viability in shBcl-2-expressing and shGFP-expressing gliomas treated with S-TRAIL was significantly lower ( $P < .02$ ) in shBcl-2-expressing cells than in shGFP cells (Figure 4D). These results strongly suggest that downregulation of Bcl-2 sensitizes EGFRvIII-expressing glioma cells to S-TRAIL-mediated apoptosis.

To monitor the therapeutic efficacy of S-TRAIL *in vivo*, Gli36-EGFRvIII cells were transduced with LV-Fluc, and the resulting Gli36-EGFRvIII-Fluc cells were shown to retain the same proliferative capacity as Gli36-EGFRvIII glioma cells (Figure 5A). Different concentrations of Gli36-EGFRvIII-Fluc cells (ranging from  $5 \times 10^4$  to  $5 \times 10^6$ ) were implanted subcutaneously, and mice were imaged for Fluc activity 24 hours later. Bioluminescent signal correlated linearly with the number of implanted Gli36-EGFRvIII-Fluc cells in the ranges tested (Figure 5B), thus allowing the quantification of cells *in vivo* in mouse models of glioma. To demonstrate the effect of Bcl-2 downregulation on S-TRAIL-mediated apoptosis *in vivo*, we also transduced Gli36-EGFRvIII-Fluc-expressing shGFP or shBcl-2 glioma cells in culture with LV-S-TRAIL, mixed 20% of LV-S-TRAIL-transduced cells with 80% of Gli36-EGFRvIII-Fluc cells, and implanted them subcutaneously into nude mice. The combined effect of Bcl-2 downregulation and S-TRAIL secreted by transduced cells on tumor growth was followed by Fluc bioluminescence imaging on days 4, 6, and 8 after implantation (Figure 5C). A significant decrease in Fluc signal was seen as early as day 4 ( $P < .02$ ) in shBcl-2-downregulated gliomas; on day 8, the tumors were eradicated in shBcl-2-downregulated gliomas compared to shGFP-downregulated controls treated with S-TRAIL. The results show that downregulation of Bcl-2 and subsequent S-TRAIL treatment result in a substantial decrease in tumor growth compared to implanted tumors with intact Bcl-2 (Figure 5D).

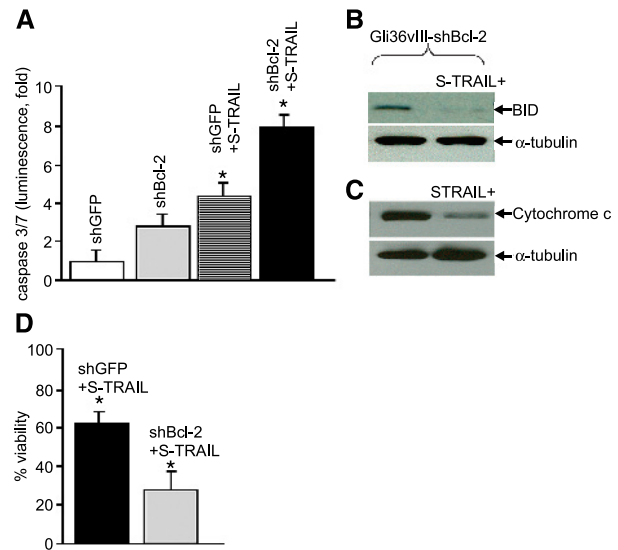
To verify whether S-TRAIL induced the upregulation of caspase-3 in glioma cells, we also performed cleaved caspase-3 staining on shBcl-2 and control tumor sections. A majority of glioma cells in the periphery of the tumor were

cleaved caspase-3-positive (Figure 6, A–D), indicating the induction of apoptosis by secreted S-TRAIL from the 20% LV-S-TRAIL-implanted cells. Immunostaining revealed a higher number of cleaved caspase-3-positive cells in Bcl-2-downregulated gliomas than in control gliomas (Figure 6E).

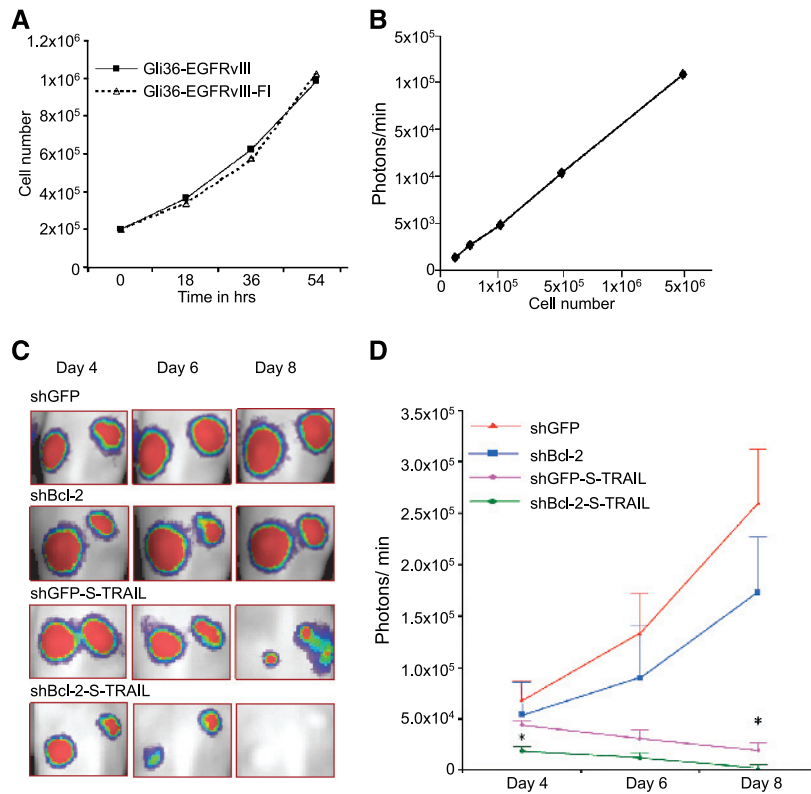
**Discussion**

In this study, we have engineered lentiviral vectors with shRNA, apoptosis-inducing agents, and *in vivo* marker genes, and we have shown that downregulation of antiapoptotic Bcl-2 enhances apoptosis mediated by S-TRAIL in a malignant model of glioma both *in vitro* and *in vivo*. Furthermore, we demonstrate that the enhanced apoptosis-inducing ability in Bcl-2-downregulated gliomas can be followed in real time by bioluminescence imaging in live animals.

There is emerging evidence that supports the role of key proteins in controlling cell proliferation, apoptosis, and angiogenesis in the pathogenesis and progression of cancer [24]. Among these proteins, Bcl-2 has been regarded as a potential therapeutic target on the basis of its ability to disrupt apoptosis and to confer resistance to chemotherapy and radiotherapy in cancer cells [12,25–28]. Human glioma cells express a variety of antiapoptotic and proapoptotic Bcl-2 family proteins [29], and overexpression of Bcl-2 has been shown to induce complex changes in glioma cell phenotype in that it not only protects glioma cells from various proapoptotic stimuli [30] but also enhances their motility [31]. We



**Figure 4.** Combined effect of the downregulation of Bcl-2 and S-TRAIL on glioma cells. (A) Gli36-EGFRvIII glioma cells expressing shBcl-2 or shGFP were treated with 40 ng/ml TRAIL; 24 hours later, caspase-3 and caspase-7 activity was determined by using ApoONE Homogeneous Caspase 3/7 Assay. (B) S-TRAIL-treated cells were harvested, and proteins were resolved by SDS-PAGE and immunoblotted with anti-BID antibody. (C) Cytochrome c assays were performed on S-TRAIL-treated cells by isolating mitochondrial fractions resolving the proteins by SDS-PAGE and immunoblotting by anti-cytochrome c antibody. (D) Gli36-EGFRvIII glioma cells expressing shBcl-2 or shGFP were treated with 40 ng/ml TRAIL; 16 hours later, cell viability was determined by using a luminescent ATP-based assay ( $*P < .02$ ).



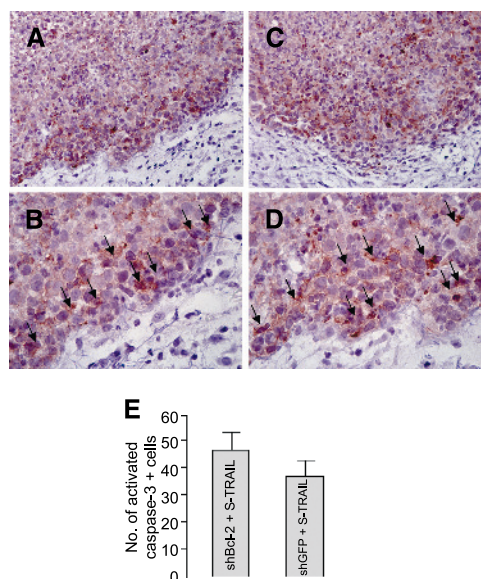
**Figure 5.** Enhanced S-TRAIL-induced apoptosis in Bcl-2-downregulated gliomas in vivo. (A) Gli36-EGFRvIII and Gli36-EGFRvIII-FI cells were grown in culture, and growth over time was compared. (B) Different concentrations of Gli36-EGFRvIII-FI cells were implanted subcutaneously; 24 hours later, mice were injected intraperitoneally with *D*-luciferin and imaged for Fluc activity, and the co-relation between the number of implanted cells and photon intensity was plotted. (C) Mice bearing subcutaneous gliomas: (i) Gli36-EGFRvIII-FI-shGFP, (ii) Gli36-EGFRvIII-FI-shBcl-2, (iii) Gli36-EGFRvIII-FI-shGFP-S-TRAIL, and (iv) Gli36-EGFRvIII-FI-shBcl-2-S-TRAIL were injected intraperitoneally with *D*-luciferin and imaged for Fluc activity on days 4, 6, and 8 after implantation. One representative mouse from each group is shown, and each image represents a scan time of 1 minute. A pseudocolor image represents the spatial distribution of photon counts produced by active luciferase within the tumor (D). Fluc bioluminescence intensity of tumors over time, given as the average of tumors in 10 animals, is shown (\* $P < .02$ ).

have employed shRNA to inhibit Bcl-2 gene expression as they show features superior to those of antibodies and inhibitors, in part because they are easily applicable to their target, including intracellular factors and even transcription factors. Furthermore, they promise potent gene inhibition with exquisite selectivity, even down to the level of single-nucleotide polymorphisms [32]. Our Bcl-2 immunoblotting results reveal that there is no downregulation of Bcl-2 at an MOI of 1; with higher MOI, this downregulation is specific and significant, and influences the proliferation capacity of the cells. Our findings on the effect of S-TRAIL on Bcl-2-downregulated glioma cells are in line with those of other studies showing the added effect of Bcl-2 downregulation with chemotherapy *in vitro* and *in vivo* in other cell types, including non-Hodgkin's lymphoma, Epstein-Barr virus-associated lymphoproliferative disease, malignant melanoma, prostate carcinoma, renal carcinoma, non-small cell lung carcinoma, breast carcinoma, and gastric carcinoma [3].

The use of death ligands for tumor therapy in animal models supports their potential for use in the clinical setting. TRAIL, a type II transmembrane protein, has become an attractive molecule for the treatment of cancers because of its potential to specifically kill tumor cells [8,33]. For efficient tumor therapy, it is important that the tumor cells producing the therapeutic protein not only undergo apop-

toxis by themselves but also induce apoptosis in surrounding cells. For this purpose, we have engineered a secretable version of TRAIL [9] and have shown that this form of TRAIL (S-TRAIL) has more potent apoptotic effects both *in vitro* and *in vivo* [9,10,34]. In this study, we have treated Bcl-2-downregulated gliomas with S-TRAIL and have shown that these gliomas have a significantly enhanced induction of apoptosis both in culture and *in vivo*. We [10,19,22] and others [35–37] have shown that intratumoral delivery of TRAIL has antitumor activity but requires multiple injections to have an antitumor effect [22]. To circumvent the use of multiple injections and to quantify the subtle changes in tumor growth in S-TRAIL-treated gliomas expressing shBcl-2, we implanted a mix of a defined number of LV-S-TRAIL-transduced glioma cells and nontransduced cells. LV-S-TRAIL-transduced cells produce S-TRAIL within the tumor and induce apoptosis in surrounding tumor cells. The production of S-TRAIL within the tumor allows to quantify differences between Bcl-2-downregulated and control GFP-downregulated gliomas *in vivo*. In this study, we have shown that 20% of TRAIL-expressing cells in gliomas is able to significantly reduce tumor burden in Bcl-2-downregulated gliomas *in vivo*.

The limited availability of noninvasive imaging methods to monitor molecular events has been one of the main



**Figure 6.** Immunohistochemistry detects activated caspase-3 in glioma cells. Mice implanted with a mix of LV-S-TRAIL or control vector–transduced and nontransduced Gli36-EGFRVIII-FI-shGFP or Gli36-EGFRVIII-FI-shBcl-2 cells (Figure 5) were sacrificed on day 6 after tumor cell implantation, and tumors were sectioned and stained with anti-caspase-3 antibodies. The stained sections were counterstained with hematoxylin. (A and B) Sections from S-TRAIL–expressing Gli36-EGFRVIII-FI-shGFP gliomas. (C and D) S-TRAIL–expressing Gli36-EGFRVIII-FI-shBcl-2 gliomas. Caspase-3–stained cells are shown by arrows. (E) The number of activated caspase-3 positive cells was calculated by counting the positive cells in randomly selected field of views under a microscope. Original magnification,  $\times 10$  (A and C) and  $\times 40$  (B and D).

limitations in testing the efficacy of various tumor therapy paradigms. In our previous studies, we have shown that we can quantify glioma burden *in vivo* using noninvasive bioluminescence imaging [22,34]. In the present study, we further demonstrate the sensitivity of luciferase-based bioluminescence imaging and show that we can follow the effect of S-TRAIL on Bcl-2–downregulated gliomas in real time, thus allowing us to quantify the combined effects of Bcl-2 downregulation and the induction of apoptosis by S-TRAIL. Furthermore, the presence of activated caspase-3–positive cells reveals the induction of apoptosis in gliomas through the S-TRAIL–mediated pathway. Most of the glioma is necrotic, except for cells in the tumor border that are still undergoing apoptosis and those cells to which the anti-cleaved caspase-3 antibody binds. The apoptotic process is a dynamic continuum of specific molecular events, and it is difficult to quantitate significant changes in caspase-3 activation between shBcl-2–downregulated and shGFP–downregulated gliomas at a particular time point.

In conclusion, we show that downregulation of Bcl-2 using lentivirally delivered shRNA enhances the apoptosis of glioma cells by S-TRAIL both *in vitro* and *in vivo*. The enhanced apoptosis in Bcl-2–downregulated gliomas can be monitored in real time by bioluminescence imaging in live animals. The engineering of clinically approved viral vectors expressing regulatable versions of S-TRAIL and shBcl-2 will be highly useful in tuning up this therapeutic approach.

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