

# The G-rich promoter and G-rich coding sequence of basic fibroblast growth factor are the targets of thalidomide in glioma

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## Abstract

Thalidomide is considered to be a potent antiangiogenic and immunomodulatory drug for cancer therapy. Earlier clinical studies have found that patients responding to this drug often had high plasma levels of basic fibroblast growth factor (bFGF). This cytokine is a proangiogenic factor overexpressed in many tumors and is also a regulator of limb development; hence, it might be a target of thalidomide. Using U-87 MG cell lines, we found that thalidomide, especially when encapsulated in a liposome, down-regulated the transcription and translation of the *FGF-2* gene by interacting with G-rich regions present in the promoter and the internal ribosome entry site of its transcript at concentrations much lower than therapeutic serum concentrations. Thalidomide treatment also dramatically suppressed the anchorage-independent growth of U-87 MG and other glioma cells by over a thousand fold without affecting its anchorage-dependent growth, which may be accomplished by knocking down endogenous bFGF expression in these cells. Accordingly, the addition of recombinant bFGF partially restored the anchorage-independent growth of these cells. Our data suggest that by targeting the G-rich regions of bFGF, thalidomide (at 0.1  $\mu\text{g}/\text{mL}$ ) can reduce cellular bFGF levels and affect tumor anchorage-independent growth, the hallmark of tumorigenicity. Our results are promising for future clinical investigations using low doses of thalidomide. [Mol Cancer Ther 2008;7(8):2405–14]

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## Introduction

Despite the very high risk of teratogenicity, thalidomide is emerging as a treatment for cancer and inflammatory diseases (1). Thalidomide is considered to be an effective drug for treating refractory multiple myeloma due to its antiangiogenic and immunomodulatory activities (2). In addition to myelomas, thalidomide has been widely tested on various types of tumors, such as renal cell carcinoma, prostate cancer, glioma, and Kaposi's sarcoma (3). Clinical efficacy in some inflammatory conditions, including graft-versus-host disease after allogeneic bone marrow transplantation and renal transplantation, further supports the immunomodulatory properties of thalidomide (3). Although current data provide much promise for the use of thalidomide in the treatment of these diseases, its mechanism of action is still incompletely understood. In addition, many clinical studies have searched for indicators for monitoring responses to thalidomide treatment in cancer patients. Some reports show a correlation between high pretreatment plasma basic fibroblast growth factor (bFGF) levels and a positive response to thalidomide treatment in glioma and multiple myeloma (4–6). The changes in serum bFGF levels during therapy imply that bFGF may be the target of thalidomide.

bFGF belongs to the FGF gene family and is a potent autocrine and paracrine mitogen that is ubiquitously expressed. Secretion of bFGF is independent of the traditional ER-Golgi pathways (7). Besides the secreted form, which is translated using the first AUG codon, there exist four nuclear target forms of bFGF. These four forms are translated differently from upstream in-frame CUG codons through an internal ribosome entry site (IRES)-dependent mechanism. Due to the different intracellular distribution and the NH<sub>2</sub>-terminal extension of high molecular weight (HMW) bFGFs, the functions of these HMW bFGFs compared with the low molecular weight (LMW) bFGFs are believed to be different (8). bFGF is overexpressed in various types of tumors, such as glioma and renal cancer, and increased expression of bFGF has been found to be correlated with disease progression (9, 10). The expression of bFGF transcripts is under the control of a G-rich promoter, which may be capable of forming secondary structures such as G-quadruplexes. These complex nucleic acid structures are targeted by some DNA-binding drugs, which interact with and subsequently alter promoter activity (11). In addition to transcriptional regulation by the G-rich promoter, the NH<sub>2</sub>-terminal-extended bFGF coding sequence is also G-rich, which may function to regulate translation of different isoforms (12, 13). This RNA region may also serve as a target for some DNA-binding drugs and consequently modulate expression of the isoforms.

The teratogenic activity of thalidomide was proposed to be mediated by its binding to both the DNA and RNA of the fetus when administered either p.o. or i.p. Consequently, binding of the thalidomide glutarimide moiety to DNA might alter the secondary structure of the DNA (14, 15). Drucker et al. (16) reported that thalidomide at relative high concentrations (>25  $\mu\text{g}/\text{mL}$ ) could down-regulate transcription for genes with GC-rich promoters. Additionally, Stephens et al. (17) proposed that thalidomide may inhibit insulin-like growth factor–induced and bFGF-induced limb genesis because both genes were under the control of GC-rich promoters. The transcriptional and the translational regulation of bFGF are under the control of G-rich–containing sequences, which can interact with thalidomide. Considering that bFGF is a ubiquitous growth factor involved in many biological activities (18), we hypothesized that the G-rich sequences of bFGF are the major targets of thalidomide. The present study was to determine the possible molecular mechanism of thalidomide as well as to highlight the feasibility of using low-dose thalidomide to treat glioma in a clinical setting.

## Materials and Methods

### Cell Culture and Stable Transfection

U-87 MG, C6, and 293T cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. GBM 8401 cells were obtained from Bioresources Collection and Research Center and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. For stable transfections,  $2 \times 10^5$  cells were plated in six-well plates 24 h before transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's suggestion. Stable transfectants were selected using 800  $\mu\text{g}/\text{mL}$  geneticin (Merck Biosciences) for 1 mo.

### Thalidomide Treatment

Thalidomide (a gift by TYY Biopharm) was dissolved in DMSO to a stock concentration of 50  $\text{mg}/\text{mL}$ . Further dilutions to the desired drug concentration were carried out in culture medium. The maximum final concentration of DMSO in all cultures was 0.02%. For the preincubation test, thalidomide was diluted to the indicated concentrations in culture medium alone and incubated in the  $\text{CO}_2$  incubator for 9 h before it was added to the cells.

### RNA Isolation and Real-time PCR

Total RNA was extracted using RNA-Bee RNA isolation solvent (Tel-Test) and 5  $\mu\text{g}$  RNA was then converted into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega). Real-time PCR primers targeting human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and bFGF were designed using Primer Express software (Applied Biosystems) and the sequences were shown in Supplementary Table S1.<sup>3</sup> The ABI Prism 7700

Sequence Detection System and the SYBR Green Master Mix kit (both from Applied Biosystems) were used for the real-time PCR analysis of the reverse-transcribed cDNA samples. The expression level of human GAPDH was used as an internal reference. Relative gene expression levels were calculated with the  $2^{-\Delta\Delta\text{CT}}$  method.

### Liposome-Encapsulated Thalidomide Preparation

Liposome-encapsulated thalidomide was prepared according to the method described previously (19) with minor modifications. To determine the thalidomide content within the liposomes, liposomal thalidomide was dissolved in methanol and the UV absorbance at 230 nm was used to quantify the thalidomide concentration.

### Immunofluorescence

Cells grown on glass coverslips were fixed in PBS containing 4% paraformaldehyde for 15 min and then permeabilized with 0.01% Triton X-100 for 30 min at room temperature. The cells were subsequently treated with 0.5  $\mu\text{g}$  of a polyclonal rabbit anti-human bFGF peptide, amino acids 40 to 63, antibody (Abcam) for 30 min at room temperature. Cells were then washed and stained with FITC-conjugated goat anti-rabbit IgG for another 30 min (1:200; Jackson ImmunoResearch). The cells were then washed and visualized with a fluorescence microscope (Olympus Optical Co.).

### Immunoblot and ELISA

Cell lysates were prepared using a radioimmunoprecipitation assay lysis buffer. For immunoblot analyses, equal amounts (2  $\mu\text{g}$ ) of protein samples were resolved on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane before probing with an anti-bFGF polyclonal rabbit antibody (Abcam) at 1:200 dilution. Anti-GAPDH (Abcam) at 1:10,000 dilution was also used as an internal control. Proteins were stained using horseradish peroxidase–conjugated anti-IgG secondary antibody and enhanced chemiluminescence for the detection (Amersham). For ELISA analysis, 1  $\mu\text{g}$  of total cellular protein was used and the bFGF ELISA was done according to the manufacturer's protocol (R&D Systems).

### Cell Proliferation Assay

For proliferation assays, cells were treated with or without thalidomide for 72 h and the relative cell number was evaluated using a resazurin-based proliferation assay (20).

### Colony-Forming Assay

A total of 1,000 cells were plated in 0.3 mL of DMEM with 0.3% low-melting agarose and 10% fetal bovine serum, laying on top of 0.3 mL/well in a 24-well plate of DMEM with 0.5% agarose and 10% fetal bovine serum. After 2 wk, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich), plates were photographed, and colony numbers were counted. The results represent the mean of three individual experiments.

### Hanging Drop Assay

Cell suspension (20  $\mu\text{L}$ ;  $5 \times 10^4$  cells/mL) was spotted on the lid of a 6-cm culture dish. The lid was then placed back on the culture dish, with the cell suspension droplet facing down over the bottom of the dish, which contained 5 mL of culture medium for maintaining moisture during

<sup>3</sup>Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

the incubation. The spheroid formed 48 h later. Each spheroid was photographed by phase-contrast microscopy, and the aggregate percentage was assayed by calculating the aggregation ability from 20 spheroids for each assay condition.

#### PCR Amplification and Plasmid Construction

Genomic DNA was purified from U-87 MG cells. About 500 ng genomic DNA was used as a template and the amplification was done using an ABI 2700 Thermocycler and Taq polymerase (Genet Bio). The PCR fragments were amplified according to the following variables: 1 cycle for 10 min at 96°C; followed by 35 cycles of 40 s at 95°C, 40 s at 58°C, and 1 min at 72°C; followed by 1 cycle for 7 min at 72°C that was then maintained at 4°C. The primers used to amplify the bFGF promoter, LMW-IRES, or HMW-IRES are shown in Supplementary Table S1.<sup>3</sup> For plasmid construction, PCR products were T/A cloned into a pGEMT-easy vector (Promega). The desired fragment was then either subcloned into a pEGFP-N2 vector (BD Biosciences Clontech) with *EcoRI* and *NotI* sites to generate a pbFGF-EGFP plasmid or subcloned into a bicistronic vector system (Promega) with a *MluI* site to form either pLMW-IRES or pHMW-IRES.

#### Dual Luciferase Assay

We carried out the luciferase assays using a Dual Luciferase Reporter Assay System according to protocols specified by the manufacturer (Promega), and the luciferase activity was quantified by scintillation counting in a Victor2 1420 Multilabel Counter (Wallac, Perkin-Elmer). The relative IRES activity was represented as an index of the ratio of *Renilla* luciferase to firefly luciferase normalized to the untreated control. All experiments were done in triplicate and repeated at least twice.

#### DNA Fragment Synthesis and UV-VIS Spectrophotometry

The G-rich and control fragments selected from the bFGF cDNA sequence were synthesized and purified by high-performance liquid chromatography (ScioPharm). Next, 0.3 μmol/L thalidomide in water was incubated with 0.96 μmol/L of the control DNA fragment or 0.52 μmol/L of the G-rich DNA fragment at room temperature for 2 h. The G-rich fragment had one G residue per molecule of thalidomide. The UV-VIS absorbance was assayed using a Hitachi U2000 Spectrophotometer with the scanning range from 330 to 190 nm.

#### Preparations of bFGF Knockdown U-87 MG Cells

For lentivirus production, 293T cells were transfected with 20 μg pLKO.1-puro lentiviral vectors expressing nontarget control short hairpin RNA (shRNA) or one of three different bFGF shRNAs (shRNA #1, #2, and #3; Supplementary Table S2)<sup>3</sup> along with 6 μg of envelope plasmid pMD.G and 15 μg of packaging plasmid pCMVΔR8.91 (National RNAi Core Facility of Academia Sinica, Taipei, Taiwan). Virus was collected 40 and 64 h after transfection according to the guidelines provided by the National RNAi Core Facility. To prepare bFGF knockdown cells, U-87 MG cells were infected with 100 μL lentivirus for 24 h. Fresh medium containing 1 μg/mL puromycin

(Sigma-Aldrich) was replaced every 3 d for 2 wk for drug-resistant cell selection. After selection, one U-87 MG clone for each shRNA target was chosen for further analysis. The names of the knockdown clones followed a scheme such that clone #1 represented the clone derived from shRNA #1-targeted U-87 MG stable cell and so forth.

#### Tumor Xenograft

Fifteen 8-wk-old athymic female mice were implanted s.c. with control knockdown cells ( $1 \times 10^6$  per mouse) in the left flank and then randomly divided into three groups. Three bFGF knockdown clones ( $1 \times 10^6$  cells per mouse) were then implanted s.c. in the right flank. The tumor size was calculated by external caliper measurements every other day.

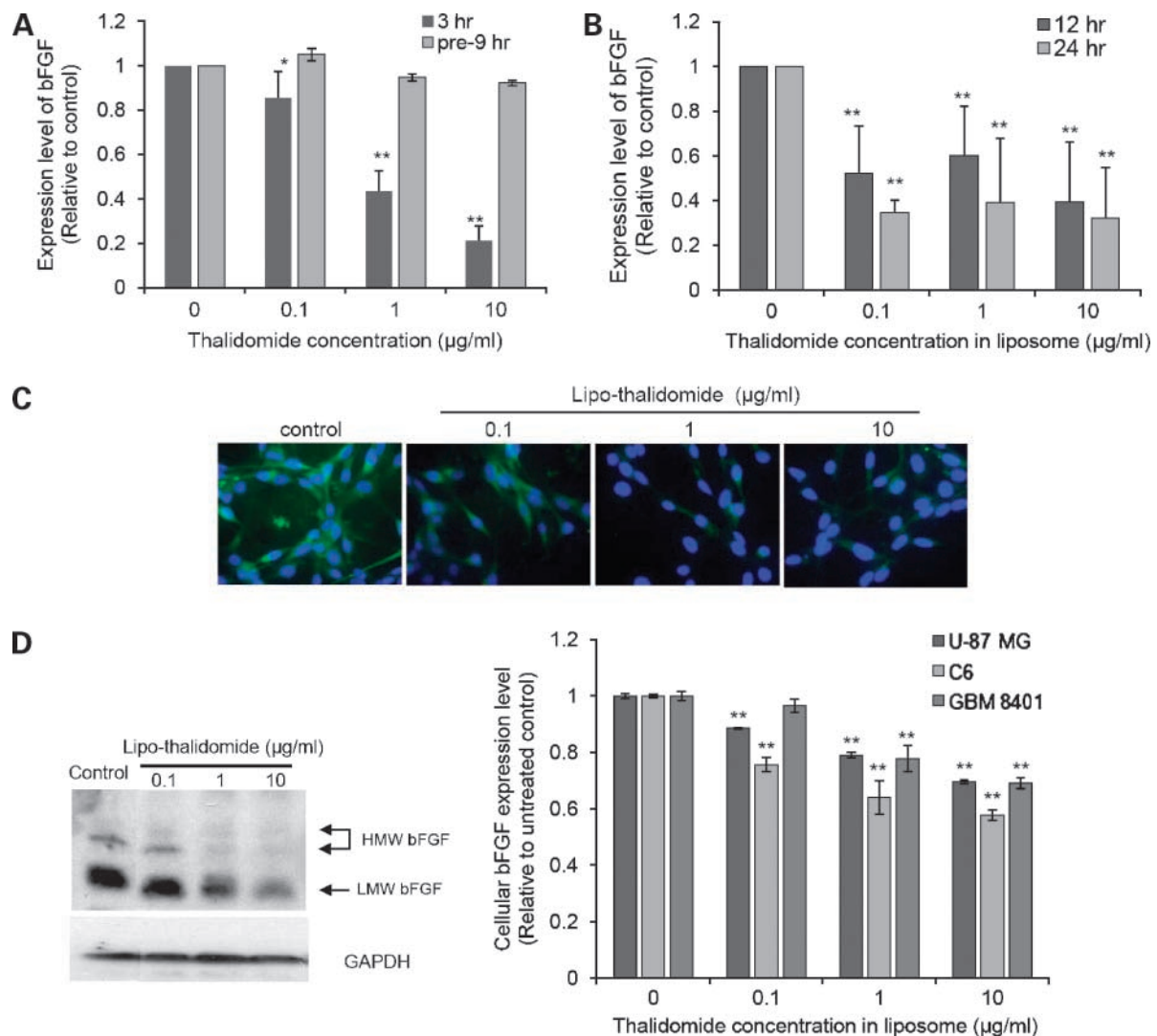
#### Calibration of Cell Number in Spheroid by Methylene Blue Staining

The spheroids were first transferred into a 96-well microplate. After attachment for about 12 h, the spheroids were stained with methylene blue in methanol (200 μL of 5 g/L) for 30 min. The wells were washed five times with tap water to remove excess dye and the plates were allowed to dry overnight (25°C). The stained spheroids were solubilized with 200 μL of 2% SDS for 24 h (25°C). The absorbance of the spheroid lysate, which was proportional to the number of cells in the spheroid, was measured at 650 nm using the PowerWave HT 340 (BioTek).

## Results

### Thalidomide Regulates bFGF Expression and Cellular Distribution in Glioma Cells

To examine the antitumor effect of thalidomide, we used U-87 MG cells, which are a high-grade human glioblastoma cell line expressing high basal levels of bFGF (21). These cells were used as our model because patients who showed a positive clinical response to this drug always showed a high plasma bFGF level before treatment, which decreased significantly during therapy (4–6). Real-time reverse transcription-PCR analysis was used to assess the mRNA levels of bFGF in U-87 MG cells with and without thalidomide treatment. As shown in Fig. 1A, bFGF mRNA levels in U-87 MG cells were markedly reduced after being treated with thalidomide for 3 h even at concentrations lower than the reported therapeutic dose (3–6 μg/mL; ref. 22). However, when cells were treated with thalidomide for longer periods, the inhibitory effect on bFGF expression diminished (data not shown). Because thalidomide has been reported to be highly susceptible to hydrolysis in solution (23), it was not surprising to find that this drug completely lost its activity even after a short (9 h) incubation in culture medium (Fig. 1A). To increase the stability of thalidomide in aqueous solution, we encapsulated it in liposomes formed from a mixture of phosphatidylcholine and cholesterol (4:1) as previously described (19). Interestingly, a significant inhibition of bFGF expression in U-87 MG cells by thalidomide was detected even 24 h after drug addition, but the dose response was no longer seen (Fig. 1B). Because the 5'-end of the bFGF transcript is GC-rich, we postulated that translation of bFGF may also be affected by thalidomide.



**Figure 1.** Thalidomide regulates bFGF expression and cellular distribution in glioma cells. *bFGF* mRNA expression relative to *GAPDH* in U-87 MG cells treated with freshly prepared thalidomide or thalidomide with a 9-h preincubation with culture medium for 3 h (**A**) or treated with liposome-encapsulated thalidomide for 12 or 24 h (**B**) was analyzed by real-time PCR. Data were collected from at least three independent experiments. *Columns*, relative index of untreated or empty liposome-treated control; *bars*, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's *t* test. **C**, immunofluorescence detection of bFGF in U-87 MG cells treated with liposome-encapsulated thalidomide for 12 h. Cellular distribution of bFGF was studied by fluorescence microscopy. DNA was stained with H33258 as a nuclear marker (see also Supplementary Fig. S2). Magnification,  $\times 400$ . **D**, protein extracts from U-87 MG cells treated liposome-encapsulated thalidomide for 12 h were subjected to Western blot analysis of bFGF expression. *Left*, GAPDH was used as an internal control. U-87 MG, C6, and GBM 8401 cells were treated with liposome-encapsulated thalidomide for 24 h. Protein extracts (1  $\mu$ g) were used to determine cellular bFGF levels by ELISA. *Right*, data were normalized with empty liposome-treated control.

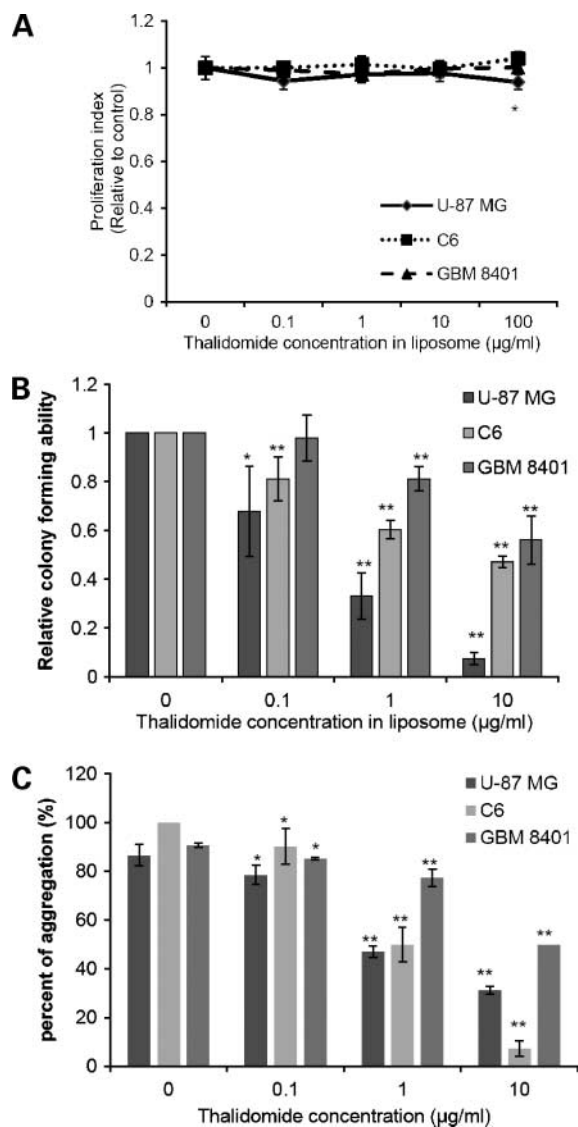
Indeed, our immunofluorescence staining data showed that thalidomide down-regulated cellular bFGF content especially at the nuclear level (Fig. 1C; Supplementary Figs. S1 and S2).<sup>3</sup> Because HMW bFGFs were the major isoforms localized in the nucleus (24), a decrease in signal intensity in this compartment may reflect reduced expression of HMW bFGFs. Western blot analysis was therefore done to analyze the levels of the various bFGF isoforms, and GAPDH was used as the internal control because the level of GAPDH is not affected by thalidomide. Although both HMW and LMW bFGFs are translated from the same transcripts, thalidomide decreased the level of HMW bFGFs more than

the LMW bFGF levels (Fig. 1D, *left*). To understand whether the down-regulation of bFGF levels in U-87 MG cells is cell line specific or a general event, we used ELISA to assess bFGF expression in another two glioma cell lines, C6 and GBM 8401. These data showed that, as in U-87 MG cells, thalidomide significantly diminished the expression of bFGF in the other two glioma cells in a dose-dependent manner (Fig. 1D, *right*; Supplementary Table S3).<sup>3</sup>

#### Effects of Thalidomide on Cell Proliferation and Anchorage-Independent Growth

Because thalidomide could down-regulate bFGF expression in glioma cells, we next evaluated its effects on growth

of these cells for that expression of bFGF in glioma cells has been reportedly correlated with cell proliferation and colony formation in soft agar in glioma cells (25). As shown in Fig. 2A, only a high concentration (100  $\mu\text{g}/\text{mL}$ ) of



**Figure 2.** Effects of thalidomide on cell proliferation and anchorage-independent growth. **A**, cell proliferation ability for U-87 MG, C6, and GBM 8401 cells treated with indicated concentrations (0, 0.1, 1, 10, and 100  $\mu\text{g}/\text{mL}$ ) of liposome-encapsulated thalidomide for 72 h was assayed using a resazurin assay. Points, relative index versus empty liposome-treated cells; bars, SE. \*,  $P < 0.05$ , Student's  $t$  test. **B**, colony-forming ability of U-87 MG, C6, and GBM 8401 cells seeded in culture medium containing 10% FCS and varied concentrations of liposome-encapsulated thalidomide plus 0.3% agar (size,  $>0.1$  mm) were counted 14 d after treatment. Data were collected from three independent experiments, with each experiment repeated six times. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test. **C**, aggregation ability of U-87 MG, C6, and GBM 8401 cell suspension cultured in 20  $\mu\text{L}$  culture medium contained various concentrations of thalidomide (0, 0.1, 1, and 10  $\mu\text{g}/\text{mL}$ ). Cells were imaged using phase-contrast microscopy and the aggregation percentage was counted by calculating 20 droplets of each experiment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test.

thalidomide encapsulated in a liposome could slightly reduce the proliferation of U-87 MG cells, but it did not affect the proliferation of C6 and GBM 8401 cell lines. Because bFGF has been shown to promote cell transformation (26), a soft agar colony formation assay and hanging drop technique (27) were used to assess the effects of thalidomide on anchorage-independent and three-dimensional growth abilities of U-87 MG cells, respectively. Our soft agar data showed that down-regulation of colony number was correlated with a diminishment of cellular bFGF expression level in cells after thalidomide treatment (Fig. 2B; Supplementary Fig. S3).<sup>3</sup> Consistent with this observation, cell aggregates formed in spheroid culture were also abolished by thalidomide in a dose-dependent manner and could be restored by adding exogenous recombinant bFGFs (Fig. 2C; Supplementary Fig. S4).<sup>3</sup>

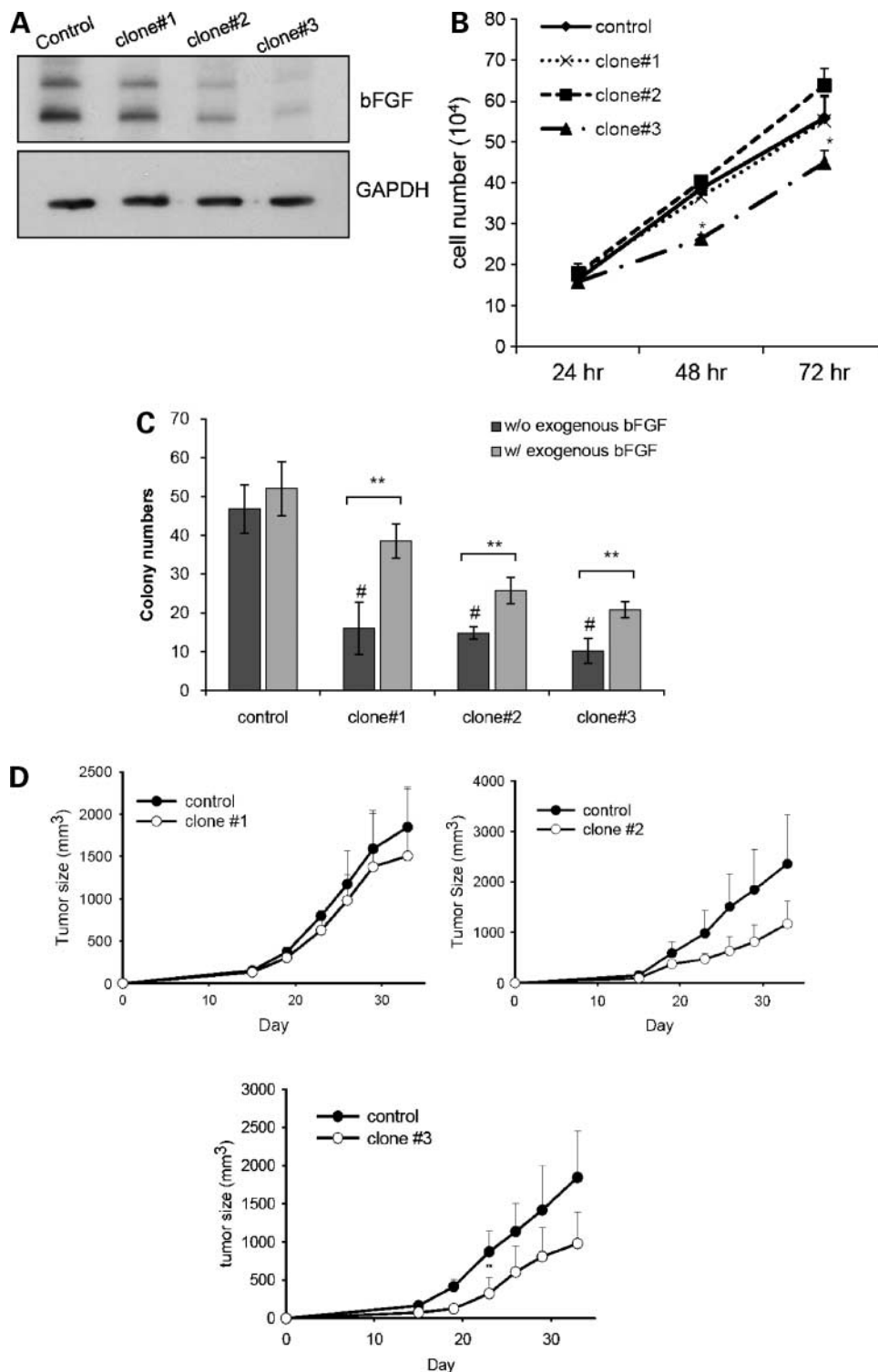
#### Anchorage-Independent Growth of U-87 MG Cells Is Suppressed by Knocking Down bFGF Expression

Having shown that thalidomide down-regulated bFGF expression and inhibited colony formation of glioma cells in soft agar, we next examined whether the tumorigenicity of these cells was reduced by down-regulation of bFGF expression. Three different bFGF shRNAs (#1, #2, and #3) or control shRNA was introduced into U-87 MG cells by lentivirus infection to generate three bFGF knockdown clones. We selected one clone for each shRNAs, called clone #1, #2, and #3, which had different efficacies in down-regulating the expression of endogenous bFGF (Fig. 3A). The doubling time of each target clone showed no significant difference from that of the control clone except clone #3 (Fig. 3B). However, the doubling time of the latter could be restored by the addition of recombinant bFGF (data not shown). Not surprisingly, the most significant inhibition of anchorage-independent growth in U-87 MG cells and in the tumor xenograft test was achieved by clone #3 (Fig. 3C and D). To distinguish between the contribution of LMW and HMW bFGF to anchorage-independent growth of U-87 MG cells, the aforementioned clones were incubated with recombinant LMW bFGF before colonies formed in soft agar, which were then counted. Although the number of colonies formed from the bFGF down-regulating cells was increased significantly by LMW bFGF supplementation, the anchorage-independent growth abilities were only partially restored by this treatment (Fig. 3C; Supplementary Table S4).<sup>3</sup> These findings suggest that nuclear bFGF (HMW ones) also plays an important role in cell transformation.

#### The Three-Dimensional Growth of U-87 MG Cells Was Diminished after Knocking Down Cellular bFGF Levels

Although the growth rates on microplate cultures of each knockdown clone, except clone #3, did not change (Fig. 3B), it was still not known whether a bFGF knockdown would affect formation of a spheroid by U-87 MG cells. By using the hanging drop method to force tumor cells to grow into spheroids, the time knockdown clones needed to aggregate was longer than the control's time (data not shown), and the spheroid size was significantly smaller (Fig. 4A). Because it was difficult to completely dissociate the formed spheroid into single cells, we used methylene blue staining

## 2410 G-Rich Sequence of bFGF Target of Thalidomide in Glioma



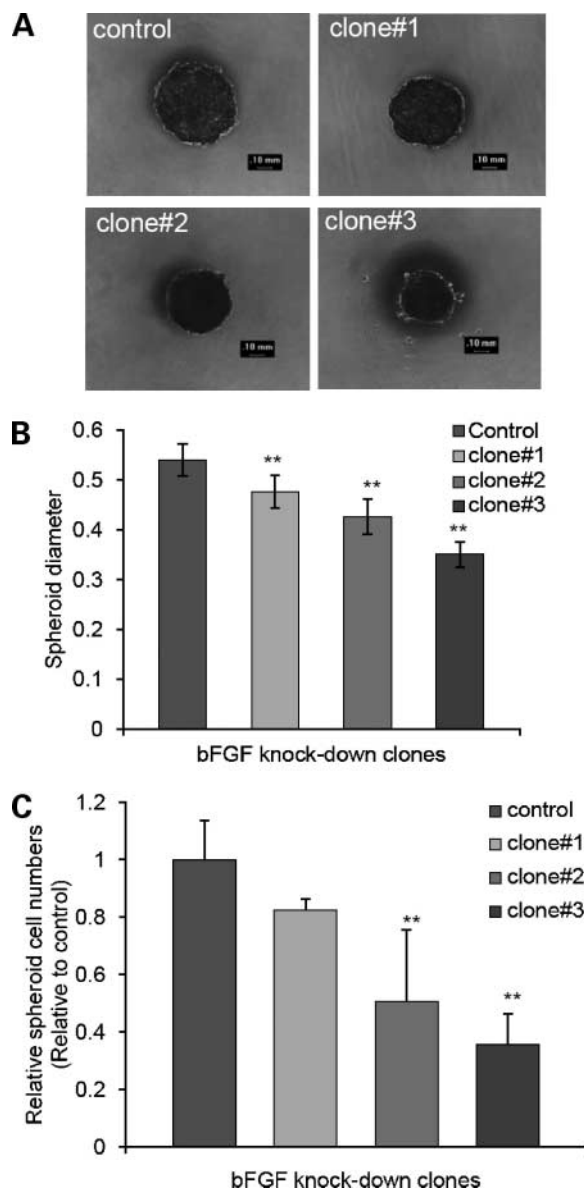
**Figure 3.** Down-regulating bFGF expression level is sufficient to decrease the anchorage-independent growth of U-87 MG cells, and exogenous bFGF can partially rescue the effect. **A**, cellular bFGF protein expression level in bFGF knockdown and control cells was assayed by Western blot. **B**, cell proliferation ability of each clone. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test. **C**, colony-forming ability of each knockdown clones in the presence or absence of exogenous bFGF protein. Colony-forming ability (size,  $>0.1$  mm) was measured 14 d later. Data were collected from three independent experiments, with each experiment repeated six times. #,  $P < 0.01$  versus control clone; \*\*,  $P < 0.01$  versus without exogenous bFGF condition, Student's  $t$  test. **D**, tumor growth of control and bFGF knockdown clones. Tumor size was determined by the following formula:  $1/2$  (length  $\times$  width<sup>2</sup>). Data were represented as mean values from three to five mice.

to measure the number of cells per spheroid. We found that the cell number also correlated with the cellular level of bFGF (Fig. 4B) and exogenous bFGF could accelerate cell proliferation in a dose-dependent manner (Supplementary

Table S5).<sup>3</sup> The recovery of spheroid size after adding exogenous bFGF revealed that the ability of U-87 MG cells to grow into a three-dimensional spheroid, unlike in soft agar, is likely dependent on the endocrine machinery of bFGF.

### Thalidomide Down-regulates bFGF Transcription and Translation by Regulating Its G-Rich Promoter and IRES Activity, Respectively

To evaluate the effect of thalidomide on transcription driven by the bFGF promoter, a pbFGF-EGFP plasmid, containing a portion of the bFGF promoter to drive the expression of EGFP, was stably transfected in U-87 MG cells to give U-87-bFGF-EGFP cells. Cells were treated with thalidomide (0.1–10  $\mu\text{g}/\text{mL}$ ) for different time intervals,



**Figure 4.** Down-regulating bFGF expression level in U-87 MG cells affects the growth rate in a three-dimensional spheroid culture and exogenous bFGF restores their proliferation ability. **A**, spheroid morphology of bFGF knock-down clones. **B**, the diameter of each spheroid was measured using Image-Pro Plus software on day 4. Scale bar, 0.1 mm. **C**, methylene blue uptake by each of the bFGF knockdown clones as an index of spheroid cell numbers. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test.

and then transcript levels and relative fluorescence indices were measured to evaluate the effect of thalidomide on the expression of EGFP. Thalidomide diminished EGFP levels in a dose-dependent pattern after a 3-h treatment but did not do so after longer incubation periods (Fig. 5A; Supplementary Fig. S5;<sup>3</sup> data not shown). Because down-regulation of the HMW bFGFs by thalidomide was more significant than LMW bFGF, we asked whether IRES-dependent translation of bFGF, which was reported to regulate the expression of different isoforms (8), was also affected by this drug. HMW and LMW bFGF IRES fragments were inserted into bicistronic vectors, as previously described (28), to generate pHMW-IRES and pLMW-IRES plasmids (Fig. 5B). Both plasmids were then stably transfected into U-87 MG cells to give U-87-HMW-IRES cells and U-87-LMW-IRES cells. After treating with liposome-encapsulated thalidomide for 12 h, IRES activity was estimated by calculating the ratio between firefly luciferase activity and *Renilla* luciferase activity (Fig. 5C). The former represented the translational efficiency of the upstream cistron, and the latter represented that of the individual bFGF IRES. We found that treating with thalidomide altered the IRES activity in a dose-dependent manner for both LMW-IRES and HMW-IRES.

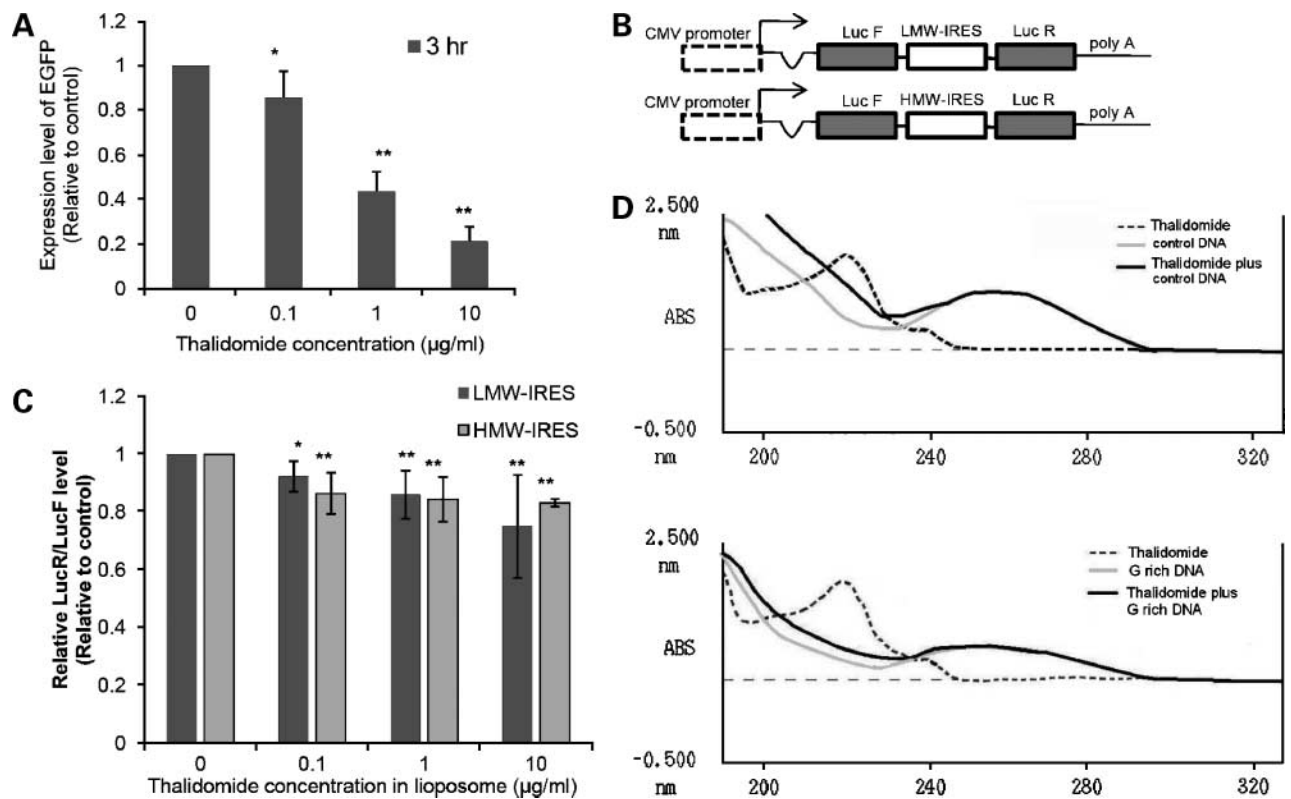
### The UV-VIS Absorbance of Thalidomide Is More Effectively Quenched by a G-Rich DNA Fragments

To examine whether thalidomide interacted preferentially with the G-rich coding sequence of bFGF, we measured the UV-VIS absorbance of thalidomide after incubation with a G-rich (nucleotides 363–428) or a non-G-rich (nucleotides 673–768) DNA fragment derived from bFGF. We hypothesized that the absorbance of thalidomide may be diminished more dramatically by the secondary structure of a DNA fragment that was bound more tightly by this drug (29). As shown in Fig. 5D, absorbance at 230 nm was quenched to a greater extent on thalidomide treatment when it was incubated with a G-rich DNA fragment, suggesting that this drug may bind preferentially with nucleic acids that have a high content of guanosine.

### Discussion

Thalidomide has been used and studied for more than 50 years, but its mechanisms of action are not fully understood. Many clinical trials using this drug have been conducted based on its antiangiogenic and immunomodulatory activities (30). Interestingly, positive responses to thalidomide treatment from some cancer patients have been shown to correlate with the changes in serum concentrations of angiogenic factors, such as vascular endothelial growth factor (VEGF), bFGF, and hepatocyte growth factor (4, 5, 22, 31). In our study, we found that a low concentration of thalidomide was sufficient to down-regulate bFGF transcript levels in U-87 MG cells in a dose-dependent manner. However, the inhibitory effect of thalidomide was lost quickly, which is most likely due to rapid hydrolysis in aqueous solution. To overcome this problem, we encapsulated the drug in a liposome that significantly prolonged the

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**Figure 5.** Thalidomide down-regulates bFGF transcription and translation by targeting its G-rich promoter and IRES region. **A**, EGFP mRNA expression relative to GAPDH in pbFGF-EGFP stably transfected U-87 MG cells treated with thalidomide for 3 h was analyzed by real-time PCR. Data represent at least three independent experiments. Columns, relative index of the untreated control; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test. **B**, schematic representation of LMW-IRES and HMW-IRES plasmids. **C**, IRES activity of U-87-LMW-IRES and U-87-HMW-IRES cells treated with indicated concentrations of liposome-encapsulated thalidomide for additional 12 h. Cells were lysed, and luciferase activity was assayed using a Dual Luciferase Assay kit. IRES activity was determined by calculating the *Renilla* luciferase (*LucR*) to firefly luciferase (*LucF*) ratio. Columns, index of the ratio of *Renilla* luciferase to firefly luciferase normalized to the empty liposome-treated control; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Student's  $t$  test. **D**, UV-VIS spectrum of thalidomide incubation with non-G-rich control DNA (top) or G-rich DNA (bottom).

inhibitory effects. Because thalidomide has been reported to bind DNA directly (32), we examined whether it might directly target the G-rich promoter of the *bFGF* gene. Our reporter expression and DNA-binding analyses (Fig. 5) suggested that the down-regulation of bFGF transcript levels by thalidomide is likely to be mediated by its direct interaction with the G-rich promoter of this gene. Intriguingly, the effective concentrations of thalidomide we found were much lower (0.1 and 1 µg/mL) than those used by other to suppress the G-rich human telomere reverse transcriptase promoter (12.5 and 25 µg/mL; ref. 16). Additionally, decreased bFGF protein levels were found in these cells after thalidomide treatment, which was associated with a change of nuclear localization of HMW bFGFs (Fig. 1). Surprisingly, the IRES activity present in both HMW and LMW bFGF transcripts (33) was shown to be down-regulated by thalidomide in a dose-dependent manner (Fig. 5). It has been suggested that cellular IRES activities may contribute to the development of several pathologic conditions in human, such as diabetes, cardiovascular disease, and the development and progression of cancer (34). Our results provide the rationale for future clinical

investigations into the clinical use of low-dose liposome-encapsulated thalidomide for treatment of diabetes, cardiovascular disease, and cancers, with the advantage of improving on current high-dose regimens, which lead to various side effects.

It is well known that solid tumors grow *in vivo* as multicellular masses in which a proportion of cells are deprived of normal contact with the basement membrane and are anoikis resistant. Cell lines derived from such solid tumors are capable of growing in an anchorage-independent manner as colonies in soft agar or suspension culture (35). The acquisition of anchorage independence is an important hallmark of cancer cells and is thought to be one of the critical factors in the growth and metastasis of cancer. Although certain signaling pathways have been proposed to abrogate the requirement for integrin-extracellular matrix-mediated signaling functions for anchorage-independent growth of cancer cells, the precise molecular mechanism is not fully understood (36, 37). In contrast to the ineffectiveness in suppressing the proliferation of glioma cells (Fig. 2A), thalidomide efficiently inhibited the anchorage-independent growth and aggregation of these cells at a low dose (Fig. 2B



and C), suggesting a novel tumor-suppressing mechanism of this drug. In this respect, positive correlations between the expression levels of bFGF and anchorage-independent growth of human fibroblasts, prostatic epithelial cells, and melanocytes have been reported (38–41). Therefore, the down-regulation of glioma colony formation in soft agar by thalidomide might be attributed to a decreased bFGF expression. This hypothesis was supported by our shRNA-mediated bFGF knockdown study, which clearly showed a positive correlation between cellular bFGF levels and the colony-forming ability of these cells in soft agar and *in vivo* tumor growth (Figs. 2B and 3C and D). However, because the addition of recombinant human bFGF only partially rescued the loss of the soft agar colony-forming ability of knockdown cells (Supplementary Table S3),<sup>3</sup> the contribution of intracrine signaling of HMW bFGFs to cell transformation was postulated. Although the precise role of nuclear bFGF in glioma cells is unclear, stimulation of fibroblast growth in low serum by nuclear bFGF has been reported (42). In addition, the nuclear accumulation of bFGFs or the increased ratio of HMW bFGFs to LMW bFGF has been reported as an indicator for tumor progression (43). Moreover, nuclear accumulation of bFGF in human astrocytic tumors has been shown as a useful predictor of patient survival (43). Integrating previous studies and our data, we thought that bFGF may function as an upstream regulator modulating adhesion molecules and other factors for anchorage-independent growth. Moreover, HMW bFGFs may also translocate into the nucleus and induce gene transcription in tumor cells for adapting to a more stringent environment (Supplementary Fig. S6).<sup>3</sup> In line with our findings, thalidomide may offer insight into a new strategy for the development of novel anticancer drugs based on the mechanisms of anchorage independence, instead of conventional anchorage dependence. Moreover, the cell growth features in tumors are somewhat similar to that seen in embryo development. Thus, our data may highlight a unified molecular mechanism for teratogenicity in embryonic development from anchorage-independent tumors.

The antiangiogenic activity was found for it can suppress bFGF-induced cornea vasculogenesis (44). In addition to bFGF, VEGF, hepatocyte growth factor, and IL-6 were important factors being considered in thalidomide treatment (4, 5, 31); however, the data were controversial among these studies and the regulatory mechanisms are still unknown. Like bFGF, the expression of VEGF is also under the control of a GC-rich promoter. Our preliminary data have found that thalidomide can also regulate the GC-rich promoter of VEGF in low-dose treatment. Furthermore, it has been shown that bFGF could induce VEGF expression through autocrine and paracrine mechanisms (45). We suggest that the down-regulation of cellular bFGF levels by thalidomide in glioma might also lead to the sequential down-regulation of gene expression for VEGF.

In conclusion, our results showed that thalidomide down-regulated bFGF expression in high bFGF-expressing glioma cells and it mediated this effect by interacting with the G-rich sequences present in both the promoter

region and the transcripts of bFGF. Due to the reduction of this crucial cytokine, anchorage-independent growth was markedly suppressed by thalidomide, which was confirmed by our small interfering RNA-mediated bFGF knockdown study. The daily dose of thalidomide used clinically results in serum concentrations of 1.8 to 10  $\mu\text{g}/\text{mL}$  (30). According to our data, the dose needed for patients with elevated bFGF serum levels might be much lower than what is currently used, which might reduce side effects. Additionally, with proper drug delivery systems, such as liposomes, for preventing rapid hydrolysis in serum, the clinical side effects of thalidomide may be greatly reduced. Although bFGF is not the only angiogenic factor reported to be affected by thalidomide, it serves as an upstream regulator for angiogenesis (45–47). The activity of thalidomide in cancer patients might be due not only to its down-regulation of the growth of tumor cells with high pretreatment bFGF expression levels but also for its suppression of bFGF-regulated angiogenesis (Supplementary Fig. S7).<sup>3</sup>

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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