The International Journal of Biochemistry & Cell Biology 42 (2010) 1164–1173

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Knockdown of hTERT and concurrent treatment with interferon-gamma inhibited proliferation and invasion of human glioblastoma cell lines

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ARTICLE INFO

Article history:
Received 8 September 2009
Received in revised form
31 December 2009
Accepted 5 April 2010
Available online 13 April 2010

Keywords:
Cell invasion
Glioblastoma
hTERT
IFN-γ
Telomerase

ABSTRACT

Human telomerase reverse transcriptase (hTERT) is the catalytic component of telomerase that facilitates tumor cell invasion and proliferation. Telomerase and hTERT are remarkably upregulated in majority of cancers including glioblastoma. Interferon-gamma (IFN-γ) modulates several cellular activities including cell cycle and multiplication through transcriptional regulation. The present investigation was designed to unravel the molecular mechanisms of the inhibition of cell proliferation, migration, and invasion of human glioblastoma SNB-19 and LN-18 cell lines after knockdown of hTERT using a plasmid vector based siRNA and concurrent treatment with IFN-γ. We observed more than 80% inhibition of cell proliferation, migration, and invasion of both cell lines after the treatment with combination of hTERT siRNA and IFN-γ. Our studies also showed accumulation of apoptotic cells in subG1 phase and an increase in cell population in G0/G1 with a reduction in G2/M phase indicating cell cycle arrest in G0/G1 phase for apoptosis. Semiquantitative and real-time RT-PCR analyses demonstrated significant downregulation of c-Myc and upregulation of p21 Waf1 and p27 Kip1. Western blotting confirmed the downregulation of the molecules involved in cell proliferation, migration, and invasion and also showed upregulation of cell cycle inhibitors. In conclusion, our study demonstrated that knockdown of hTERT and concurrent treatment with IFN-γ effectively inhibited cell proliferation, migration, and invasion in glioblastoma cells through downregulation of the molecules involved in these processes and cell cycle inhibition. Therefore, the combination of hTERT siRNA and IFN-γ offers a potential therapeutic strategy for controlling growth of human glioblastoma cells.

1. Introduction

Glioblastomas are mostly primary brain tumors characterized by their invasive infiltration and destruction of surrounding normal tissue, making complete surgical resection of these tumors virtually impossible. The prognosis for patients diagnosed with glioblastoma is very poor, with a mean survival of 9–12 months even after surgery, radiation, and chemotherapy (Castro et al., 2003). Glioblastomas remain highly refractive to therapy, and current treatments produce no long-term survivors in patients with these tumors. Limited efficacy of current treatment modalities call for the development of novel therapeutic approaches targeting the specific biological features of glioblastomas (George et al., 2007).

Telomerase is responsible for the addition of telomere hexameric (5′-TTAGGG-3′) repeats to the 3′ end of chromosomes, a crucial event that enables sustained tumor progression. Telomerase is remarkably upregulated in majority of cancers, including glioblastoma, to support the continuous multiplication of cancer cells and make them immortal (Masutomi and Hahn, 2003; Carpentier et al., 2007). Human telomerase reverse transcriptase (hTERT) is the catalytic component that regulates telomerase activity (Cohen et al., 2007). So knockdown of the expression of hTERT would be an effective way to inhibit telomerase activity and thus to prevent cell cycle progression and tumor growth.

Interferon-gamma (IFN-γ) is a pleiotropic cytokine produced by T cells and natural killer cells. It is known to play pivotal roles in eliciting immune responses to control tumors in vivo (Stark et al., 1998). IFN-γ modulates several cellular activities, including cell cycle and growth, through transcriptional regulation (Boehm et al., 1997; Schroder et al., 2004). It regulates over 200 genes, producing a variety of cellular and physiological responses (Boehm et al., 1997). Furthermore, IFN-γ has antiviral, anti-proliferative, pro-apoptotic, and anti-tumor properties (Sen, 2001; Maher et al., 2007; Wang et al., 2008). Recently, we showed that N-(4-hydroxyphenyl)

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doi:10.1016/j.biocel.2010.04.002

retinamide repressed telomerase and cell cycle to potentiate IFN-γ treatment for increasing apoptosis in human glioblastoma cells (Janardhanan et al., 2008). Previously, it has been demonstrated that injection of lentiviral vector encoding hTERT small interfering RNA (siRNA) can significantly inhibit the growth of glioblastoma U87MG xenograft tumors (Zhao et al., 2007). So we thought that a novel therapeutic modality involving combination of hTERT siRNA and IFN-γ would be ideal for efficient and successful growth inhibition of glioblastoma cells.

The introduction of siRNAs directly as oligonucleotides (George et al., 2009a) or their expression through a plasmid vector encoding specific siRNA (George and Tsutsumi, 2007; George et al., 2009b) is a very powerful technique to knockdown a particular mRNA molecule and subsequently the protein level of the targeted gene. The introduction of hTERT siRNA into tumor cells through a mammalian expression vector can provide unlimited number of siRNA molecules to downregulate the hTERT mRNA, the cognate protein level, and thereby the endogenous telomerase activity. The aim of our present investigation was to elucidate the molecular mechanisms of the inhibition of proliferation, migration, and invasion of human glioblastoma cells following knockdown of hTERT and concurrent treatment with IFN-γ.

2. Materials and methods

2.1. Cell culture conditions

Human glioblastoma SNB-19 cell line was procured from the National Cancer Institute (Frederick, MD). The other human glioblastoma LN-18 cell line was purchased from the American Type Culture Collection (Manassas, VA). We have selected the SNB-19 and LN-18 cell lines because of their different status of the tumor suppressor phosphatase and tensin homolog located on chromosome 10 (PTEN), which is mutated and not expressed in SNB-19 cell line but PTEN is wild type in LN-18 cell line. We propagated SNB-19 cells in 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 and LN-18 cells in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics in a humidified incubator containing 5% CO_2 at 37 °C. Human IFN-γ (Sigma, St. Louis, MO) was diluted in serum-free medium to a final concentration of 10 ng/ml.

2.2. Construction of hTERT siRNA expression vectors

The hTERT siRNA cDNA with sense and antisense strands was constructed into a mammalian expression vector, pRNAT-CMV3.2/Neo (GenScript, Piscataway, NJ), between BamHI and XhoI sites. We prepared three siRNA sequences and the most effective one was selected based on percent knockdown of hTERT both at the mRNA and protein levels. The selected siRNA sequence began at nucleotide 2035 (NM_198253), 5′-GGC ACT GTT CAG CGT GCT CTT-3′ (sense) and 3′-GAG CAC GCT GAA CAG TGCC-5′ (antisense). The scrambled siRNA sequence contained 5′-CCG TCG ACG CGT ACT TGG TTT-3′ (sense) and 3′-CGG TCC AGA GCA TCA ACGG-5′ (antisense). The selected hairpin sequence was 5′-TTG ATA TCC G-3′. The linear siRNA construct (containing the sense and antisense strand, hairpin, termination signal, and BamHI and XhoI restriction sites) was annealed with the complimentary strand and ligated into the plasmid vector between the BamHI and XhoI sites. In this vector, the powerful cytomegalovirus (CMV) promoter drives the expression of siRNA and the SV40 promoter drives the expression of the neomycin resistance gene. This vector also carries coral green fluorescein protein (cGFP) for tracking of transfection efficiency in cell cultures. The validity of siRNA sequence was confirmed by DNA sequencing using a forward sequencing primer for CMV (5′-GTA CGG TGG GAG GTC TAT AT-3′) and a reverse sequencing primer for pRNA (5′-TAG AAG GCA CAG TCG AGG-3′). The plasmid encoding hTERT siRNA was transformed into the JM109 competent cells (Promega, Madison, WI) and the positive colonies were screened using miniprep plasmid DNA purification kit (Qiagen, Valencia, CA). The highly expressing colony was selected and propagated in LB broth containing neomycin. The plasmid vector encoding hTERT siRNA was purified using maxiprep (Qiagen, Valencia, CA) and employed in the transfection experiments.

2.3. Treatment of SNB-19 and LN-18 cells with hTERT siRNA plasmid vector and IFN-γ

About 80% confluent cultures of SNB-19 and LN-18 cells were transfected with the plasmid encoding hTERT siRNA or treated with 10 ng/ml IFN-γ (10 ng IFN-γ was equivalent to100 units) or both in combination in serum-free medium. The plasmid vector with Fugene HD (Roche Diagnostics, IN) was used for transfection. Transfection efficiency in the form of expression of cGFP was monitored using an inverted fluorescent microscope (Olympus IX71, Tokyo, Japan). A set of cultures was also transfected with the plasmid encoding for hTERT scrambled siRNA. After 24 h, the medium was replaced with regular serum-containing medium, and the cultures were incubated for another 24 h. A dose of 10 ng/ml IFN-γ was selected based on a dose–response study for cell viability as determined by MTT assay (Mosmann, 1983) and cell survival and proliferation based on clonogenic assay (Franken et al., 2006). Higher concentrations of IFN-γ did not significantly decrease cell viability and proliferation, while lower concentrations were found to be less effective.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting to examine downregulation of hTERT after treatment with hTERT siRNA

RT-PCR and Western blotting were carried out to examine the downregulation of hTERT mRNA and protein levels, respectively, after knockdown of hTERT or/and treatment with IFN-γ. The following primer sequences were used for PCR amplifications of hTERT (NM_198253) gene (forward 5′-CAC CAA GAA GTT CAT CTCC-3′ and reverse 5′-CAA GTG CTG TCT GAT TCC-3′) and GAPDH (NM_002046) gene (forward 5′-CCA CCC ATG GCA AAT TCC-3′ and reverse 5′-CAG GAG GCA TTG CTG ATG AT-3′). The primers were transcribed with 300 ng of total RNA using a single-step RT-PCR kit (Invitrogen, Carlsbad, CA) on a PCR cycler (Eppendorf, Westbury, NY) with annealing at 56 °C. Western blotting was carried out with hTERT antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for determining hTERT protein levels as described below. Both RT-PCR and Western blotting images were quantified using Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD).

2.5. MTT assay for cell viability

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) was performed to determine cell viability after transfection with hTERT siRNA or/and treatment with IFN-γ. Approximately 10^4 cells were seeded into each well of a 96-well plate and incubated for 48 h in an incubator with 5% CO_2 at 37 °C. The MTT assay was performed as per manufacturer's instructions (Chemicon International, Temecula, CA). The MTT formazan was dissolved by the addition of isopropanol and the final blue color was measured using an ELISA reader (BioTek, Winooski, VT).

Table 1
Sequences of the primers used in semiquantitative and real-time RT-PCR.

Transcript	GeneBank number	Primer sequence	Position	Length (mer)	Product size (bp)
CDK2	BC003065	5′-CATTCCTCTTCCCCTCATCA-3′	454F	20	173
		5′-CAGGGACTCCAAAAGCTCTG-3′	626R	20	
CDK4	BC010153	5′-TGGTGTCGGTGCCTATGG-3′	177F	18	128
		5′-GAACTGTGCTGATGGGAAGG-3′	304R	20	
p27 Kip1	BT019553	5′-CTGAGGACACGCATTTGG-3′	374F	18	211
		5′-CTTCTGAGGCCAGGCTTCTT-3′	584R	20	
p21 Waf1	NM_000389	5′-GACACCACTGGAGGGTGACT-3′	290F	20	172
		5′-CAGGTCCACATGGTCTTCCT-3′	461R	20	
c-Myc	V00568	5′-CCTACCCTCTCAACGACAGC-3′	1145F	20	248
		5′-CTCTGACCTTTTGCCAGGAG-3′	1392R	20	
GAPDH	NM_002046	5′-CCACCCATGGCAAATTCC-3′	254F	18	304
		5′-CAGGAGGCATTGCTGATGAT-3′	557R	20	

F, forward primer; R, reverse primer.

2.6. Clonogenic assay for cell survival and proliferation

Clonogenic assay or colony formation assay (Franken et al., 2006) was carried out using SNB-19 and LN-18 cells to study the effect of hTERT siRNA or/and IFN-γ. Both SNB-19 and LN-18 cells were transfected with hTERT siRNA, treated with IFN-γ, or combination of both for 48 h. The cells were harvested using TrypLE Express (Invitrogen, Carlsbad, CA) and 200 cells were seeded into Corning 6-well plates. The cells were allowed to attach for 3–5 h and again treated with hTERT siRNA or/and IFN-γ for 48 h. The cultures were terminated on day 10, fixed with 6% (v/v) glutaraldehyde, stained with 0.5% (w/v) crystal violet, and counted using a stereomicroscope. Small colonies formed from less than 50 cells were not scored for survival.

2.7. Cell migration from spheroids

Cell migration assay (McElwain and Pettet, 1993) was performed to assess the effect of hTERT siRNA or/and IFN-γ on cell migration from spheroids. SNB-19 and LN-18 cells, which were stably transfected with a red fluorescent protein (Clontech, Mountain View, CA), were transfected with hTERT siRNA, treated with IFN-γ, or both together for 24 h. The cells were then washed twice with serum-free medium, harvested, and seeded (5×10^4 cells/well) into 96-well low attachment culture plates (Corning Life Sciences, Corning, NY) coated with 1% agarose. Cells were incubated overnight in presence of 5% CO_2 at 37 °C with constant shaking at 75 rpm. The spheroids formed uniformly were transferred to the center of wells of a 24-well culture plate and incubated in presence of 5% CO_2 at 37 °C for 24 h. The migration of cells from the spheroids was examined under a fluorescent microscope (Olympus IX71, Tokyo, Japan) and photographed. The percentage of migration of cells from the center of the spheroids to the monolayers was measured using a microscope calibrated with a stage and ocular micrometer and used as an index of cell migration.

2.8. Matrigel invasion assay

Matrigel invasion assay (Tolboom and Huizinga, 2007) was performed to assess the effects of hTERT siRNA or/and IFN-γ on the invasive properties of SNB-19 and LN-18 cells. Transwell inserts (12-well, 12 mm with 12.0 μm pore size) from Corning (Corning, NY) were coated with 200 μl of matrigel (final concentration, 1.0 mg/ml in ice-cold serum-free medium) (BD Biosciences, San Jose, CA) and allowed to dry at 37 °C for 3–5 h. At 24 h post-treatment, the control and treated cells were washed twice with serum-free medium, trypsinized, and 200 μl of cell suspension (2×10^5 cells) from each sample was added to each well in triplicate. After 48 h incubation in presence of 5% CO_2 at 37 °C, the membranes were collected and stained with HEMA stain (Fisher Scientific, Pittsburg, PA). The cells that migrated to the undersurface of the membrane were examined under a microscope, counted in 10 randomly selected microscopic fields, and photographed.

2.9. Fluorescent activated cell sorting (FACS) analysis

We carried out FACS analysis to examine the alterations in cell cycle and determine the cell population in G0/G1 phase and G2/M phase after the treatments. Cells were harvested using TrypLE (Invitrogen) and washed twice with cold PBS. The cells were then fixed with 70% cold ethanol on ice for 15 min. After sedimentation, the cells were dispersed in 0.5 ml of propidium iodide (50 μg/ml) (Biosure, Grass Valley, CA) with gentle vortex and incubated for 20 min in dark at 4 °C. The cells were sorted on a FACS machine (Beckman Epics XL, Fullerton, CA) and analyzed based on the red fluorescence at 488 nm. Data were presented as FACS histograms and the percent changes in cell population in G0/G1 phase and G2/M phase.

2.10. Semiquantitative and real-time RT-PCR to determine expression of cell cycle regulators

To elucidate the molecular mechanism of the results obtained in cell migration, invasion, and cell cycle analysis, we determined the expression of cyclin-dependent kinase 2 (CDK2), CDK4, p27 Kip1, p21 Waf1, and c-Myc at the transcriptional level employing both semiquantitative RT-PCR and quantitative real-time RT-PCR. Total cellular RNA was isolated using the Aurum kit (Bio-Rad, Hercules, CA). The quality of the isolated RNA was validated with UV spectrometry. Gene-specific primers (Table 1) were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA). All the targeted genes were transcribed with 300 ng of isolated total RNA using a single-step RT-PCR kit (Invitrogen, Carlsbad, CA) on a thermocycler (Eppendorf, Westbury, NY) with annealing at 56 °C. The RT-PCR products were separated on agarose gels and visualized using a UV transilluminator (Alpha Innotech, San Leandro, CA). The expression of GAPDH was used as an internal control.

We also used the same primer sequences for quantitative real-time RT-PCR analysis for mRNA expression of CDK2, CDK4, p27 Kip1, p21 Waf1, and c-Myc. Real-time RT-PCR was carried out using a one-step RT-PCR kit with SYBR green (Bio-Rad, Hercules, CA) on a real-time PCR machine (iCycler iQ5, Bio-Rad, Hercules, CA) with the following reaction conditions: cDNA synthesis, 10 min at 50 °C; reverse transcriptase inactivation at 95 °C for 5 min; PCR cycling

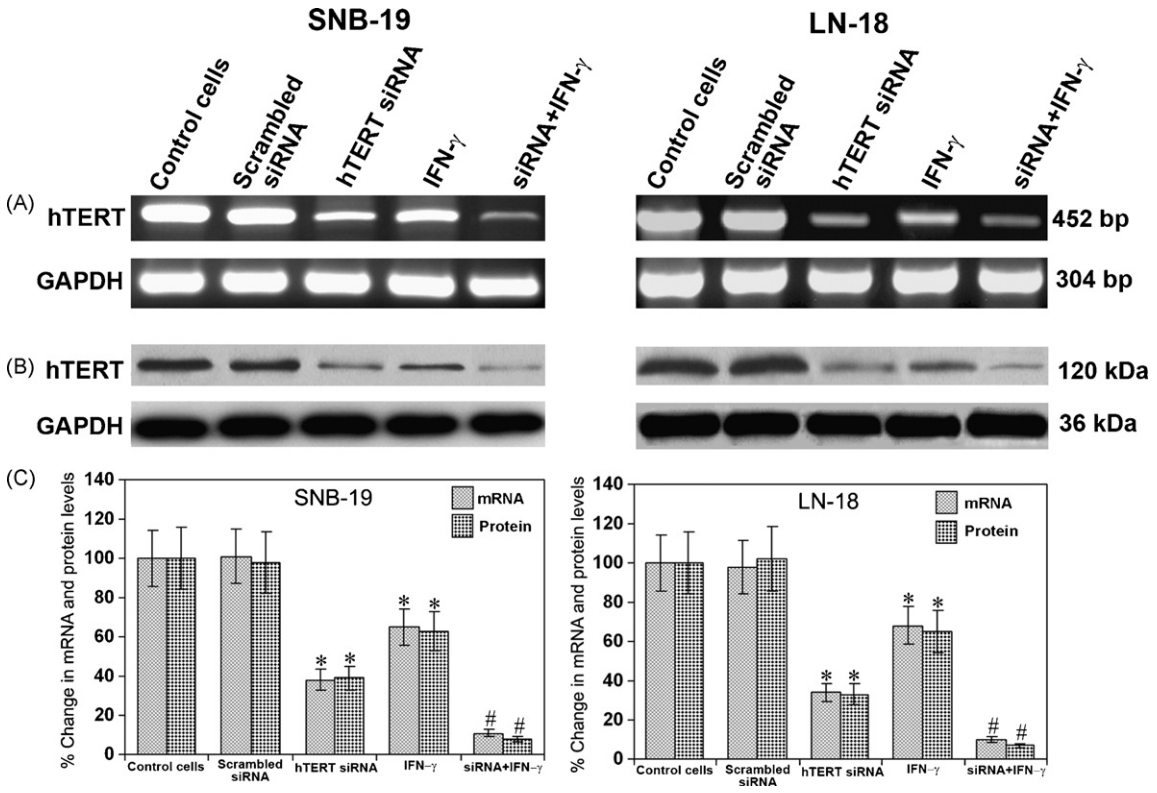

Fig. 1. Levels of expression of hTERT mRNA and protein in SNB-19 and LN-18 cells after transfection with a plasmid encoding hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) Semiquantitative RT-PCR for hTERT. Expression of GAPDH mRNA was used as an internal control. The data are representative of 5 independent experiments. (B) Western blotting for hTERT. The blots were reprobed for GAPDH content to demonstrate that all lanes loaded contain equal amounts of protein. The data are representative of 5 independent experiments. (C) Determination of hTERT mRNA and protein levels after the treatments with hTERT siRNA or/and IFN-γ. Data are mean ± SD of 5 independent experiments ($^{*}p < 0.001$ compared with the control mean values and $^{\#}p < 0.001$ compared with hTERT siRNA or IFN-γ mean values).

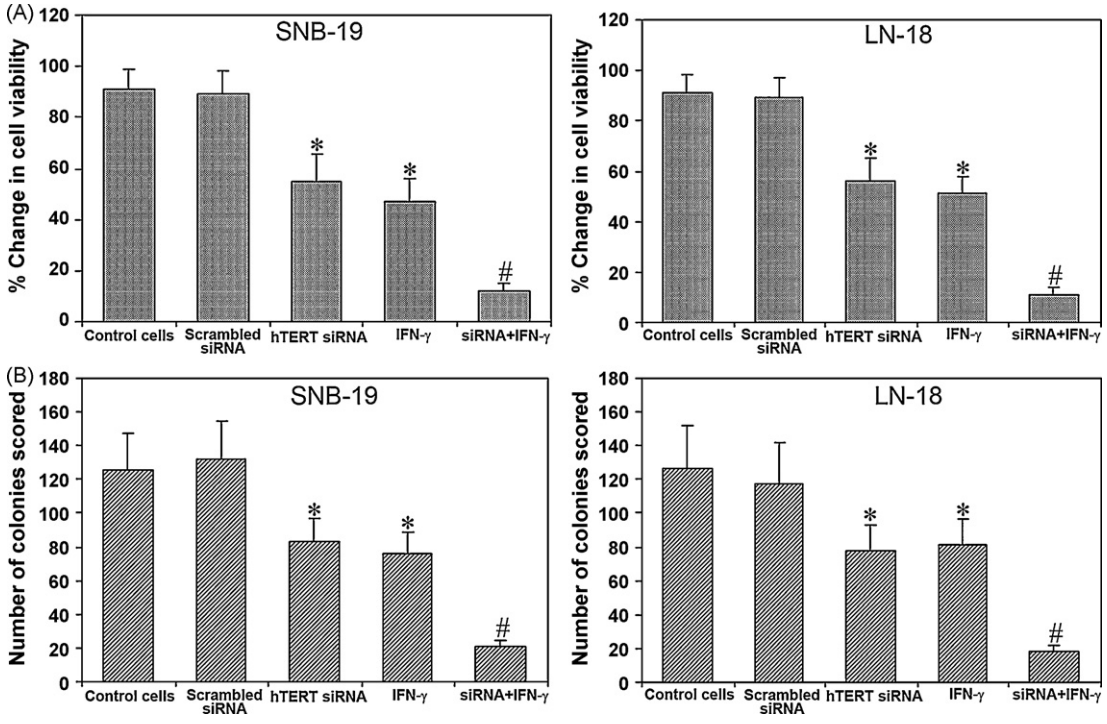

Fig. 2. Determination of residual cell viability and colony forming ability of SNB-19 and LN-18 cells after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) MTT assay for determination of residual cell viability. Data are mean ± SD of 6 independent experiments in duplicate ($^{*}p < 0.001$ compared with the control mean values and $^{\#}p < 0.001$ compared with the hTERT siRNA or IFN-γ mean values). (B) Clonogenic assay for evaluation of cell survival and proliferation. Following treatments, cells were harvested and 200 cells were seeded/well on 6-well plates. The seeded cells were again treated for 48 h after attachment. The cultures were terminated on day 10, stained with crystal violet, and the colonies were counted using a stereomicroscope. Data are mean ± SD of 6 independent experiments ($^{*}p < 0.001$ compared with the control mean values and $^{\#}p < 0.001$ compared with hTERT siRNA or IFN-γ mean values).

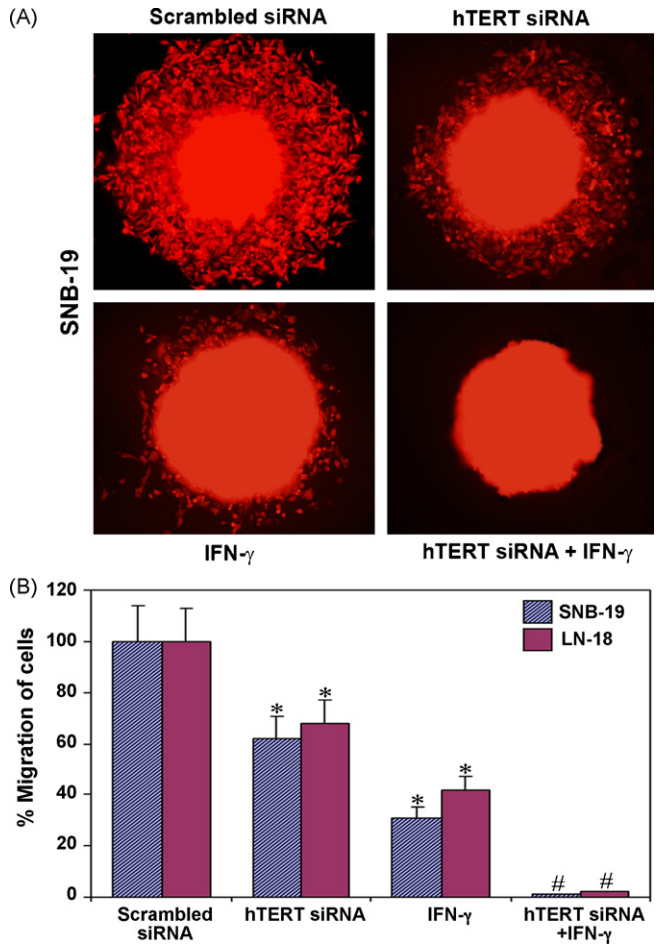

Fig. 3. Determination of migration of SNB-19 and LN-18 cells from spheroids after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) Representative inhibition of SNB-19 cell migration from spheroids. Spheroids were prepared from SNB-19 control and treated cells stably expressing cGFP, transferred to a 24-well chamber, and allowed to migrate for 24 h. The migration of cells from the spheroids were observed under a fluorescent microscope and photographed. (B) Percentage of migration of SNB-19 and LN-18 cells from the center of the spheroids to the monolayers. The data are presented as mean ± SD of 6 independent experiments from each group (*$p < 0.001$ compared with the control mean values and #$p < 0.001$ compared with hTERT siRNA or IFN-γ mean values).

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.

2.11. Western blotting for molecules involved in cell proliferation, invasion, and cell cycle regulation

In order to confirm the results obtained in semiquantitative RT-PCR and quantitative real-time RT-PCR, we used Western blotting to examine the protein levels of the molecules involved in cell proliferation, invasion and related processes. Both control and treated cells were washed twice with ice-cold PBS and scraped with 1 ml of freshly prepared radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors [50 mM Tris–HCl, pH 7.4, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (activated), 1 mM sodium fluoride, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, and 5 μg/ml pepstatin]. Cells were centrifuged at 4000 rpm for 10 min at 4 °C in an Eppendorf centrifuge. The supernatant was discarded, and the cell pellet was suspended in RIPA buffer with protease inhibitors according to the size of the pellet and sonicated gently in a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ). Cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were collected. The protein concentration in the supernatant was determined using Coomassie plus protein assay (Pierce Biotechnology, Rockford, IL). The samples were stored at −20 °C until assayed.

The proteins prepared from the cell lysates were resolved on 4–20% polyacrylamide gradient gels (Bio-Rad, Hercules, CA) and electroblotted to the activated PVDF membranes (Millipore, Bedford, MA). After blocking the non-specific binding sites, the membranes were incubated overnight on a rocker at 4 °C with specific antibodies. The primary IgG antibodies for CDK2, CDK4, cyclin D1, and c-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), matrix metalloproteinase-9 (MMP-9), p27 Kip1 and p21 Waf1 were procured from Cell Signaling Technology (Danvers, MA), and proliferating cell nuclear antigen (PCNA) was from BD Biosciences (San Jose, CA). After incubation, the membranes were washed and treated with horse-radish peroxidase (HRP) conjugated respective secondary IgG antibodies (Biomeda, Foster City, CA) at room temperature for 2 h. The membranes were washed again, treated with chemiluminescence reagent (Amersham, Buckinghamshire, UK), exposed to autoradiography film (BioMax XAR, New Haven, CT), and developed. The membranes were reprobed for expression of GAPDH to show that equal amounts of protein samples were loaded in all lanes.

2.12. Statistical analysis

Mean and standard deviation (SD) were calculated for all quantitative data. The results were statistically evaluated using one-way analysis of variance (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. The difference between two values was considered statistically significant at $p < 0.05$. Pearson's correlation coefficient analysis was used to evaluate the correlation between the proportions of cells in subG1 phase in the FACS analysis and also the percentage of dead cells observed in the MTT assay.

3. Results

3.1. Downregulation of hTERT mRNA and protein levels in SNB-19 and LN-18 cells

We examined the changes in expression of hTERT after the treatments (Fig. 1). Transfection with a plasmid encoding hTERT siRNA resulted in marked downregulation of cognate mRNA and protein levels in both SNB-19 and LN-18 cells (Fig. 1A and B). But hTERT mRNA and protein levels were not altered after transfection with hTERT scrambled siRNA. Treatment with IFN-γ alone resulted in significant downregulation of hTERT mRNA and protein levels, and treatment with the combination of both agents showed remarkable knockdown of hTERT mRNA and protein levels in both cell lines. Expression of GAPDH was used as an internal control for both mRNA and protein analyses. We presented the quantitative measurement of the expression of hTERT mRNA and protein levels after the treatments with hTERT siRNA or/and IFN-γ (Fig. 1C). More than 90% inhibition of hTERT expression was observed at both mRNA and protein levels after the combination treatment.

3.2. Effect of hTERT siRNA and IFN-γ on cell viability and colony forming ability

We determined cell viability and colony forming ability of the cells after the treatments (Fig. 2). We observed a significant ($p < 0.001$) decrease in the percentage of cell viability in both SNB-19 and LN-18 cells after knockdown of hTERT or treatment with

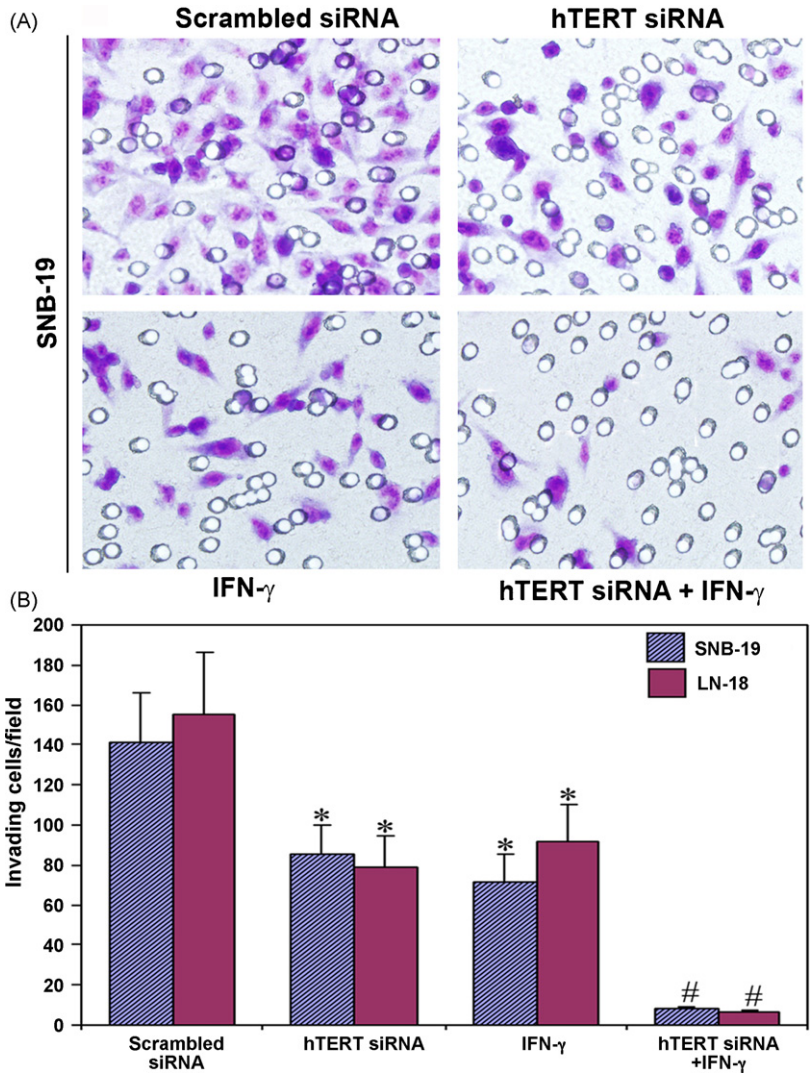

Fig. 4. Cell invasion assay after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) Representative cell invasion assay for SNB-19 cells. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 μm pores coated with 200 μl of 0.1% matrigel. After 48 h incubation period, the membranes were collected and stained. A significant reduction in the number of invaded cells indicated the decrease in invasive capacity. (B) Quantitative evaluation of matrigel invasion assay. The data represented are mean ± SD of 10 randomly selected microscopic fields from 3 independent wells ($^{*}p < 0.001$ compared with the control mean values and $^{\#}p < 0.001$ compared with hTERT siRNA or IFN-γ mean values).

IFN-γ (Fig. 2A). The combination of both agents reduced the mean cell viability to 12% and 11% in SNB-19 and LN-18 cells, respectively. Clonogenic assay is based on the ability of a single cell to form a multicellular colony of a minimum 50 cells within in a particular time frame. The colony forming ability of the cells after hTERT siRNA or IFN-γ treatment was significantly decreased ($p < 0.001$) when compared with the untreated control or scrambled siRNA transfected cells (Fig. 2B). After the combination of both agents, the colony forming ability of SNB-19 and LN-18 cells was reduced to 17% and 14%, respectively, when compared with the untreated control cells.

3.3. Combination of hTERT siRNA and IFN-γ inhibited tumor cell migration

We assayed the rate of cell migration from spheroids prepared from SNB-19 and LN-18 control cells, cells transfected with a plasmid encoding hTERT siRNA, treated with IFN-γ, and both agents (Fig. 3). The hTERT siRNA and IFN-γ treated cells did not migrate significantly from the spheroids, compared with the untreated controls or scrambled siRNA transfected cells (Fig. 3A). We observed complete inhibition of cell migration after treatment with combination of hTERT siRNA and IFN-γ for both SNB-19 and LN-18 cells. Quantitative evaluation of the percentage of migration of cells from the center of the spheroids to the monolayers was measured using an ocular micrometer (Fig. 3B). The migrations of cells treated with hTERT siRNA, IFN-γ, and combination of both agents were, respectively, 62%, 31%, and 0% in SNB-19 cells and 68%, 42%, and 2% in LN-18 cells. Since there was no difference in cell migration between untreated controls and scrambled siRNA transfected samples, only the scrambled siRNA data were presented.

3.4. Marked reduction in tumor cell invasion

We evaluated cell invasion after the treatments (Fig. 4). Matrigel invasion assays demonstrated remarkable reductions in the invasive properties of both SNB-19 and LN-18 cells after treatment with hTERT siRNA and IFN-γ (Fig. 4A). The staining of invaded cells through the polycarbonate membrane demonstrated that the number of cell invasion was significantly reduced in cells treated with hTERT siRNA or IFN-γ, compared with that of untreated control or scrambled siRNA transfected cells. The treatment with the combi-

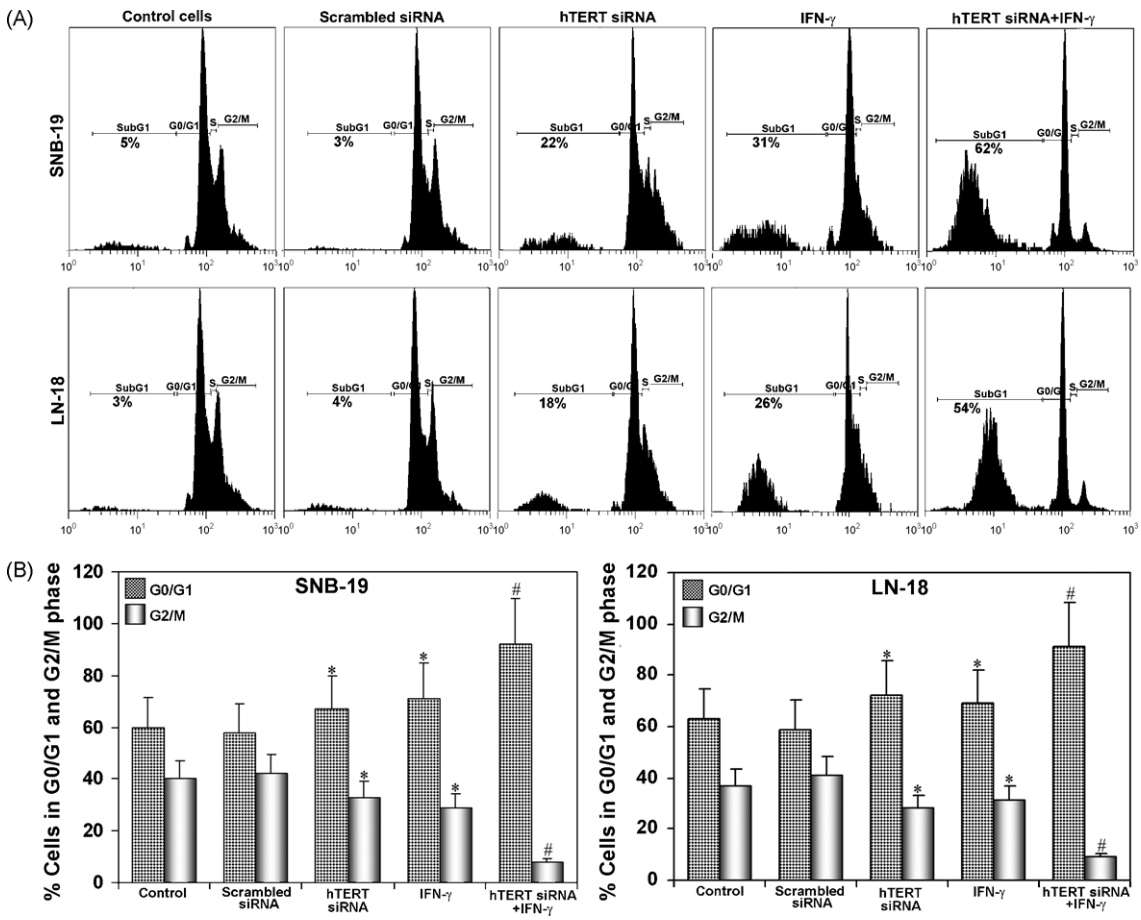

Fig. 5. Cell cycle analysis after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) FACS histograms of SNB-19 and LN-18 cells to show cell cycle arrest. The cells were treated with 50 μg/ml propidium iodide for 20 min at 4 °C in dark prior to cell cycle analysis. An increase in cell population in G0/G1 phase indicated cell cycle arrest. (B) Quantitative presentation of FACS data for population of cells in G0/G1 phase and G2/M phase. Data are representative of 6 independent experiments (*$p < 0.001$ compared with the mean values of scrambled siRNA treated cells and #$p < 0.001$ compared with the mean values of hTERT siRNA or IFN-γ treated cells).

nation of hTERT siRNA and IFN-γ resulted in a dramatic reduction in tumor cell invasion, compared with either treatment alone. Quantitation of the invaded cells using Image-Pro Discovery software showed that the cells treated with hTERT siRNA, IFN-γ, and both together caused, respectively, 60%, 51%, and 6% invasion in SNB-19 cells and 51%, 59%, and 4% invasion in LN-18 cells, compared with the scrambled siRNA transfected cells (Fig. 4B). Since there was no difference in tumor cell invasion between untreated control cells and scrambled siRNA transfected cells, the scrambled siRNA data were considered as the controls.

3.5. Treatment with combination of hTERT siRNA and IFN-γ resulted in cell cycle arrest

In order to obtain more information about the mechanism of the inhibition of cell proliferation, migration, and invasion, cell cycle analyses were carried out using both SNB-19 and LN-18 cells after treatments (Fig. 5). The results showed an increase in apoptotic subG1 phase after transfection with hTERT siRNA or treatment with IFN-γ and subG1 phase was markedly elevated after treatment with combination of both agents (Fig. 5A). There was significant increase in the population of cells in G0/G1 phase in both SNB-19 and LN-18 cells after treatment with hTERT siRNA or IFN-γ (Fig. 5A). Treatment with combination of both agents resulted in a marked increase in the population of cells in G0/G1 phase and a decrease in G2/M phase, indicating the cell cycle arrest in G0/G1 phase (Fig. 5A). There was no alteration in the cell population either in subG1, G0/G1, or G2/M phase after transfection with a scrambled siRNA vector. We performed quantitative evaluation of percent changes in the population of SNB-19 and LN-18 cells in G0/G1 phase and G2/M phase after knockdown of hTERT siRNA or/and treatment with IFN-γ (Fig. 5B). The results revealed that 60%, 58%, 67%, 71%, and 92% of SNB-19 cells while 63%, 59%, 72%, 69%, and 90% of LN-18 cells in G0/G1 phase after treatment with nothing, scrambled siRNA, hTERT siRNA, IFN-γ, and combination of both agents, respectively. There was a positive correlation between the proportion of subG1 cells and the percentage of dead cells (as observed in the MTT assay) in both SNB-19 ($r = 0.983$) and LN-18 ($r = 0.992$) cell lines.

3.6. Combination of hTERT siRNA and IFN-γ upregulated mRNA expression of cyclin-dependent kinase inhibitors

In order to elucidate the molecular mechanism of inhibition of glioblastoma cell proliferation, migration, and invasion, we measured the mRNA expression of CDK2, CDK4, p27 Kip1, p21 Waf1, and c-Myc after the treatments (Fig. 6). Qualitative RT-PCR experiments showed no notable alteration in the mRNA expression of CDK2 and CDK4 in both SNB-19 and LN-18 cells. Treatment with IFN-γ alone or combination of hTERT siRNA and IFN-γ resulted in a remarkable increase in the expression of p27 Kip1 and p21 Waf1 in both cell lines. However, transfection of cells with hTERT alone did not alter the mRNA expression of both p27 Kip1 and p21 Waf1 (Fig. 6A). The transcriptional expression of c-Myc was markedly downregulated after transfection with hTERT siRNA, treatment with IFN-γ,

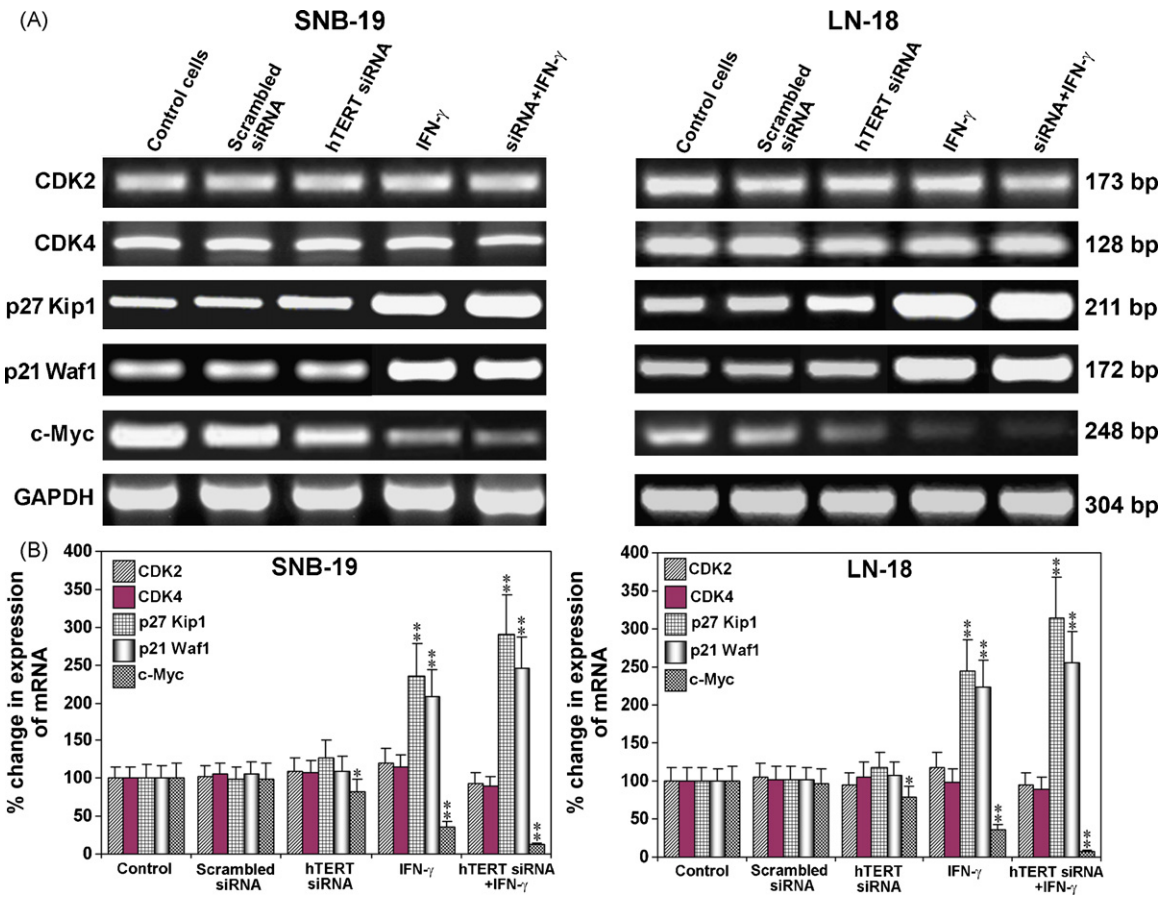

Fig. 6. Determination of levels of transcriptional expression of cell cycle regulatory molecules after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) Semiquantitative RT-PCR for levels of expression of CDK2, CDK4, p27 Kip1, p21 Waf1 and c-Myc mRNA. The isolated total RNA was amplified using a single-step RT-PCR. Expression of GAPDH mRNA was used as internal control. (B) Quantitative real-time RT-PCR analysis using SYBR green for levels of expression of CDK2, CDK4, p27 Kip1, p21 Waf1 and c-Myc mRNA. Data are mean ± SD of 6 samples (*$p < 0.01$ and **$p < 0.001$ compared with the mean values of scrambled siRNA treated samples).

and combination of both agents. The results from the quantitative real-time RT-PCR analysis for the mRNA expression of CDK2, CDK4, p27 Kip1, p21 Waf1, and c-Myc showed a pattern similar to qualitative RT-PCR analysis (Fig. 6B). Transfection of the scrambled hTERT siRNA vector did not alter the mRNA expression of any molecules studied either by qualitative RT-PCR or quantitative real-time RT-PCR in both cell lines.

3.7. Combination of hTERT siRNA and IFN-γ downregulated the molecules involved in cell proliferation, invasion, and cell cycle

In order to confirm the molecular mechanism of the inhibition of cell proliferation, migration, and invasion after knockdown of hTERT siRNA or/and treatment with IFN-γ, we determined the protein levels of the important molecules involved in these processes (Fig. 7). We have observed dramatic downregulation of PCNA and MMP-9 (the molecules involved in cell proliferation and migration, respectively) after knockdown of hTERT and treatment with IFN-γ in both SNB-19 and LN-18 cells (Fig. 7A). The molecules involved in cell cycle (CDK2, CDK4, and cylcin D1) were remarkably downregulated after treatment with IFN-γ alone or combination of hTERT siRNA and IFN-γ (Fig. 7A). However, transfection of hTERT siRNA alone did not alter the protein levels of any of these molecules. The expression of the putative oncoprotein c-Myc was highly reduced in both cell lines after treatment with hTERT or/and with IFN-γ. The expression of the major CDK inhibitors such as p27 Kip1 and p21 Waf1 were dramatically upregulated after treatment with IFN-γ alone or combination of hTERT siRNA and IFN-γ (Fig. 7A). However, knockdown of hTERT did not increase the protein expression of these molecules. There was no apparent difference in the expression of all these molecules in the cells transfected with a plasmid encoding scrambled hTERT siRNA compared with the untreated cells and, therefore, cells treated with scrambled hTERT siRNA were considered as the treated controls. Reprobing for expression of GAPDH demonstrated equal loading of protein samples in all lanes. Then, we performed the quantitative determination of the protein levels of PCNA, MMP-9, c-Myc, p27 Kip1 and p21 Waf1 after the treatments (Fig. 7B). The protein levels of p27 Kip1 and p21 Waf1 were increased to 215% and 220%, respectively, in SNB-19 cells and 210% and 193%, respectively, in LN-18 cells after the treatment with combination of hTERT siRNA and IFN-γ.

4. Discussion

Our study demonstrated that the combination of hTERT siRNA and IFN-γ effectively inhibited human glioblastoma cell proliferation, migration, and invasion through transcriptional and/or translational downregulation of molecules involved in these processes and cell cycle arrest. Treatment with the combination of hTERT siRNA and IFN-γ also resulted in a marked upregulation of p27 Kip1 and p21 Waf1/Cip1, the CDK inhibitors. In the present study, we employed a PTEN mutant cell line (SNB-19) and a PTEN wild type cell line (LN-18) to evaluate the effect of combination of hTERT knockdown and IFN-γ treatment. We did not observe any significant difference between these two cell lines with regard to the effects of various treatments. Combination of hTERT knock-

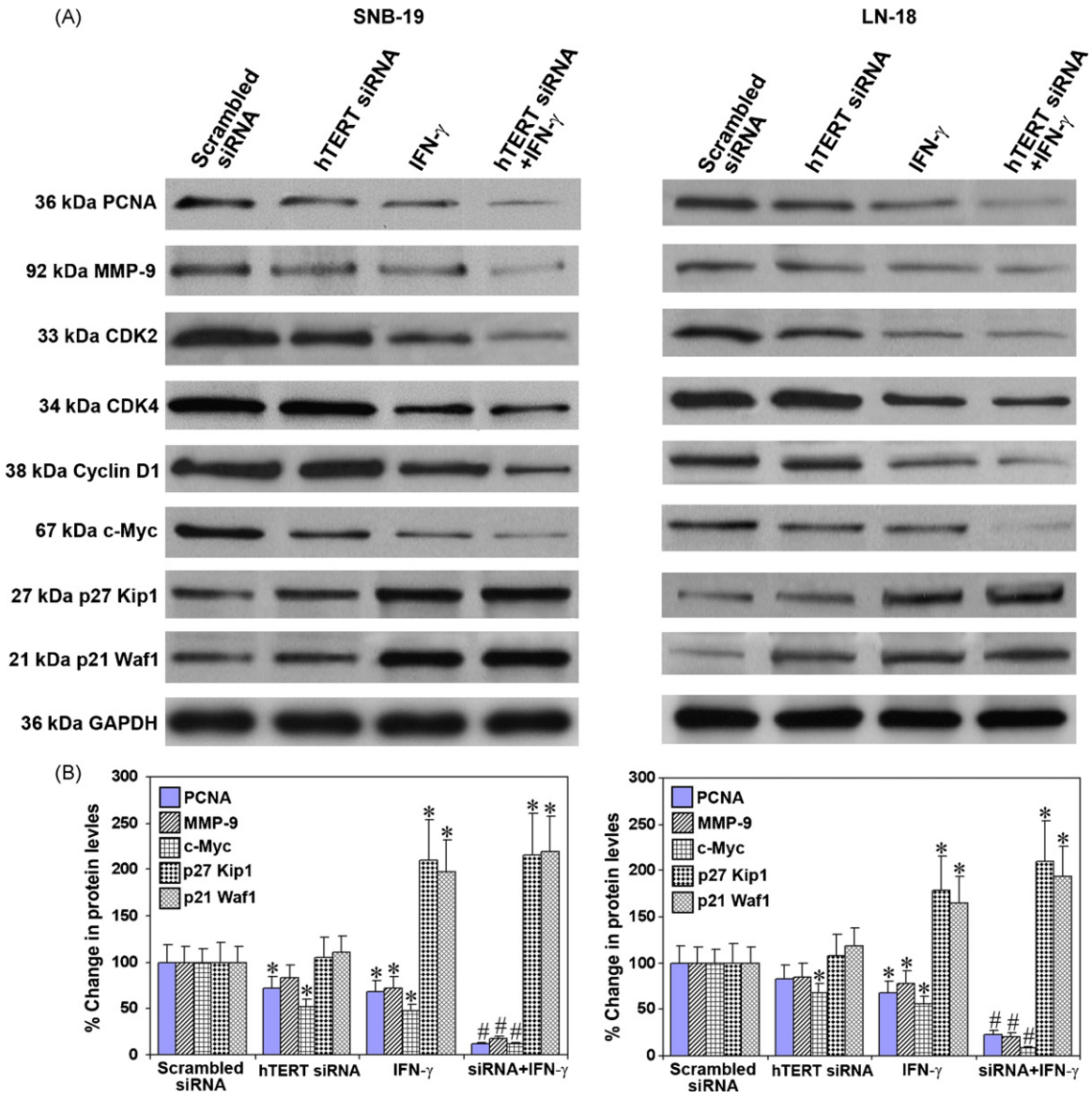

Fig. 7. Determination of levels of translational expression of MMP-9 and cell cycle regulatory molecules after transfection with hTERT siRNA or treatment with IFN-γ or both agents together for 48 h. (A) Western blotting for levels of PCNA, MMP-9, CDK2, CDK4, cyclin D1, c-Myc, p27 Kip1 and p21 Waf1 in the cell lysate of SNB-19 and LN-18 cells. The nitrocellulose membranes were reprobed for GAPDH content to demonstrate that same amount of protein was loaded in each lane. (B) Quantitative presentation of Western blot data. The Western blots were quantified using Gel-Pro analyzer software. Data are mean ± SD of 4 independent experiments ($^{*}p < 0.001$ compared with the control mean values and $^{\#}p < 0.001$ compared with hTERT siRNA or IFN-γ mean values).

down and IFN-γ treatment was highly effective for inhibition of proliferation and invasion of not only of PTEN wild type cell line but also of PTEN mutant cell line.

Telomerase plays an important role in regulating telomere length in tumor cells, maintaining proliferative capacity and tumor progression in more than 90% of human cancers (Shay et al., 2001; Maher et al., 2007). Telomerase and its major catalytic subunit hTERT are upregulated in most cancers, including glioblastomas (Hiraga et al., 1998). Moreover, hTERT expression was correlated with poor survival in glioblastomas (Tchirkov et al., 2003; Wang et al., 2006). Furthermore, telomerase activity and expression of hTERT correlated well with cell proliferation in meningiomas and malignant brain tumors in vivo (Cabuy and de Ridder, 2001). Thus, targeting telomerase has enormous potential for diagnostic, prognostic, and therapeutic applications to various cancers (Kirkpatrick and Mokbel, 2001; Shay et al., 2001). In the present study, siRNA directed against hTERT resulted in more than 70% suppression and this treatment with combination of IFN-γ resulted in more than 95% suppression of hTERT at the mRNA and protein levels. The down-regulation of hTERT was correlated with decrease in cell viability and proliferation, tumor cell migration, and invasion. Furthermore, we noticed that PCNA and MMP-9, the major molecules involved in these processes were also remarkably decreased after the knock-down of hTERT. The PCNA is directly involved in DNA replication and cell multiplication, while MMP-9 paves the way for cell proliferation and angiogenesis through degradation of the connective tissue matrix.

Investigating the effects of IFN-γ during silencing of hTERT expression is a novel strategy for inhibition of glioblastoma cell proliferation and invasion. Increased telomerase activity in various tumor cells, including glioblastomas has been implicated in resistance to anticancer drugs and radiation. Wild type hTERT transfected HeLa cells with longer telomeres exhibited resistance to radiation and chemotherapeutic agents (Xi et al., 2006). Telomerase activity in cultured human pancreatic carcinoma cells correlates with their potential for migration and invasion and

reflects the aggressive behavior of the tumor cells (Sato et al., 2001). In addition, siRNA targeted against hTERT significantly inhibited cell proliferation and increased apoptosis by downregulating hTERT expression and decreasing telomerase activity in SGC-7901 human gastric cancer cells (Qian et al., 2008). It was observed that IFN-γ downregulates telomerase activity and hTERT expression in human cervical cancer through upregulation of p27 Kip1 (Lee et al., 2005). In the present study, we noticed significant downregulation of hTERT at mRNA and protein levels after IFN-γ treatment. The siRNA mediated knockdown of hTERT together with IFN-γ treatment resulted in a remarkable reduction in cell viability and proliferation, cell migration from spheroids, and cell invasion on matrigel. IFN-γ mediated transcriptional regulation of related cellular activities would have also played a significant role in the modulation of these processes. Inhibition of hTERT had no short-term effect on cell survival and growth presumably because telomere shortening caused by inhibition of hTERT required several cell division (Zhao et al., 2007). The combined inhibitory effect of hTERT siRNA and IFN-γ on telomerase activity and cell proliferation could be responsible for the quick loss of cell survival, growth, and invasion capabilities of glioblastoma cells in the present study.

It is known that IFN-γ induces cell cycle arrest and cell death in primary culture of hepatocytes (Kano et al., 1997, 1999). In addition IFN-γ induces cell cycle arrest and apoptosis in ovarian cancer (Burke et al., 1997). It was demonstrated that prolonged treatment with IFN-γ induced cellular senescence in human endothelial cells as confirmed by G0/G1 cell cycle arrest, and upregulation of p53 and p21 protein levels (Sangfelt et al., 1999; Kim et al., 2009). In the present investigation, we have observed that up to 90% tumor cells are committed to cellular death within 48 h after the treatment with combination of hTERT siRNA and IFN-γ. The surviving cells after 48 h of combination treatment were very weak and not capable of regaining growth. Therefore, they were not capable of migrating from the spheroids.

In conclusion, our study demonstrated that knockdown of hTERT and concurrent treatment with IFN-γ effectively inhibited cell proliferation, migration, and invasion of human glioblastoma cells through downregulation of molecules involved in these processes and cell cycle arrest at G0/G1 phase. Therefore, the combination of hTERT siRNA and IFN-γ offers a potential therapeutic regimen for effectively controlling growth of human glioblastoma cells.

Acknowledgements

This work was supported in part by the R01 grants (CA91460 and NS57811 to S.K.R.) from the National Institutes of Health (Bethesda, MD).

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