

Combination of hTERT Knockdown and IFN- γ Treatment Inhibited Angiogenesis and Tumor Progression in Glioblastoma

Joseph George,¹ Naren L. Banik,² and Swapan K. Ray¹

Abstract **Purpose:** The limitless invasive and proliferative capacities of tumor cells are associated with telomerase and expression of its catalytic component, human telomerase reverse transcriptase (hTERT). IFN- γ modulates several cellular activities, including signaling pathways and cell cycle, through transcriptional regulation.

Experimental Design: Using a recombinant plasmid with hTERT siRNA cDNA, we downregulated hTERT during IFN- γ treatment in human glioblastoma SNB-19 and LN-18 cell lines and examined whether such a combination could inhibit angiogenesis and tumor growth in nude mice. *In vitro* angiogenesis assay was done using coculture of tumor cells with human microvascular endothelial cells. *In vivo* angiogenesis assay was done using diffusion chambers under the dorsal skin of nude mice. *In vivo* imaging of intracerebral tumorigenesis and longitudinal solid tumor development studies were conducted in nude mice.

Results: *In vitro* and *in vivo* angiogenesis assays showed inhibition of capillary-like network formation of microvascular endothelial cells and neovascularization under dorsal skin of nude mice, respectively. We observed inhibition of intracerebral tumorigenesis and s.c. solid tumor formation in nude mice after treatment with combination of hTERT siRNA and IFN- γ . Western blotting of solid tumor samples showed significant downregulation of the molecules that regulate cell invasion, angiogenesis, and tumor progression.

Conclusions: Our study showed that the combination of hTERT siRNA and IFN- γ effectively inhibited angiogenesis and tumor progression through the downregulation of molecules involved in these processes. Therefore, the combination of hTERT siRNA and IFN- γ is a promising therapeutic strategy for controlling the growth of human glioblastoma. (Clin Cancer Res 2009;15(23):7186–95)

Glioblastomas are highly invasive and aggressive brain tumors with a dismal prognosis (1). In the United States, >20,000 new cases of primary malignant brain tumors are diagnosed every year, accounting for 1.4% of all cancers and 2.3% of all cancer deaths (2). Because malignant brain tumor cells often infiltrate deep into the normal tissue, complete surgical removal of the brain tumor is almost impossible, contributing to the high incidence of recurrence (3). Although understanding of the glioblastoma pathophysiology has increased significantly over

the past few years, an effective treatment has not yet been developed for this devastating cancer. Advance gene therapy in combination with traditional treatment techniques to prolong the lifespan of cancer patients and control or cure the disease is very promising (4, 5).

Tumor invasion, angiogenesis, and metastasis are complex mechanisms that involve a variety of biochemical and cellular processes, including proteolytic degradation of the extracellular matrix (6). Studies focusing on matrix metallo-proteinases (MMP), especially MMP-9, have shown that the overexpression of these proteolytic enzymes actively involves the degradation of extracellular matrix proteins, thereby promoting tumor invasion, angiogenesis, and metastasis of most solid tumors, including brain tumors (7, 8). Extracellular matrix degradation simultaneously stimulates expression of vascular endothelial growth factor (VEGF) and angiogenesis (9). Inhibition of these processes may not only suppress tumor growth and invasion but also improve the prognosis for recurrent malignant brain tumors. Methods to inhibit cell invasion and angiogenesis would likely prevent the growth of glioblastomas.

Telomerase adds repeats of specific DNA sequence (TTAGGG) to the 3' end of DNA strands in the telomere regions. Human telomerase is upregulated in >85% of primary

Authors' Affiliations: ¹Department of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina and ²Department of Neurosciences, Medical University of South Carolina, Charleston, South Carolina

Received 6/3/09; revised 7/31/09; accepted 8/4/09; published OnlineFirst 11/24/09.

Grant support: R01 grants (CA-91460 and NS-57811) from the NIH. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Swapan K. Ray, Department of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, 6439 Garners Ferry Road, Columbia, SC 29209. Phone: 803-733-1593; Fax: 803-733-3192; E-mail: swapan.ray@uscmed.sc.edu.

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doi:10.1158/1078-0432.CCR-09-1425

Translational Relevance

The invasive and proliferative abilities of tumor cells are dependent on telomerase, which is tightly regulated by expression of human telomerase reverse transcriptase (hTERT). Telomerase is active in 90% human tumors, including glioblastoma, but inactive in most normal cells, making it an ideal target for cancer therapy. Because IFN- γ modulates >200 molecules at transcriptional and translational level, downregulation of hTERT in glioblastoma in the presence of IFN- γ may reduce angiogenesis and tumor growth. In our study, we downregulated hTERT in two human glioblastoma cell lines using plasmid vector-based siRNA during IFN- γ treatment and found significant inhibition of angiogenesis *in vitro* and *in vivo*. There was marked inhibition of orthotopic tumorigenesis and solid tumor formation in the s. c. region of immunocompromised mice after the treatment with combination of hTERT siRNA and IFN- γ . Therefore, the downregulation of hTERT during IFN- γ treatment is a promising therapeutic strategy to control the malignant growth of human glioblastoma.

cancers, including glioblastomas, and its activity is tightly controlled by expression of human telomerase reverse transcriptase (hTERT; refs. 10–12). Knockdown of hTERT can be an appropriate strategy to control the growth of glioblastomas because telomerase plays the key role in conferring cellular immortality.

IFN- γ is a multifunctional cytokine produced by T cells and natural killer cells. IFN- γ modulates several cellular activities, including signaling pathways, through transcriptional regulation (13, 14). It regulates >200 genes, producing a variety of physiologic and cellular responses (13). One of the key elements of all tumor cells is evasion from immunosurveillance. Many investigators have indicated that either neutralization of IFN- γ or inhibition of IFN- γ -mediated pathways promotes spontaneous tumor formation *in vivo* (15, 16), strongly supporting the involvement of IFN- γ in the process of immunosurveillance. Therefore, exposure of cancer cells to IFN- γ would be an ideal strategy to regulate tumor cell growth.

Using the technique of RNA interference, successful gene silencing can be achieved either through introduction of synthetic siRNA oligonucleotides (17) or their expression through a plasmid vector carrying a specific siRNA cDNA (18). The purpose of this investigation was to downregulate telomerase activity through the knockdown of hTERT using a plasmid vector carrying the cognate siRNA cDNA in combination with IFN- γ treatment in two highly invasive human glioblastoma SNB-19 and LN-18 cell lines and to examine whether such a combination could inhibit angiogenesis and tumor growth in nude mice. Furthermore, we wanted to elucidate the molecular mechanisms of inhibition of angiogenesis and tumor growth *in vivo* after treatment with hTERT siRNA and IFN- γ .

Materials and Methods

Cell culture conditions. Human glioblastoma SNB-19 cells were procured from the National Cancer Institute. Human glioblastoma LN-18 cells were purchased from American Type Culture Collection. We have selected these cell lines because PTEN is mutated and not expressed in SNB-19 cells and PTEN is wild type in LN-18 cells. We propagated SNB-19 cells in a 50:50 mixture of DMEM and Ham's F12 whereas LN-18 cells in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics in a humidified incubator containing 5% CO₂ at 37°C. Human IFN- γ (Sigma) was diluted in serum-free medium to a final concentration of 10 ng/mL.

Construction of hTERT siRNA expression vector. The hTERT siRNA cDNA with sense and antisense strands was constructed into a mammalian expression vector, pRNAT-CMV3.2/Neo (GenScript) between *Bam*HI and *Xho*I sites. We prepared three siRNA sequences to select the most effective one on the basis of percent knockdown of hTERT at the mRNA and protein levels. The selected siRNA sequence targeting human hTERT mRNA began at nucleotide 2035 (NM_198253): 5'-GGC ACT GTT CAG CGT GCT CTT-3' (sense) and 3'-GAG CAC GCT GAA CAG TGC C-5' (antisense). The scrambled siRNA sequence used was 5'-CCG TCG ACG CGT ACT TGG TTT-3' (sense) and 3'-CGG TCC AGA GCA TCA ACG G-5' (antisense). The loop selected was 5'-TTC AAG AGA-3'. The linear siRNA construct (with the sense and antisense strand, loop, termination signal, and *Bam*HI and *Xho*I restriction sites) was annealed to the complementary strand and ligated into the siRNA expression vector (pRNAT-CMV3.2/Neo) between the *Bam*HI and *Xho*I sites. In this vector, the cytomegalovirus promoter drives the expression of siRNA, and the SV40 promoter drives the expression of the resistance gene neomycin. This vector also carries coral green fluorescence protein for tracking of transfection efficiency in cell cultures. The siRNA sequence was confirmed by DNA sequencing. The plasmid vector carrying the hTERT siRNA cDNA was transformed into JM109 competent cells (Promega), and the positive colonies were screened using the Qiagen miniprep plasmid DNA purification kit (Qiagen). The highly expressing colony was selected and propagated in Luria-Bertani broth containing neomycin. The plasmid vector expressing hTERT siRNA was purified using maxiprep plasmid DNA purification kit (Qiagen) and used for cell culture and animal experiments.

Treatment of glioblastoma cells with hTERT siRNA plasmid vector and IFN- γ . Approximately 80% confluent cultures of SNB-19 and LN-18 cells were transfected with the plasmid vector carrying hTERT siRNA cDNA or treated with a final concentration of 10 ng IFN- γ per milliliter (10 ng IFN- γ corresponded to 100 units) or both agents together in serum-free medium. The plasmid vector was transfected with Eugene HD (Roche Diagnostics) in a mixture of 3:1 (3 μ L Eugene and 1 μ g DNA). Transfection efficiency was monitored with the expression of coral green fluorescence protein using a phase contrast fluorescent microscope (Olympus IX71). A set of cultures was also transfected with the plasmid vector carrying the scrambled siRNA cDNA sequences for hTERT. A dose of 10 ng/mL IFN- γ was selected based on a dose-response study for cell viability, as determined by the MTT assay (19). Higher concentrations of IFN- γ did not significantly decrease cell viability, whereas lower concentrations were found to be less effective. After 24 h, the medium was replaced with regular serum medium and the cultures were incubated for another 24 h.

Real-time reverse transcriptase-PCR (RT-PCR) for hTERT mRNA. We carried out real-time RT-PCR experiments to determine the downregulation of hTERT mRNA after transfection with the plasmid vector carrying hTERT siRNA cDNA or treatment with IFN- γ or both agents together. Total cellular RNA was isolated using the Aurum kit (Bio-Rad). We used the following primer sequences for PCR amplification of hTERT gene (NM_198253): forward, 5'-CAC CAA GAA GTT CAT CTC C-3'; and reverse, 5'-CAA GTG CTG TCT GAT TCC-3'. Real-time RT-PCR was carried out using a one-step RT-PCR kit with Synergy Bands (SYBR) green dye (Bio-Rad) on a real-time PCR machine (iCycler iQ5, Bio-Rad) with the following reaction conditions: cDNA synthesis

at 50°C for 10 min; reverse transcriptase inactivation at 95°C for 5 min; PCR cycling and detection at 95°C for 10 s; and data collection at 56°C for 30 s. We used 100 ng of total isolated RNA for transcription.

Western blotting for hTERT. Western blotting was carried out for hTERT protein levels using hTERT antibody (Santa Cruz Biotechnology), as described below. The membranes were reprobed with Western reprobe buffer (Gbiosciences) and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content using a GAPDH monoclonal antibody (Novus Biologicals). The Western blotting images were quantified using Gel-Pro analyzer software (Media Cybernetics).

In vitro angiogenesis assay. *In vitro* angiogenesis assay (20) was carried out to evaluate the effect of hTERT siRNA and/or IFN- γ on the network formation of human microvascular endothelial cells (Cascade Biologicals) in coculture. SNB-19 and LN-18 cells (1×10^4) were seeded into four-well chamber slides. After 24 h, the cells were transfected with the plasmid vector carrying hTERT siRNA cDNA or treated with IFN- γ or both agents together in serum-free medium. The cells were incubated for 24 h and then cocultured with 2×10^4 human microvascular endothelial cells in a 50:50 mixture of serum-free medium and human microvascular endothelial medium (Medium 131, Cascade Biologicals). The cocultures were terminated after 72 h; cells were fixed in 95% cold ethanol and treated with the von Willebrand factor (factor VIII) antibody, followed by biotinylated secondary antibody. After washings, the slides were further treated with horseradish peroxidase-labeled streptavidin. The final stain was developed using 3% 3-amino-9-ethylcarbazole in *N,N*-dimethylformamide. The cells were viewed under a microscope (Olympus BH2) and were photographed. The images were quantified for network formation using Image-Pro Discovery software (Media Cybernetics).

In vivo angiogenesis assay (dorsal skin fold chamber model). Dorsal skin fold chamber model (21) was used to examine the antiangiogenic effect of hTERT siRNA and/or IFN- γ on SNB-19 and LN-18 cells. The diffusion chamber rings (Millipore) were prepared with Millipore membrane filters (0.45 μ m), sterilized by UV irradiation, and then injected with 200 μ L of cell suspension (2×10^5 cells) after treatment with either the hTERT siRNA plasmid vector or IFN- γ or both agents together for 48 h. The opening of the chamber was sealed with sterile bone wax, and the chambers were surgically implanted under the dorsal skin of nude mice (Charles River Laboratories). After 10 days, the chambers were removed surgically, and the superficial fascia exposed to the chamber was harvested. The formation of new vasculature (neovascularization) was distinguished from pre-existing vessels as curved thin structures in zigzag pattern using a stereomicroscope (Olympus SZX12) equipped with a Spot RT Slider digital camera (Meyer Instruments) and was photographed. The tumor-induced neovascularization was measured and quantified with the help of an ocular micrometer.

Orthotopic tumorigenesis in nude mice. SNB-19 and LN-18 cells were stably transfected with a mammalian expression vector carrying the luciferase gene *phCMV-FSR* (Genlantis) and were propagated in a medium containing G-418 (500 μ g/mL). The cells were highly homogeneous and were propagated from a single cell. The cells were harvested and counted, and 1×10^6 cells were suspended in 10 μ L of serum-free medium and were injected into the intracerebrum of nude mice, using a 25- μ L Hamilton syringe with the help of a digital stereotaxic apparatus (Stoelting), after drilling a small hole on the skull. The animals were left for 3 days without any treatment. Afterward, the mice received intrathecal injection of either the hTERT siRNA plasmid vector (5 μ g DNA per injection per mouse) or IFN- γ (10^3 units per injection per mouse) or both agents on alternate days for 20 days. The plasmid vector carrying hTERT siRNA cDNA was suspended in RNase-free sterile water (25 μ g DNA/10 μ L) and was mixed (v/v, 1:4) with i-Fect transfection reagent (Neuromics) to obtain 5 μ g DNA/10 μ L of injection volume. The injections were given using a Hamilton syringe with the help of a stereotaxic apparatus at the site of tumor cell plantation. In the case of combination treatment, IFN- γ was injected first (10 μ L in serum-free medium), followed by siRNA plasmid vector with an interval of 10 min. One set of mice received similar injections of scrambled siRNA vector, whereas another set was left untreated. On day 21, the mice

were injected i.p. with 100 μ L (50 mg/mL) luciferin (Genlantis). After 10 min, the mice were visualized for luciferase activity using the Xenogen IVIS-200 imaging system. Six mice were used in each group. All animal experiments were done in compliance with our Institutional Animal Care and Use Committee.

Solid tumor development in nude mice. The effect of hTERT siRNA and/or IFN- γ on solid tumor development under the dorsal skin of nude mice was studied. Because SNB-19 cells grow very slowly to form a s.c. solid tumor, we have used only LN-18 cells to study s.c. solid tumor development. Approximately 80% confluent cultures of LN-18 cells were harvested and counted, and 6×10^7 cells suspended in 300 μ L of serum-free medium were mixed with 300 μ L of high-concentration Matrigel (22.1 mg/mL; BD Biosciences). We used 100 μ L of this cell suspension (1×10^7 cells) in Matrigel to inject under the dorsal skin of nude mice. The animals were left for 4 weeks without any treatment for development of visible tumors with approximate volume of 150 to 300 mm³. Animals were then divided into five groups of six mice each. The mice were injected at the tumor site either with the hTERT siRNA plasmid vector (50 μ g DNA per injection per mouse) or IFN- γ (10^4 units per injection per mouse) or both agents on alternate days for 6 weeks. The siRNA plasmid vector was mixed with *in vivo*-jetPEI (Polyplus-transfection) in 5% glucose solution (100 μ L per injection). In the case of combination treatment, the animals received IFN- γ injections first, followed by siRNA plasmid vectors with an interval of 10 min. One group of animals received scrambled siRNA vector and was considered as the treated controls. Tumor volume was measured beginning from week 4 using a digital vernier caliper. Tumor volume was calculated using the formula [(smallest diameter² $\times$ widest diameter) / 2], and the growth curves were plotted for each treatment group (22). At the end of the 10th week, the animals were anesthetized with a mixture of ketamine and xylazine and then were photographed. The solid tumors were surgically removed, tumor weight was recorded, and the tumors were photographed. Tumor samples were stored at -80°C for further analysis.

Western blotting for molecules involved in cell proliferation, angiogenesis, and cell cycle regulation in solid tumors. To elucidate the molecular mechanisms of the inhibition of angiogenesis and tumor growth by knockdown of hTERT and/or treatment with IFN- γ , we assayed protein levels of the molecules involved in these processes in the solid tumors. The tumor samples were weighed, cut into small pieces, and homogenized using a Omni Ruptor 400 Ultrasonic homogenizer (Omni International). The homogenized tumor samples were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein concentration in the supernatant was determined using the Coomassie-Plus protein assay (Pierce Biotechnology), and the samples were stored at -20°C until used. The proteins were resolved on 4% to 20% polyacrylamide gradient gels (Bio-Rad) and electroblotted to an activated polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked with 5% nonfat milk, and the membranes were incubated overnight on a rocker at 4°C with specific antibodies for various protein molecules. The antibodies for proliferating cell nuclear antigen (PCNA) and CD31 were obtained from BD Biosciences; VEGF, basic fibroblast growth factor (bFGF), and antibodies for cyclin-dependent kinase (CDK) 2, CDK4, and cyclin D1 were purchased from Santa Cruz Biotechnology; and the antibodies for MMP-9, p27Kip1, and p21Waf1 were procured from Cell Signaling Technology. After incubation, the membranes were washed and treated with horseradish peroxidase-conjugated respective secondary antibodies (Biomed) at room temperature for 2 h. The membranes were washed again, treated with chemiluminescence reagent (Amersham), and exposed to Kodak film (BioMax XAR) for autoradiography. The membranes were reprobed using Western reprobe buffer (Gbiosciences) for GAPDH content with a GAPDH monoclonal antibody (Novus Biologicals) to show that equal amount of protein was loaded in each lane. The Western blotting images were quantified using Gel-Pro analyzer software (Media Cybernetics).

Statistical analysis. Arithmetic mean and SD were calculated for all quantitative data. The results were statistically evaluated using one-way

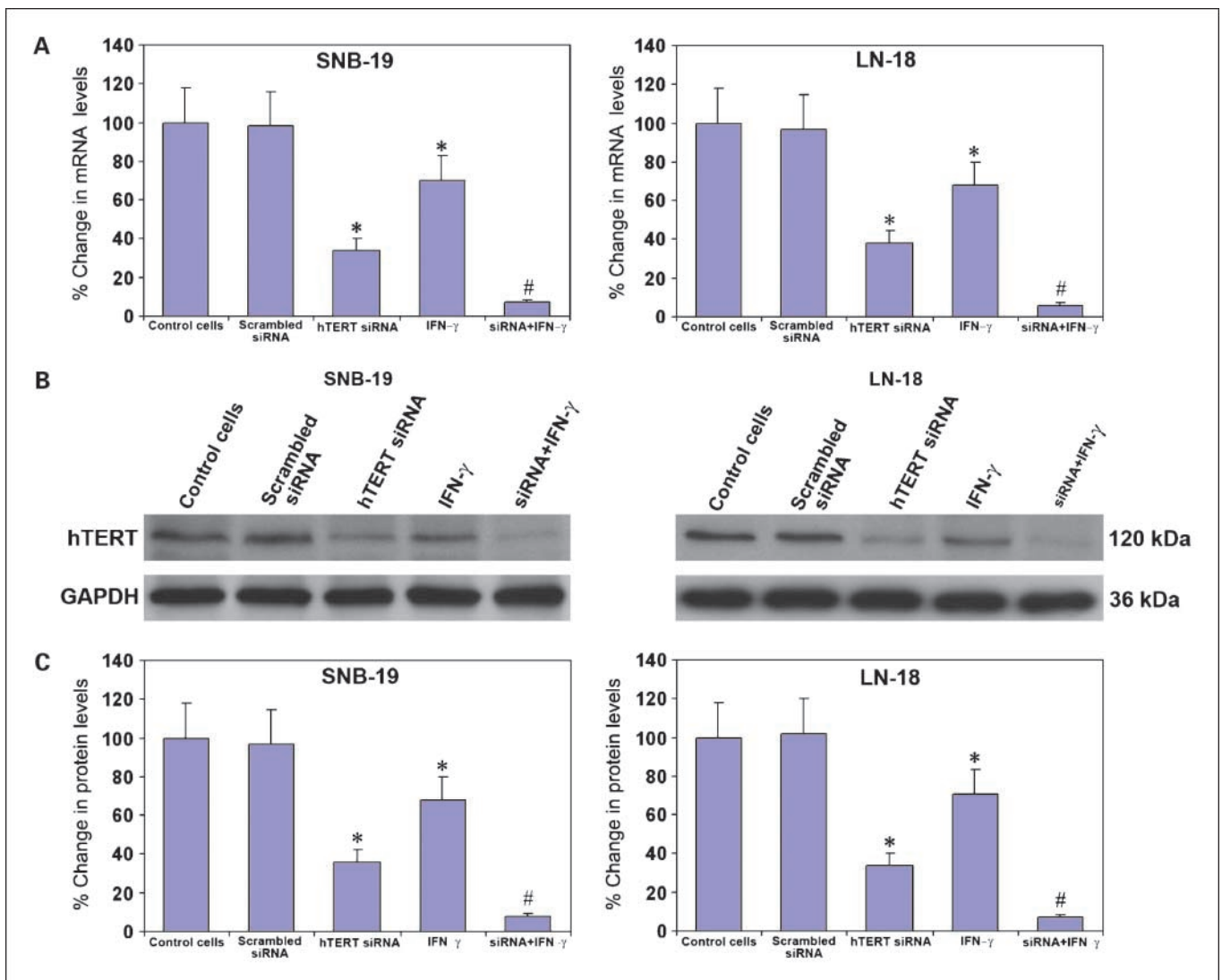


Fig. 1. Alteration in hTERT mRNA and protein levels in SNB-19 and LN-18 cells after transfection with a plasmid vector carrying hTERT siRNA cDNA or treatment with IFN- γ (10 ng/mL) or both agents together for 48 h. **A**, real-time RT-PCR analysis for hTERT mRNA levels. Values are means $\pm$ SD of six assays in each group. *, $P < 0.001$ compared with the control mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values. **B**, Western blotting for hTERT protein levels. The data are representative of six independent experiments. **C**, quantitative evaluation of Western blotting. Values are means $\pm$ SD of six assays in each group. *, $P < 0.001$ compared with the control mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values.

ANOVA. The least significant difference method was used to compare the mean values of control or scrambled siRNA-treated groups with those of hTERT siRNA- or IFN- γ -treated groups. The individual hTERT siRNA or IFN- γ mean values were also compared with the combination treatment mean values. A value of $P < 0.05$ was considered as statistically significant.

Results

Knockdown of hTERT mRNA and protein in glioblastoma SNB-19 and LN-18 cells. We examined the knockdown of hTERT mRNA and protein levels in glioblastoma SNB-19 and LN-18 cells (Fig. 1). Transfection of SNB-19 and LN-18 cells with a plasmid vector carrying hTERT siRNA cDNA resulted in a marked downregulation of cognate mRNA (Fig. 1A) and protein (Fig. 1B) levels in both cell lines. Transfection of cells with a plasmid vector carrying the scrambled hTERT siRNA cDNA did not alter hTERT mRNA and protein levels. Treatment with

IFN- γ also resulted in significant downregulation of hTERT mRNA and protein levels. Treatment with combination of both agents depicted remarkable knockdown of hTERT mRNA and protein levels in both cell lines. GAPDH was used as a loading control for protein levels. Quantification of Western images showed >90% knockdown of hTERT protein levels after the combination treatment (Fig. 1C).

Combination of hTERT siRNA and IFN- γ inhibited *in vitro* angiogenic network formation. We examined the *in vitro* angiogenic network formation in coculture of human microvascular endothelial and glioblastoma cells after the treatments (Fig. 2). The von Willebrand factor is a characteristic marker for endothelial cells. The coculture of human microvascular endothelial cells with SNB-19 or LN-18 cells or cells transfected with scrambled siRNA produced capillary-like network formation of human microvascular endothelial cells (Fig. 2A). Such a network formation of endothelial cells was significantly reduced when cocultured with SNB-19 and LN-18 cells transfected

with a plasmid vector expressing hTERT siRNA or treated with IFN- γ (Fig. 2A). Similar network formation was almost completely inhibited after treatment with a combination of both agents. Quantitative evaluation of *in vitro* network formation of human microvascular endothelial cells showed that treatment with hTERT siRNA, IFN- γ , and combination of both agents caused, respectively, 37%, 52%, and 95% inhibition in the case of SNB-19 cells and 42%, 48%, and 95% in the case of LN-18 cells (Fig. 2B). Because there was no difference between untreated controls and the scrambled siRNA treated samples, only the scrambled siRNA results were considered as the controls.

hTERT siRNA and IFN- γ inhibited *in vitro* angiogenesis. The effect of hTERT siRNA and/or IFN- γ on neovascularization was studied under the dorsal skin of nude mice (Fig. 3). The implantation of diffusion chambers containing SNB-19 and LN-18 untreated cells and cells transfected with a plasmid vector carrying hTERT scrambled siRNA cDNA resulted in the development of microvessels, as indicated by the thin and curved structures arising from the pre-existing vessels in a zig-zag manner (Fig. 3A). The formation of such microvessels was significantly reduced in the cells treated with hTERT siRNA or IFN- γ and almost completely inhibited in the cells treated with both agents together (Fig. 3A). This inhibition of neovascularization indicated that the cells treated with a combination of hTERT siRNA and IFN- γ failed to secrete the potent angiogenic factors such as VEGF and bFGF. Quantitative measurement revealed that treatment with hTERT siRNA, IFN- γ , and both agents together caused, respectively, 55%, 62%, and 96% inhibition of neovascularization in SNB-19 cells (Fig. 3B) and 47%, 54%, and 99% inhibition in LN-18 cells (Fig. 3B). The scrambled siRNA results were considered as the treated controls. We showed a mouse bearing surgically implanted diffusion chamber for *in vivo* angiogenesis assay (Fig. 3C). The diffusion of angiogenic factors through the chamber membrane induces neovascularization in the superfi-

cial fascia of nude mice. This process was effectively inhibited due to the treatment of cells with a combination of hTERT siRNA and IFN- γ .

Inhibition of orthotopic tumorigenesis in nude mice. The effect of hTERT siRNA and/or IFN- γ on the inhibition of orthotopic tumorigenesis in nude mice was evaluated (Fig. 4). We stably transfected SNB-19 and LN-18 cells with the luciferase gene, injected into the intracerebrum of nude mice, and allowed the cells to multiply for 3 weeks during treatment. The untreated group of mice and the mice injected with scrambled siRNA vector showed multiplication and tumorigenesis, as indicated by the large bioluminescence image produced by the tumor cells carrying the luciferase gene (Fig. 4A). Production of this bioluminescent image was partially inhibited in animals injected with hTERT siRNA plasmid vector or treated with IFN- γ and was almost completely inhibited in animals treated with both agents together (Fig. 4A). Quantitative evaluation of the inhibition of intracerebral tumorigenesis showed 47.8%, 62.5%, and 94.8% inhibition in SNB-19 cells and 41%, 58%, and 96.2% inhibition in LN-18 cells, respectively, after the treatment with hTERT siRNA, IFN- γ , and combination of both agents (Fig. 4B). The scrambled siRNA-treated mice were considered as the treated controls.

Inhibition of solid tumor growth in nude mice. We studied the effect of hTERT siRNA and/or IFN- γ on s.c. solid tumor development in nude mice (Fig. 5). The control and scrambled siRNA groups of mice developed large tumors within a period of 10 weeks (Fig. 5A and B). The tumor size was considerably reduced in the animals treated with hTERT siRNA or IFN- γ . The treatment with a combination of both agents resulted in a remarkable reduction in the solid tumor size (Fig. 5A and B). At the end of the 10th week, there was no visible tumor, except a reddish patch, after treatment with combination of both agents. Longitudinal measurement of tumor volume revealed steady-state growth of solid tumors in control and the scrambled siRNA vector-transfected animals (Fig. 5C). The growth curve was

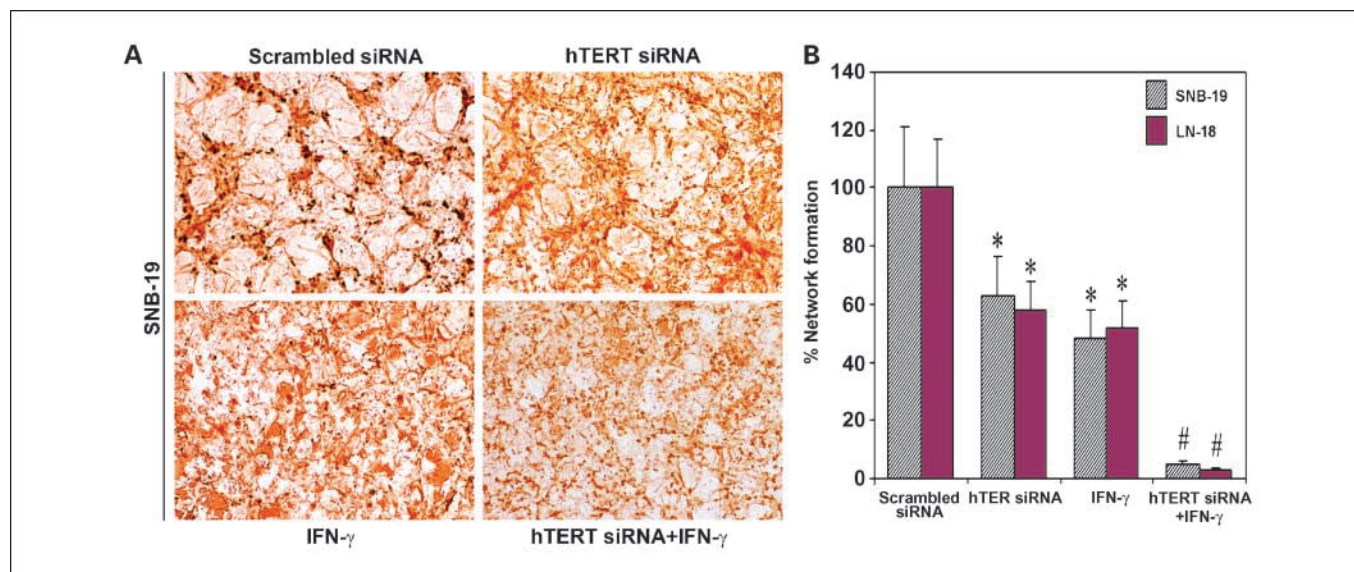


Fig. 2. Effect of hTERT siRNA or/and IFN- γ on *in vitro* network formation of human microvascular endothelial cells. **A**, SNB-19 cells on chamber slides were transfected with hTERT siRNA vector or treated with IFN- γ or both agents together. After 24 h, human microvascular endothelial cells were cocultured with glioblastoma cells. The cocultures were terminated at 72 h and immunohistochemically stained for the von Willebrand factor. **B**, quantitation of *in vitro* network formation by human microvascular endothelial cells. Values are means $\pm$ SD of six experiments in each group of SNB-19 and LN-18 cells. *, $P < 0.001$ compared with the control mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values.

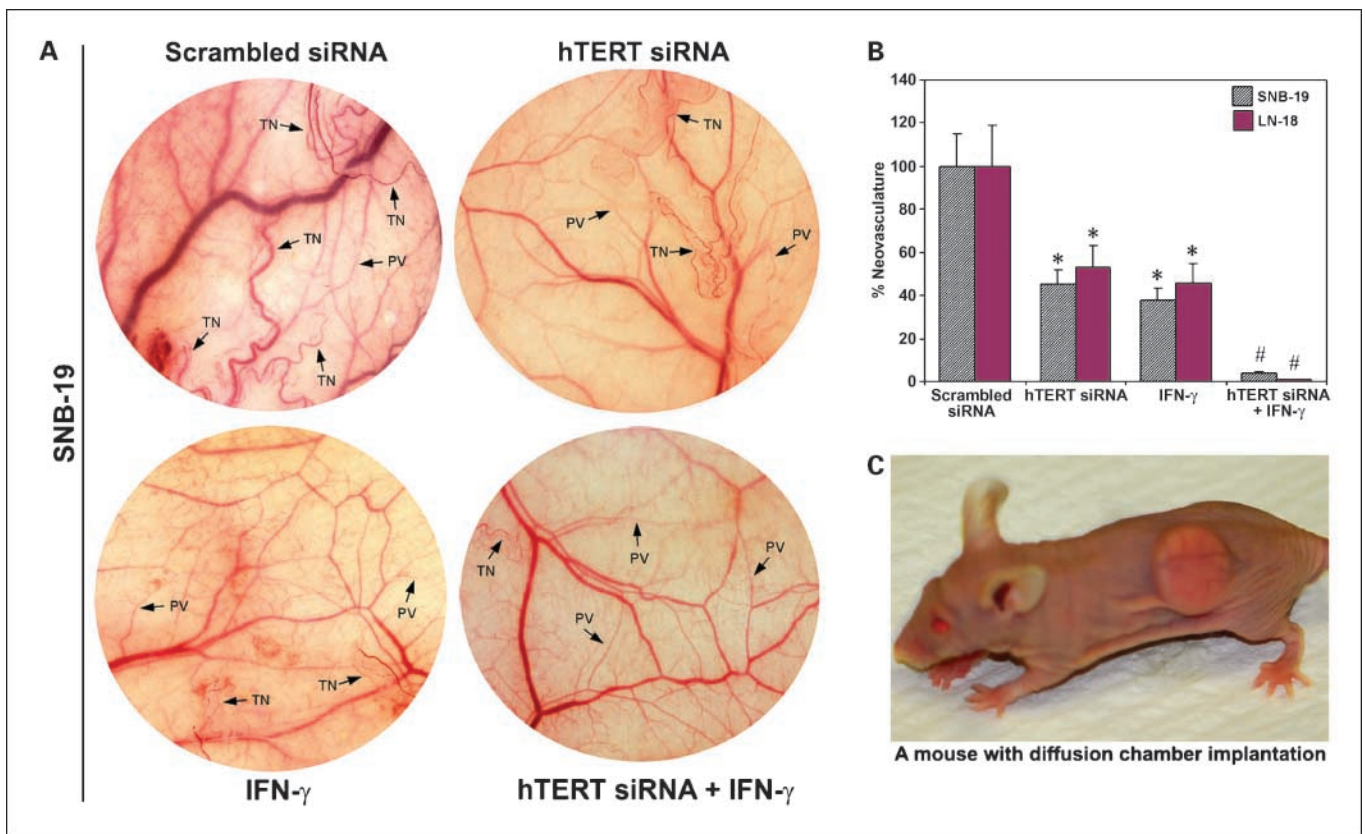


Fig. 3. *In vivo* angiogenesis assay. **A**, SNB-19 cells transfected with a plasmid vector expressing hTERT siRNA or treated with IFN- γ or both agents together were suspended in 200 μ L of serum-free medium, injected into a diffusion chamber, implanted under the dorsal skin of nude mice, and left for 10 days. Strong development of tumor-induced neovasculation (TN) with curved thin structures in zigzag pattern arising from pre-existing vasculature (PV) was observed in SNB-19 control and scrambled siRNA transfected cells. **B**, quantitative presentation of neovasculation to indicate the extent of *in vivo* angiogenesis. Values show mean $\pm$ SD of six experiments in each group of SNB-19 and LN-18 cells. *, $P < 0.001$ compared with the control mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values. **C**, a nude mouse bearing a diffusion chamber implanted under the dorsal skin for the *in vivo* angiogenesis assay.

significantly switched downward after treatment with hTERT siRNA or IFN- γ and was almost straight after treatment with combination of both agents (Fig. 5C). Measurement of tumor weight showed 60% and 66% reduction after treatment with hTERT siRNA and IFN- γ , respectively (Fig. 5D). The treatment with a combination of both agents resulted in 99% reduction of tumor weight. The scrambled siRNA treated mice were considered as the treated controls.

Combination of hTERT siRNA and IFN- γ downregulated the molecules involved in cell proliferation, angiogenesis, and cell cycle in solid tumors. To elucidate the molecular mechanisms of the inhibition of glioblastoma cell invasion, angiogenesis, and tumor progression by knockdown of hTERT siRNA and/or treatment with IFN- γ , we performed Western blotting for the protein levels of the prominent molecules involved in these processes in solid tumors (Fig. 6). The hTERT protein levels in solid tumor samples showed >60% knockdown after injection with hTERT siRNA alone and >80% knockdown after treatment with combination of hTERT siRNA and IFN- γ (Fig. 6A). There was significant ($P < 0.001$) downregulation of PCNA and MMP-9, the molecules involved in tumor cell proliferation and invasion, after treatment with combination of hTERT siRNA and IFN- γ (Fig. 6A and B). The key angiogenic factors VEGF and bFGF were also markedly decreased after treatment with hTERT siRNA, IFN- γ , or both agents (Fig. 6A

and B). There was a significant reduction in CD31 molecule after treatment with IFN- γ or a combination of hTERT siRNA and IFN- γ (Fig. 6A and B), indicating a decrease in proliferation of endothelial cells and angiogenesis. The molecules CDK2, CDK4, and cyclin D1, known to be involved in progression of cell cycle, were also downregulated after treatment with IFN- γ or a combination of both agents (Fig. 6A and C). In contrast, the major CDK inhibitors p21Waf1 and p27Kip1 were remarkably upregulated after treatment with IFN- γ or a combination of both agents (Fig. 6A and C). However, no alteration was noticed in the protein level of any of these molecules after treatment with hTERT siRNA plasmid vector. There was no significant alteration in the protein levels of all the molecules in the animals injected with plasmid vector carrying the scrambled hTERT siRNA, compared with the parental cells, and thus, scrambled hTERT siRNA treated animals were considered as the treated controls. Reprobing for GAPDH showed equal loading of protein sample in each lane.

Discussion

The present study showed that treatment with a combination of hTERT siRNA and IFN- γ inhibited cell proliferation, angiogenesis, and tumor progression in glioblastomas because of the

downregulation of the molecules involved in these processes and upregulation of p27Kip1 and p21Waf1. Treatment with IFN- γ alone suppressed to some extent the expression of hTERT and other proteins involved in cell proliferation and angiogenesis. The combined effects of hTERT siRNA and IFN- γ most effectively downregulated the expression of the molecules involved in cell proliferation and angiogenesis and thus caused the inhibition of tumor progression. However, further studies are necessary to delineate the other molecular mechanisms of inhibition of glioblastomas following treatment with combination of hTERT siRNA and IFN- γ .

The ability of most tumor cells to grow indefinitely relies on the presence of functional telomerase to maintain telomere length, thus circumventing normal cellular senescence or apoptosis to promote tumor growth (23, 24). Telomerase activity in most human cancers is tightly controlled by its catalytic component hTERT. In the present study, treatment with hTERT siRNA resulted in >60% suppression, and treatment with a combination of hTERT siRNA and IFN- γ resulted in >90% suppression of hTERT mRNA and protein levels. The suppression of hTERT was correlated with decreases in angiogenesis and tumor growth. In addition, analysis of xenograft tumor samples showed

decreases in expression of PCNA, MMP-9, VEGF, and bFGF, the major molecules involved in angiogenesis and tumor progression. PCNA is directly involved in DNA replication and cell multiplication, whereas MMP-9 paves the way for tumor cell proliferation and angiogenesis through the degradation of the connective tissue matrix.

In glioblastoma, hTERT expression is a survival predictor and correlated with a poor patient survival rate (25). Thus, suppression of telomerase expression has enormous therapeutic potential in various cancers, including glioblastomas, because this enzyme plays the key role in conferring cellular immortality (26). Inhibition of telomerase activity has been shown to impair both s.c. and intracranial tumor growth in glioblastoma xenografts (27). In the present study, we have observed ~50% decrease in angiogenesis, as well as in tumor progression, after knockdown of hTERT using cognate siRNA.

Treatment with IFN- γ has been found to downregulate hTERT expression and telomerase activity in human cervical cancer due to the upregulation of p27Kip1 (28). In the present study, we confirmed a similar downregulation of hTERT mRNA and protein levels after IFN- γ treatment. This indicated that IFN- γ could downregulate hTERT expression through the

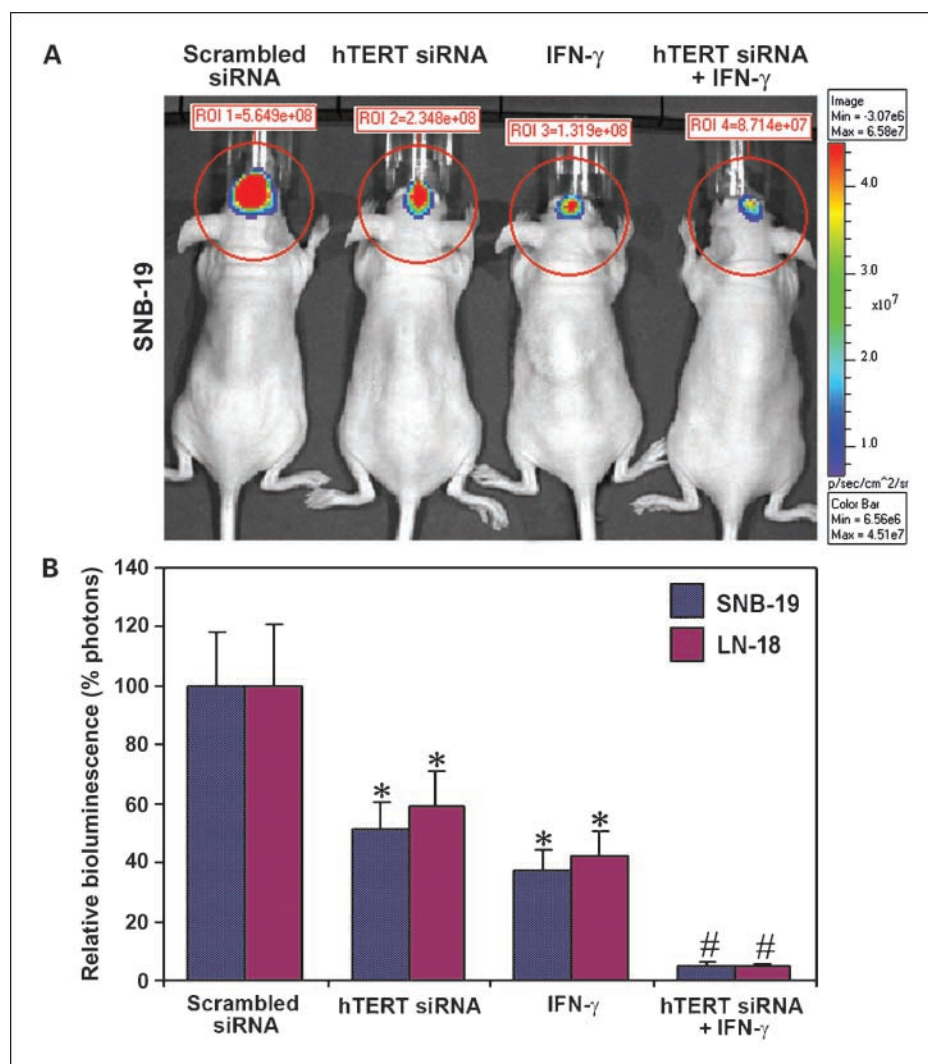
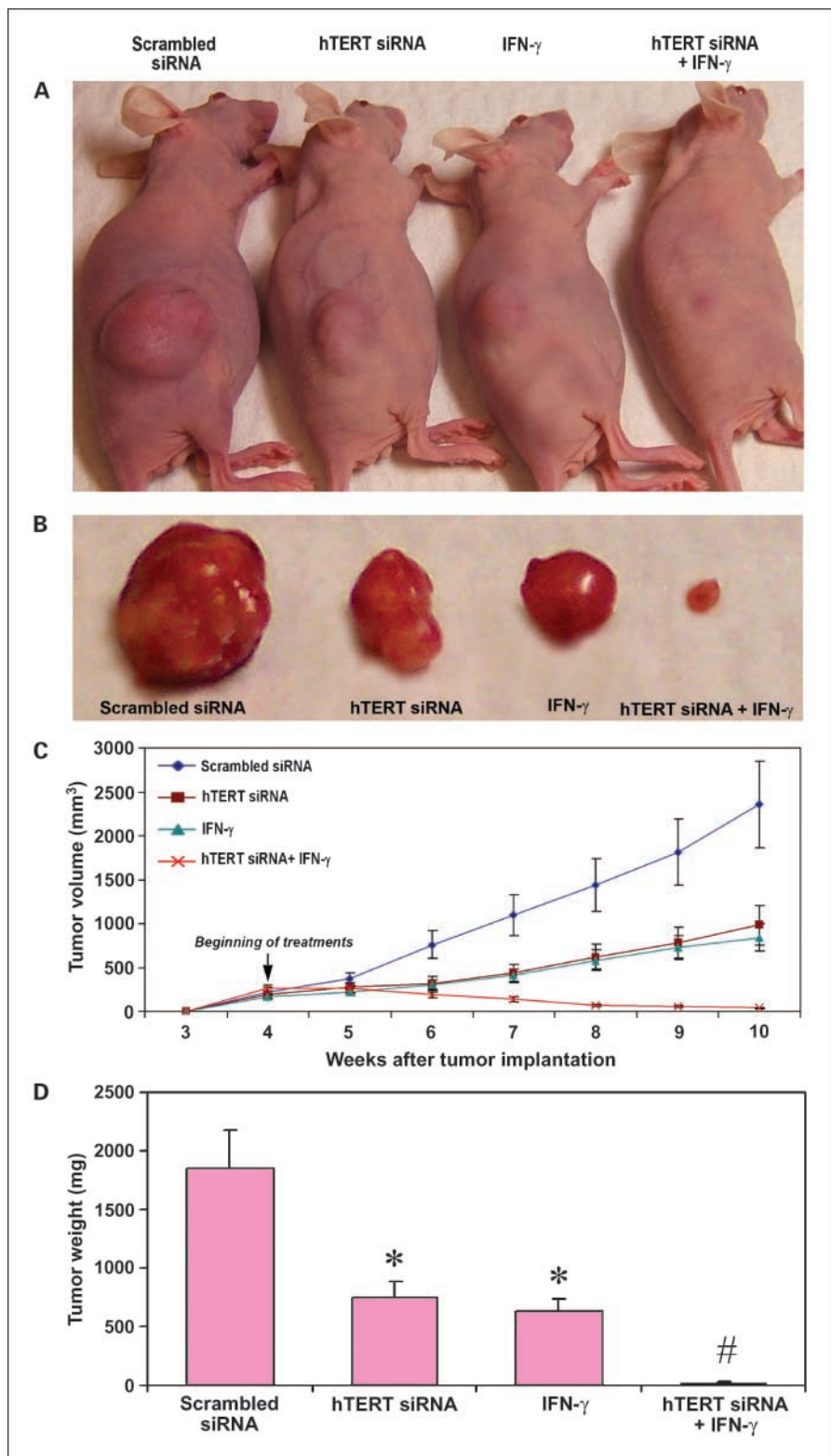


Fig. 4. Inhibition of intracerebral tumorigenesis in nude mice after treatment with hTERT siRNA or/and IFN- γ . **A**, SNB-19 cells were stably transfected with the luciferase gene and were implanted into the intracerebrum of nude mice. Beginning from day 3, the mice were injected at the site of tumor cell implantation either with hTERT siRNA vector or IFN- γ or both agents together on alternate days for 20 days. Then mice were injected with luciferin and were visualized for the effect of treatments using the Xenogen IVIS-200 imaging system. The data are representative of six animals in each group. The background signal for bioluminescence from an untreated mouse is $\sim 1.5 \times 10^5$ photons on the Xenogen IVIS-200 imaging machine. **B**, quantitation of relative bioluminescence as percent photons in SNB-19 and LN-18 cells after treatment with hTERT siRNA or/and IFN- γ . Data show mean $\pm$ SD of six animals in each group. *, $P < 0.001$ compared with the scrambled siRNA mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values.

Fig. 5. Inhibition of s.c. solid tumor development in nude mice after treatment with hTERT siRNA or/and IFN- γ . **A**, SNB-19 cells were harvested, counted, and suspended in an equal volume of high concentration Matrigel, and 100 μ L of the suspension (5×10^6 cells) was injected under the dorsal skin of nude mice. The animals were left for 4 weeks without any treatment for uniform development of visible tumors. Afterward, the mice were injected at the tumor site with either hTERT siRNA vector, IFN- γ , or both agents together on alternate days for 6 weeks. At the end of 10th week, the animals were anesthetized with ketamine and xylazine and then were photographed. **B**, the tumors were surgically removed, weighed, and photographed. The data are representative of six animals in each group. **C**, longitudinal measurement of tumor volume using a digital vernier caliper in nude mice after treatment with hTERT siRNA or/and IFN- γ . Data are means $\pm$ SD of six animals in each group. **D**, quantitation of tumor weight. Data are means $\pm$ SD of six animals in each group. *, $P < 0.001$ compared with the scrambled siRNA mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values.



upregulation of p27Kip1. Our data depicted potent effect of IFN- γ on the inhibition of angiogenesis and tumor progression in glioblastoma, coinciding with the significant decreases in the protein levels of VEGF, bFGF, and CD31. These findings prompted us to examine the dual role of hTERT knockdown

and IFN- γ treatment as a combination therapy for effectively controlling the malignant growth of glioblastoma. Our studies showed that a combination of hTERT knockdown with IFN- γ treatment resulted in a marked reduction in tumor progression of glioblastoma xenografts.

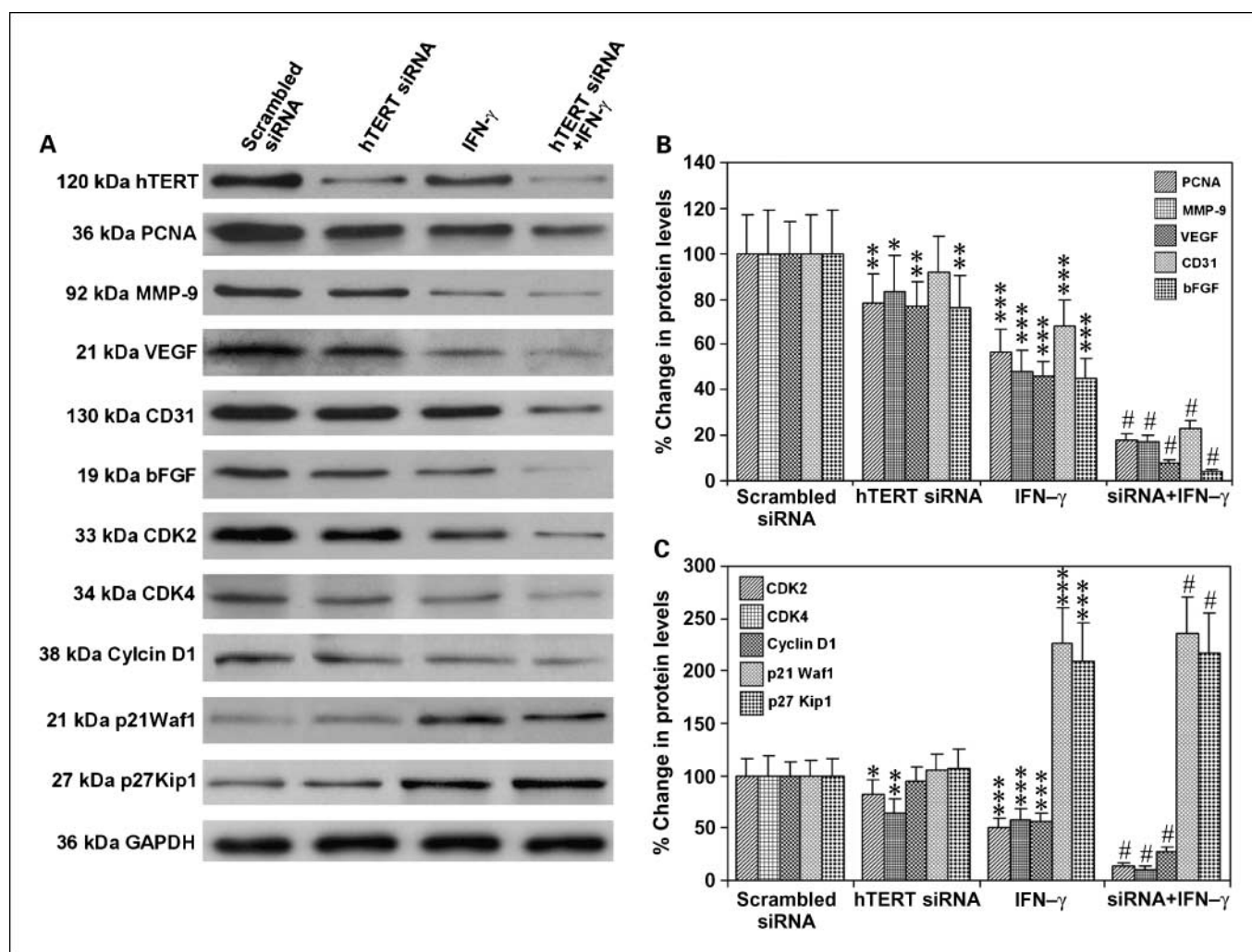


Fig. 6. Western blotting for molecules involved in cell proliferation, angiogenesis, and cell cycle in solid tumors. **A**, representative Western blots for hTERT, PCNA, MMP-9, VEGF, CD31, bFGF, CDK2, CDK4, cyclin D1, p21Waf1, and p27Kip1 in the xenograft solid tumors of LN-18 cells. **B** and **C**, quantitation of Western blots. Values are means $\pm$ SD of six experiments in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the scrambled siRNA mean values; #, $P < 0.001$ compared with hTERT siRNA or IFN- γ mean values.

Angiogenesis, the formation of new blood vessels (neovascularization) from pre-existing ones, is a significant event in cell invasion and tumor progression. Neovascularization is one of the major rate-limiting events in the invasion and progression of glioblastomas because the presence of blood vessels not only sustains tumor growth but also facilitates penetration of tumor cells deep inside normal brain tissue. Development of appropriate molecular strategies to prevent neovascularization is an important milestone in the therapeutic intervention against glioblastomas. VEGF is the key angiogenic stimulant and the major driving force behind not only tumor angiogenesis but also normal blood vessel formation (29, 30). VEGF is highly upregulated in glioblastomas and is responsible for endothelial cell proliferation and vascular permeability in primary human brain tumors (31). Vascular endothelial cells present in astrocytic tumors express telomerase and hTERT and are involved in tumor angiogenesis (32). Inhibition of telomerase in the endothelial cells disrupts tumor angiogenesis in glioblastoma xenografts (33). *In vitro* capillary-like networking of endothelial cells requires special angio-

genic factors such as VEGF released by the tumor cells in culture. In this study, we observed a marked inhibition of capillary-like network formation *in vitro* and a remarkable decrease of neovascularization under the dorsal of skin of nude mice after treatment with combination of hTERT siRNA and IFN- γ . This suggested that the cells treated with a combination of hTERT siRNA and IFN- γ failed to secrete potent angiogenic factors such as VEGF and bFGF.

There has been no previous report on the suppression of intracranial or s.c. tumorigenesis of glioblastoma after treatment with combination of hTERT siRNA and IFN- γ . However, it has been observed that the suppression of hTERT mRNA inhibits tumorigenicity and motility of HCT116 human colon cancer cells (34). In addition, replication-defective adenovirus mediated transfer of IFN- γ gene has been shown to repress brain tumor growth by inhibiting angiogenesis (35). In the present study, we observed the near-complete growth inhibition of intracranial and s.c. tumors in nude mice after treatment with a combination of hTERT siRNA and IFN- γ . Our studies also showed the downregulation of the molecules

involved in cell cycle and angiogenesis after treatment with combination of hTERT siRNA and IFN- γ .

In conclusion, the present study showed that the combination of hTERT knockdown and IFN- γ treatment effectively inhibited angiogenesis and tumor growth in glioblastomas through the downregulation of molecules involved in these

processes and could offer a potential therapeutic strategy for treatment of human glioblastomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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