Neuroprotective Signaling Mechanisms of Telomerase Are Regulated by Brain-Derived Neurotrophic Factor in Rat Spinal Cord Motor Neurons

Chenchen Niu, PhD and Henry K. Yip, PhD

INTRODUCTION

Apoptotic neuron death is a major pathologic process in many types of nervous system injury and in neurodegenerative diseases (1). Motor neurons undergo apoptosis during development and after axonal injury (2, 3) and apoptosis of spinal cord motor neurons (SMNs) is likely involved in the pathogenesis of amyotrophic lateral sclerosis (4, 5) and spinal muscular atrophy (6, 7). Apoptotic cell death in these disorders is thought to be related to a mitochondria-dependent pathway in which cytochrome c is released from mitochondria to the cytoplasm after activation of the initiator caspase-9 and the effector caspase-3 (8–10). Understanding the processes involved in SMN apoptosis is important for developing useful treatments that may promote neuron survival in these conditions.

Telomerase is a large protein complex that adds DNA repeats (TTAGGG) to the end of chromosomes to maintain telomere length. Telomerase inactivation is associated with telomere shortening and contributes to replicative senescence and cell death (11). Telomerase has 2 major components: the functional RNA component (TERC), which serves as a template for telomeric DNA synthesis, and the catalytic protein component with reverse transcriptase activity (TERT), which adds the telomeric repeats onto the end of chromosomes (12, 13). Telomerase plays important roles in the biology of both aging and neoplasia, processes that depend on telomere maintenance. However, there is accumulating evidence suggesting that telomerase has a role in the regulation of apoptosis and that is independent of its canonical function in telomere maintenance. Fu et al (14) established a stable clone of TERT-overexpressing pheochromocytoma (PC12) cells and found that ectopic TERT expression made these cells more resistant to staurosporine (STS)-, amyloid β- peptide–, and FeSO₄-induced apoptosis. They also found that TERT could protect primary hippocampal neurons against glutamate toxicity (15). In addition, ectopic expression of TERT protected mouse brain from hypoxia-ischemic injury (16) and developing SMNs from axonal injury (17). These results suggest that telomerase might be involved in promoting neuronal survival after injury.

Telomerase can be regulated by various factors, including growth factors, cytokines, natural compounds, and hormones (18). Moreover, its expression and activity are upregulated through activation of the phosphatidylinositol-3-OH kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways (19, 20). In neurons, BDNF mediates its cellular effects through activation of the PI3K/Akt and MAPK pathways (21). BDNF expression and activity increase during the early stages of development and contribute to the survival and differentiation of neurons (22). BDNF promotes neuronal survival and plasticity by activating various intracellular signaling pathways, including the PI3K/Akt and MAPK pathways (21). BDNF increases survival-promoting effect of telomerase in spinal cord motor neurons with higher telomerase levels induced by BDNF. These results suggest a role for BDNF in the survival-promoting effect of telomerase.
kinase/extracellular signal–regulated kinases 1 and 2 (MAPK/ERK1/2) signaling pathways by brain-derived neurotrophic factor (BDNF) and secreted amyloid precursor protein (15).

Brain-derived neurotrophic factor is a member of the neurotrophin family that is essential for neuronal survival during development. It is widely expressed throughout the CNS and contributes to synaptic plasticity in the adult mammalian brain. The effects of BDNF are mediated through its binding to the tyrosine kinase receptor B (TrkB) and subsequent activation of downstream signaling pathways (19). The PI3K/Akt and MAPK/ERK1/2 Trk signaling cascades mediate the neuroprotective response of BDNF (20–22). Recent works have shown that PI3K/Akt signaling pathway activation can regulate telomerase both at the transcriptional and post-transcriptional levels (23, 24). Phosphorylation of the MAPK/ERK1/2 signaling pathway can also regulate telomerase at the transcriptional level (25, 26) and at the posttranscriptional level (27). These data suggest the possibility that telomerase might be a downstream target of BDNF and that it may play a role in mediating the neuroprotective effects of BDNF.

Tyrosine kinase receptor B (TrkB) is expressed by SMNs (28). Moreover, BDNF can promote SMN survival after nerve injury in vivo (29). Because telomerase can promote SMN survival and SMNs respond to BDNF, we hypothesized that telomerase activation is a requirement for the neuronal survival-promoting effects of BDNF. In light of the induction of telomerase by BDNF and the neuroprotective role of telomerase in primary hippocampal neurons, the beneficial effects of telomerase might be extended to SMNs. To establish the relationships between BDNF signaling, telomerase, and the survival of SMNs, we investigated the regulation of telomerase by the BDNF/TrkB survival signaling pathway in SMNs. We observed antiapoptotic effects of telomerase in SMNs undergoing various cytotoxic insults. The underlying mechanisms of the antiapoptotic function of telomerase were studied using a stable clone of the TERT-overexpressing SMN cell line NSC-34.

**MATERIALS AND METHODS**

**SMN and NSC-34 Cell Cultures**

Primary SMN cultures were established from embryonic day 14.5 (E14.5) Sprague-Dawley rat embryos as previously described, with minor modifications (12). Ventral spinal cords were dissected out and collected in ice-cold Ca²⁺- and Mg²⁺-free Hanks balanced salt solution (HBSS) (Sigma, St Louis, MO). Harvested ventral spinal cords were cut into 4 to 5 pieces designated 1 spinal cord equivalent. About 4 to 5 spinal cord equivalents were pooled and incubated in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 10 minutes at 37°C. After digestion, the trypsin was carefully removed, and Neurobasal medium (Gibco, Carlsbad, CA) was added. The digested tissues were gently triturated up and down and centrifuged at 150 × g for 10 minutes at 4°C. After removing the supernatant, the cell pellet from every 4 to 5 spinal cord equivalents was resuspended in 1 ml of ice-cold HBSS and carefully layered on a cushion of ice-cold OptiPrep density gradient medium (Sigma; 1:4 in HBSS without phenol red). After centrifuging at 470 × g for 20 minutes at 4°C, SMNs were located in the turbid band at the HBSS-OptiPrep interface. The turbid bands from each tube were pooled together and diluted 1:3 with Neurobasal medium and centrifuged at 470 × g for 10 minutes at 4°C again. The cell pellet was resuspended in culture medium (F12/Dulbecco modified essential medium [DMEM, Gibco] containing 10% heat-inactivated fetal bovine serum [FBS] and 5% heat-inactivated horse serum [both from Invitrogen]) and seeded into a 4-well, 6-well, or 12-well plate coated with poly-d-lysine (10 μg/mL; Sigma) and laminin (10 μg/mL; Invitrogen) and cultured in a humidified incubator at 37°C with 95% air/5% CO₂. The cell density was 500 cells/mm². At the end of a 24-hour incubation period, the culture medium was changed to serum-free medium (Neurobasal medium containing 2% B-27 supplement and 2 mmol/L L-glutamine [Invitrogen]).

Spinal cord motor neuron cell line NSC-34 cells (Cedarlane, Burlington, Canada) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin in a humidified incubator at 37°C with 5% CO₂. The culture medium was changed every other day, and the cells were subcultured when they reached confluence. The vector containing full-length human TERT (hTERT) complementary DNA (cDNA; PLXIN-hTERT) or empty vector control (PLXIN) was transfected into NSC-34 cells using Lipofectamine 2000 (Invitrogen) for 24 hours. The pooled transfectants were grown in the presence of Geneticin (G418, 0.4 mg/mL; Invitrogen) for 2 weeks to ensure that the NSC-34 cells stably expressed hTERT.

Recombinant human BDNF, nerve growth factor (NGF), and glial-derived neurotrophic factor (GDNF) were purchased from PeproTech (London, UK). Glutamate and STS were purchased from Sigma. K252a (Sigma), LY294002 (Cell Signaling), and Bay11-7082 (Calbiochem, Darmstadt, Germany) were prepared in dimethyl sulfoxide and diluted in the culture medium to obtain the desired concentration.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (30) for 15 minutes. After washing with 0.01 mol/L PBS (pH 7.4) 3 times, the cells were blocked with 10% normal goat serum or normal donkey serum (depending on the secondary antibody) in diluent (1% bovine serum albumin and 0.3% Triton X-100 in 0.01 mol/L PBS) for 1 hour at room temperature. To assess the phenotypic characteristics of the cultured cells, immunolabeling studies were performed using the following antibodies: anti-SMI-32 (1:200; Covance, Princeton, NJ), anti-Hb9 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-β-tubulin III (1:1000; Sigma), anti-neurofilament 200 (1:1000; Sigma), anti-choline acetyl transferase (ChAT, 1:500; Chemicon, Temecula, CA), anti-p75 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-TrkB (1:500; Santa Cruz Biotechnology). Astrocytes were stained with anti-glial fibrillary acidic protein (GFAP) antibody (1:1000; Sigma). The primary antibody p65 (1:100; Cell Signaling) was used to detect nuclear translocation of p65 in the SMNs (Table 1).
The cultures were incubated with the primary antibodies in the diluent overnight at 4°C. For detection of primary antibodies, cells were then incubated in 0.01 mol/L PBS containing species-specific fluorescence-conjugated secondary antibodies (Table 2) for 1 hour at room temperature in the dark. Cell nuclei were counterstained with the nuclear dye 4′,6-Diamidino-2-phenylindole (DAPI) (1:10,000, Sigma). Negative controls were incubated with secondary antibodies only. All coverslips were mounted on slides and examined using a Leica fluorescence microscope. Images were captured by a SPOT digital camera (Diagnostic Instruments, Inc, Sterling Heights, MI). For quantification of the percentage of SMNs, the number of SMI-32−positive motor neurons was determined relative to the total number of DAPI-labeled nuclei at 3 days in vitro. For each coverslip, at least 8 randomly selected fields with more than 200 cells were counted. The counts were repeated in 3 independent samples.

### RNA Extraction and Quantitative Polymerase Chain Reaction

Both primary SMNs and NSC-34 cells were homogenized in TRIzol (Gibco). Total RNA was further isolated by chloroform (0.2 mL of chloroform per 1 mL of TRIzol reagent), precipitated in 0.5 mL of isopropanol alcohol per 1 mL of TRIzol reagent, washed in 75% ethanol for 5 minutes, dried for 10 minutes, and dissolved in 15 μL of diethyl pyrocarbonate–treated water. RNA quantity was assessed by spectrophotometric analysis using GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ). Complementary DNA was prepared by reverse transcription (RT) from 2 μg of RNA using a commercially available kit (SuperScript First-Strand Synthesis System; Invitrogen) according to the manufacturer’s instructions as the template for quantitative polymerase chain reaction (QT-PCR); 2 μL of cDNA template, 1 μL of forward primer (10 mM), 1 μL of reverse primer (10 mM), 12.5 μL of Platinum SYBR Green qPCR SuperMix (Invitrogen), and 6.5 μL of diethyl pyrocarbonate–treated water were mixed together and loaded in a 96-well plate. Quantitative PCRs were performed using the iCycler PCR system (Bio-Rad, Hercules, CA) and were repeated 3 times. Primers are listed as follows:

**Rat TERT primers** (forward 5′-AGCCATGTACGTAGC CATCC-3′ and reverse 5′-CTCTCAGCTGTTGGT GAA-3′); and

**Human TERT primers** (forward 5′-GGTTTTGGTGAT GATTC-3′ and reverse TGTACCTGCAATTT AGA-3′).

The comparative threshold cycle method (ΔΔ Ct) was used to quantify the messenger RNA (mRNA) expression levels of each gene. Amplified products of QT-PCR were loaded on a 1.0% agarose gel, electrophoresed through the gel, stained with ethidium bromide, and visualized under ultraviolet light (White/Ultraviolet Transilluminator; Ultra Violet Products, Upland, CA).

### Western Blotting

Primary SMNs and NSC-34 cells grown in 6-well plates were rinsed with PBS after different treatments and homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 2 mmol/L dithiothreitol, 2 mmol/L EDTA, 2 mmol/L EGTA, 50 mmol/L okadaic acid, 5 mmol/L sodium pyrophosphate, 1 mmol/L orthovanadate, 0.1% sodium deoxycholate, 0.5% sodium dodecyl sulfate [SDS], 1% protease inhibitor cocktail [Sigma]). The lysate was sonicated (3 × 3 seconds) and centrifuged at 12,000 rpm at 4°C for 20 minutes to separate the protein from the supernatant. Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad) and Labsystems Multiskan MS Plate Reader (Analytical Instruments, LLC, Golden Valley, MN). Equal amounts of protein were added with 5× sample buffer (250 mmol/L Tris-HCl, pH 7.5, 500 mmol/L dithiothreitol, 10% SDS, 0.5% bromphenol blue, 50% glycerol), and followed by heating at 95°C for 10 minutes. Equal amounts (30 μg) of protein at each time

### Table 1. Primary Antibodies Used for Immunocytochemistry

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**ChAT**, choline acetyltransferase; GFAP, glial fibrillary acidic protein.

### Table 2. Secondary Antibodies Used for Immunocytochemistry

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Point were separated by 10% or 12.5% SDS–polyacrylamide gel electrophoresis according to the molecular weight of different target proteins. After separation, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) for 3 hours at 400 mA at 4°C. Membranes were washed for 10 minutes with Tris buffer saline, pH 7.5 with 0.1% Tween-20 (TBST), and blocked by incubation in TBST containing 10% nonfat dry milk for 1 hour, followed by overnight incubation at 4°C with primary antibodies (Table 3). On the following day, the membranes were washed extensively in TBST before incubation for 90 minutes at room temperature with secondary antibodies: horseradish peroxidase-conjugated anti-rabbit (at 1:5000 dilution, Cell Signaling), or horseradish peroxidase-conjugated anti-mouse antibodies (at 1:5000 dilution, Cell Signaling). Membranes were then washed and exposed to a high-performance chemiluminescence film (GE Healthcare, Piscataway, NJ) and visualized with the Enhanced Chemiluminescence Western blotting Kit (GE Healthcare) (31). Images were digitally scanned and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). α-Actin was used as an internal control.

### Analysis of Telomerase Activity

Telomerase activity was determined by the telomeric repeat-amplification protocol (TRAP) assay using a TRAPeze Telomerase Detection Kit (Chemicon). Primary SMNs and NSC-34 cells cultured in 12-well plates were lysed in the CHAPS lysis buffer with RNase inhibitor (1 U/5 μL; GE Healthcare). After incubating at 4°C for 30 minutes, the lysate were centrifuged at 12,000 g at 4°C for 20 minutes, and protein concentrations of tissue extracts were determined by Coomassie Blue (Pierce, Rockford, IL) and Labsystems Multiskan MS Plate Reader (Analytical Instruments, LLC). Proteins (0.4 μg) were added to a reaction mixture containing 10× TRAP reaction buffer, 50× dNTP mixture, TS primers, TRAP primer mixture, and 5 U/μL Tag polymerase. The mixture was incubated at 30°C for 30 minutes to allow telomerase to add telomeric repeats to the TS primer and then subjected to 33 cycles of amplification (94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute), followed by a final extension step of 5 minutes at 72°C. The PCR products

### Table 3. Primary Antibodies Used for Western Blotting

<table>
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FIGURE 1. Phenotypic characterization of purified spinal cord motor neurons (SMNs). (A) Representative phase-contrast photomicrograph of SMN 3 days in vitro with typical triangle-shaped cell body and at least 2 primary neurites. (B–E) The SMNs are positive for the neuronal marker neurofilament (green) (arrow) and colabeled with Hb9 (red) (arrow) (B). They also express SMI-32 (C, green, arrow), p75 (D, red, arrow), and choline acetyl transferase (ChAT) (E, red, arrow). (F) There were also glial fibrillary acidic protein (GFAP)–positive astrocytes (green, arrow) in the cultures. Nuclei are counterstained with DAPI (blue) in C, E, and F. Scale bar = 20 μm.
were loaded on a 12.5% non-denaturing polyacrylamide gel, and DNA was electrophoresed through the gel. After staining by SYBR Gold (1:1000; Molecular Probes, Carlsbad, CA) at room temperature for 20 minutes, the gel was visualized under ultraviolet light (White/UV Transilluminator; Ultra Violet Products, Upland, CA). For quantitative analyses of relative telomerase activity in each sample, the total density of bands of the characteristic ladder (band 1 being the band immediately above the internal standard band) was quantified using ImageJ software. Values were expressed as a percentage of the control group.

**TERT Promoter Luciferase Assay**

NSC-34 cells were seeded in DMEM culture containing 1% FBS without antibiotics in 24-well plates before transfection. On the second day, cells were cotransfected with 0.5 µg of TERT-promoter (a gift from Dr.S.H. Phan, University of Michigan) (32) and 0.01 µg of pRL-luc plasmid as an internal control using Lipofectamine 2000. The pGLbasic3 plasmid was also transfected to the cells together with 0.01 µg pRL-luc plasmid as an external control. After 24 hours of transfection, a new medium was applied, and the cells were incubated for 24 hours. Finally, the cells were extracted in the passive lysis buffer, and luciferase assay was performed using the standard luciferase assay kit (Promega Corporation, Madison, WI). All experiments were carried out in triplicate.

**Analysis of Neuronal Death**

Neuronal injury was assessed by measuring lactate dehydrogenase (LDH) released into the culture medium using an LDH kit (Roche, Mannheim, Germany) after exposure to neurotoxic insults for 24 hours. Briefly, the culture medium was collected and centrifuged at 250 × g for 10 minutes to remove cells. The supernatant was then added to a 96-well plate and incubated with the assay buffer for 30 minutes at room temperature. Lactate dehydrogenase activity was analyzed by absorbance at 492 nm. Each measurement was repeated 3 times and run in triplicate. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an in situ cell death detection kit Fluorescein (Roche), in accordance with the manufacturer’s protocol. Briefly, primary SMNs and NSC-34 cells were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 in PBS for 15 minutes. The cells were incubated in the TUNEL reaction mixture for 90 minutes at 37°C. After washing with PBS, cells were counterstained with DAPI and visualized under a Leica fluorescent microscope with a ×20 objective. Representative areas (n = 8) were selected randomly, and at least 400 DAPI-positive cells were counted. The percentage of apoptotic cells was determined by the ratio of TUNEL-positive cells to the total DAPI-positive cells. The experiments were repeated using 3 independent samples. Cleaved caspase-3 immunostaining was also performed to detect the change of caspase-3 activity in SMNs after neurotoxic injury (Table 1).

**Knockdown of TERT Expression Using Short Interfering RNA**

A short interfering RNA (siRNA) duplex (5'-UAAGGA ACUGGCCCAUGUAAGCGCUG-3', 5'-CAGCCAUACAG GGCCAGUUCCUUA-3') targeting TERT (siTERT) was purchased from Invitrogen and dissolved in RNase-free distilled water. The Stealth RNAi negative control duplex (Invitrogen) was used as a negative control (siCON). NSC-34 cells were seeded at 80% to 90% confluence without antibiotics 1 day before transfection. The siRNA duplexes and Lipofectamine RNAiMAX (Invitrogen) were diluted in Opti-MEM (Invitrogen) for 5 minutes, mixed together for 20 minutes at room temperature, and then added to the culture medium for 24 hours. The knockdown efficiency of siTERT was tested by QT-PCR and Western blotting. The siRNA-transfected cells were used for RT-PCR and Western blot analyses 24 hours after transfection.

**JC-1 Mitochondrial Staining and Fluorescence Measurement in NSC-34 Cells**

JC-1 mitochondrial staining and fluorescence measurement of the NSC-34 cells were performed using a Mitochondrial Permeability Transition Detection Kit (ImmunoChemistry Technology, Bloomington, MN), according to the manufacturer’s instructions. During early apoptosis, mitochondria undergo 2 major changes: the outer mitochondrial membrane becomes permeable to proteins resulting in the release of soluble intermembrane proteins from the mitochondria and the inner mitochondrial membrane transmembrane potential (ΔΨm) is reduced. The cationic lipophilic fluorescein JC-1 can be used to detect the alteration in ΔΨm. In healthy cells, owing to the higher mitochondrial membrane potentials, JC-1 forms red fluorescent “J-aggregates,” whereas in apoptotic cells with low mitochondrial membrane potential JC-1 exists as a green fluorescent monomer. Thus, the emission of JC-1 can be used as a sensitive measurement of mitochondrial membrane potential. For JC-1 mitochondrial staining, after removing the culture medium from the chamber slides, 1 × JC-1 solution was added to cover the cells on the slide. The cells were then incubated at 37°C for 15 minutes in a CO2 incubator. In the meantime, the 1 × assay buffer was warmed to 37°C in a water bath. At the end of incubation, JC-1 solution was removed carefully and washed in the prewarmed 1 × assay buffer. Finally, the slide was mounted using a drop of 1 × assay buffer and examined under a Leica fluorescent microscope. For JC-1 fluorescence measurements, the cell pellet was collected by centrifugation at 180 × g for 5 minutes at room temperature, resuspended using 1 mL of 1 × JC-1 solution and then incubated at 37°C for 15 minutes in a CO2 incubator. At the end of incubation, 2 mL of prewarmed 1 × assay buffer was added to the tube, and the cells were centrifuged at 180 × g for 5 minutes at room temperature. Using 1 mL of 1 × assay buffer to resuspend the cell pellet and adjusting the cell number to the same among different groups, these cells were centrifuged again at 400 × g for 5 minutes at room temperature and resuspended in 1 mL of 1 × assay buffer. One hundred microliters of cell suspension was dispensed to each well of the 96-well plate, and the green fluorescence at 527 nm and the red fluorescence at 592 nm were then measured.
Phenotypic Characterization of the MAPK/ERK1/2 Cascades

BDNF Induces Telomerase Activity by TrkB

Statistical Analysis

Statistical analyses were conducted by SigmaStat software. One-way analysis of variance followed by the Tukey-Kramer multiple comparisons test was used to determine significant differences. Values are expressed as mean ± SEM from at least 3 independent experiments. p < 0.05 was regarded as significant.

RESULTS

Phenotypic Characterization of the Primary SMNs

Primary SMNs isolated and cultured for 3 days in vitro showed a triangular morphology with a minimum of 2 primary neurites under phase-contrast microscopy (Fig. 1A). Most, if not all, of the neurofilament 200–positive neurons coexpressed Hb9 (Fig. 1B). They were also SMI-32–, p75–, and ChAT-immunopositive (Figs. 1C–E). All SMI-32–positive neurons were also colabeled with p75 (Fig. 1D). Immunostaining for GFAP revealed that the cultures contained approximately 10% astrocytes (Fig. 1F). The percentage of SMI-32–positive motor neurons relative to the number of DAPI-labeled nuclei was 78.8% ± 3.1%, indicating that the purification method using OptiPrep gradient medium was reliable and highly efficient for isolating SMNs derived from the prenatal rat spinal cord.

BDNF Upregulated Telomerase Expression and Activity in SMNs

SMI-32–positive SMNs expressed the BDNF high-affinity receptor TrkB (Fig. 2A). To investigate the effect of BDNF on TERT mRNA levels and protein, primary SMNs were treated with BDNF (100 ng/mL) and assayed at 2, 6, and 24 hours by QT-PCR and Western blot analyses, respectively. Brain-derived neurotrophic factor stimulated the expression of TERT mRNA in a time-dependent manner, with a maximal increase at 6 and 24 hours versus controls (**, p < 0.001) (Fig. 2C). Using the TRAP assay, we determined that BDNF increased telomerase activity at 6 and 24 hours, a time course similar to that of TERT protein expression (Fig. 2D). To identify whether the telomerase induction was specific to BDNF, we treated the SMNs with another member of the neurotrophin family, NGF (50 ng/mL), and with a non-neurotrophin growth factor, GDNF (100 ng/mL) for 24 hours. Inhibition of TrkB with K252a abolished the increase in TERT protein expression by BDNF (Fig. 3A). We then investigated whether BDNF treatment would induce the phosphorylation of Akt and ERK1/2, the downstream effectors of PI3K and MAPK, respectively. Primary SMNs were stimulated with 100 ng/mL of BDNF for 2, 6, and 24 hours, and Western blots were performed with anti-phospho-Akt (Ser473) and anti-ERK antibodies. Western blot and densitometric analyses showed that stimulation with BDNF led to a significant increase in both Akt and ERK1/2 phosphorylation in the primary SMNs (Fig. 3B). The phosphorylation level of Akt maintained at a high level between 2 and 6 hours after the addition of BDNF and returned to the control level after 24 hours; the level of phosphorylated ERK1/2 increased at 2 hours and kept increasing until 24 hours of BDNF treatment (Fig. 3B).

BDNF Target the TERT Core Promoter Activity

The luciferase reporter assay showed that BDNF treatment for 24 hours induced a 4-fold increase in TERT core promoter activity in the SMN cell line NSC-34 cells versus the control group (Fig. 4). In addition, the increase in TERT promoter activity was abolished after neurons were pretreated with the TrkB inhibitor K252a (Fig. 4).

NF-κB, c-Myc, and Sp1 Are Major Transcription Activators of BDNF-Mediated Telomerase Regulation in SMNs

The TERT core promoter region contains binding sites for transcription factors NF-κB (33), c-Myc (34–36), and Sp1 (37), which are also the downstream targets of PI3K/Akt and MAPK/ERK1/2 signaling pathways. In addition, recent evidence showed that NF-κB could also regulate c-Myc and Sp1 (38, 39). To establish a role for NF-κB, c-Myc, and Sp1 in BDNF-mediated telomerase regulation in SMNs, we examined the effect of BDNF on the expression of these transcription factors in the primary SMNs. We found that BDNF stimulated the phosphorylation of IκB, which is required for the activation of NF-κB signaling. The increase in phosphorylated IκB emerged at 2 hours after the addition of BDNF and peaked at 6 hours; the level of phosphorylated IκB decreased at 24 hours but remained significant when compared with the control group (Fig. 5Aa). The activation of NF-κB could also translocate to the nucleus and regulate downstream target gene expression. Immunocytochemistry showed that the expression of NF-κB subunit p65 was localized in the cytoplasm of the untreated neurons;
However, 2 hours after BDNF treatment, p65 immunofluorescence was detected in the nucleus of the neurons (Fig. 5Ab). Similarly, BDNF increased c-Myc and Sp1 protein levels in the SMNs. c-Myc expression level increased significantly at 2 and 6 hours after the neurons were treated with BDNF and then decreased to the control level at 24 hours (Fig. 5B); however, the increase in Sp1 expression emerged 6 hours after BDNF treatment and was maintained until 24 hours (Fig. 5C). Inhibiting the activation of PI3K/Akt, ERK1/2, and NF-κB with LY294002, PD98059, and Bay11-7082, respectively, attenuated the increase in phosphorylated Akt, c-Myc and Sp1 expression levels induced by BDNF addition for 6 hours (Fig. 5D). Furthermore, in NSC-34 cells treated with the Bay11-7082 1 hour before incubating with BDNF for 24 hours, the stimulating effect of BDNF on TERT promoter activity was totally abolished (Fig. 5E).

**BDNF-Treated Primary SMNs With High Telomerase Levels Became More Resistant to Glutamate Toxicity**

To determine whether BDNF-induced telomerase up-regulation translated into an increase in survival of SMNs undergoing injury, SMN cultures were randomly divided into

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**FIGURE 2.** Brain-derived neurotrophic factor (BDNF) upregulates telomerase expression and activity in spinal cord motor neurons (SMNs). (A) Purified SMI-32-positive SMNs (green, arrow) express BDNF high-affinity receptor TrkB (red, arrow). Scale bar = 20 μm. (B) Telomerase with reverse transcriptase activity (TERT) mRNA expression level increases in BDNF-treated neurons. The peak of the increase was at approximately 6 hours after the addition of BDNF; it decreased after 24 hours but still was greater than control (*, p < 0.05; **, p < 0.001). (C) BDNF treatment induced TERT protein expression to 24 hours (**, p < 0.001). (D) Telomeric repeat-amplification protocol (TRAP) assay shows that telomerase activity increases in BDNF-treated SMNs. Telomerase activity continued to increase to 24 hours with BDNF treatment (*, p < 0.05) (E) TRAP assays showed that the increase in telomerase activity induced by nerve growth factor (NGF) was much weaker than that induced by BDNF after 24 hours of treatment. Glial-derived neurotrophic factor (GDNF) induced almost no increase in telomerase activity.

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**FIGURE 3.** Brain-derived neurotrophic factor (BDNF)-induced telomerase activation by TrkB signaling and via the phosphatidylinositol-3-OH kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase/extracellular signal–regulated kinases 1 and 2 (MAPK/ERK1/2) cascades in spinal motor neurons (SMNs). (A) The TrkB inhibitor K252a, the mitogen-activated protein kinase 1 (MEK1) inhibitor PD98059 and the PI3K inhibitor LY294002 abolished the effect of BDNF addition for 24 hours on telomerase protein expression in SMNs. (B) The addition of BDNF in the culture medium increased the phosphorylation levels of Akt and ERK1/2 (*, p < 0.05; **, p < 0.001). The increase in phosphorylated Akt emerged after 2 hours of stimulation and was maintained to 6 hours, then returned to the control level after 24 hours. Expression levels of phosphorylated ERK1/2 continued to increase from 2 to 24 hours of treatment.
4 groups: BDNF, BDNF plus glutamate, glutamate, and vehicle. Brain-derived neurotrophic factor was added to the culture medium for 24 hours before glutamate treatment. The morphologies of the primary SMNs with or without BDNF pretreatment were almost the same in the normal condition. Better cell morphology was observed in neurons treated with BDNF versus the nontreated control group in the presence of the cytotoxic effect of glutamate (Fig. 6A). There were more rounded cell bodies with long intact neurites in the BDNF-treated group, whereas there were more fragmented cell bodies with beaded neurites in the vehicle-treated control. The LDH assay demonstrated that the LDH levels in the culture medium released by the BDNF-treated SMNs after addition of glutamate were much lower than those in the control group not receiving BDNF (Fig. 6B). Brain-derived neurotrophic factor lowered the numbers of apoptotic cells assessed by TUNEL (Fig. 6Ca). During the 24-hour exposure period to glutamate, TUNEL-positive cells increased to 12% of the initial number of neurons, whereas only 4% of the neurons died during the 24-hour period in cultures treated with BDNF (Fig. 6Cb). The level of cleaved caspase-3 was decreased in cultures treated with BDNF versus cultures not treated with BDNF. Concomitantly, the TERT expression level was

**FIGURE 4.** Brain-derived neurotrophic factor (BDNF) targets telomerase with reverse transcriptase activity (TERT) core promoter activity. Brain-derived neurotrophic factor increased TERT promoter activity in the spinal cord motor neuron cell line NSC-34; this effect was abolished by pretreating the cells with the TrkB inhibitor K252a (**, p < 0.001). PGLBasic indicates pGL3-basic plasmid vector control.

**FIGURE 5.** Nuclear factor-κB, c-Myc, and Sp1 are the major transcription activators of brain-derived neurotrophic factor (BDNF)-mediated telomerase expression in spinal cord motor neurons (SMNs). (Aa) BDNF stimulated an increase in phosphorylated IκB at 2, 6, and 24 hours in SMNs (*, p < 0.05; **, p < 0.001). (Ab) Immunofluorescence shows nuclear translocation of p65 (red) (arrow) in BDNF-treated SMNs (β-tubulin III, green fluorescence). Scale bar = 50 μm. (B) BDNF stimulated an increase in c-Myc expression at 2 and 6 hours. After 24 hours of treatment, the c-Myc expression returned to the control level (*, p < 0.05; **, p < 0.001). (C) BDNF stimulated an increase in Sp1 expression at 6 and 24 hours (**, p < 0.001). (D) Inhibiting the activation of mitogen-activated protein kinase/extracellular signal-regulated kinases-1 and 2 (MAPK/ERK1/2), phosphatidylinositol-3-OH kinase/protein kinase B (PI3K/Akt) and NF-κB signaling pathways by the specific inhibitors PD98059, LY294002, and Bay11-7082, respectively, abolished the increased expression of phosphorylated IκB, c-Myc, and Sp1, induced by BDNF in SMNs for 6 hours. (E) Pretreating NSC-34 cells with the NF-κB inhibitor Bay11-7082 totally abolished the increase in telomerase promoter activity stimulated by the addition of BDNF for 24 hours (**, p < 0.001). PGLBasic indicates pGL3-basic plasmid vector control.
FIGURE 6. Brain-derived neurotrophic factor (BDNF)-treated primary spinal cord motor neurons (SMNs) with high telomerase levels have increased resistance to glutamate toxicity. (A) The morphologies of the primary SMNs with or without BDNF pre-treatment are almost the same under normal conditions but after the addition of glutamate for 24 hours, BDNF-pretreated SMNs were better preserved (more-rounded cell bodies with long intact neurites) than the control group (more fragmented cell bodies with beaded neurites). Scale bar = 20 μm. (B) The level of lactate dehydrogenase in the culture medium released by BDNF-treated SMNs was much lower than in the control group after treatment with glutamate (*, p < 0.05). (C) Apoptotic neurons detected by TUNEL staining (a, green). Scale bar = 50 μm. (b) TUNEL-positive cells increased to 12% of the initial number of neurons in the group without BDNF, whereas only 4% of SMNs were TUNEL-positive in the BDNF-treated group after the addition of glutamate (*, p < 0.05). (D) Representative Western blotting results show that the expression level of cleaved caspase-3 in the BDNF-treated SMNs was much lower than that in the control group after treating the neurons with glutamate for 24 hours. (E) Immunofluorescence results show that the expression level of cleaved caspase-3 (green) decreased in BDNF-treated SMNs compared with the control group after glutamate treatment for 24 hours. Scale bar = 20 μm. Neuron nuclei are counterstained with DAPI (blue) in Ca and E.
increased in the BDNF-treated SMNs versus the untreated controls (Fig. 6D). Immunocytochemistry confirmed the Western blot results showing a decrease in the numbers of SMNs with positive cleaved caspase-3 immunoreactivity in the BDNF-treated culture (Fig. 6E).

**TERT-Overexpressing NSC-34 Cells Were More Resistant to STS-Induced Apoptosis**

The TERT-overexpressing NSC-34 cells expressed the motor neuron marker ChAT (Fig. 7A). Results from the RT-PCR confirmed that NSC-34 cells stably transfected with PLXIN-hTERT (NSC-34–hTERT cells) but not the cells transfected with empty vector (NSC-34–PLXIN cells), expressed hTERT mRNA (Fig. 7B). In addition, Western blotting and TRAP assay results detected a higher expression level of hTERT protein and telomerase activity, respectively, in the TERT-overexpressing NSC-34 cells (NSC-34–hTERT cells) versus the cells transfected with vector control (NSC-34–PLXIN cells) (Figs. 7C, D).

Cleaved caspase-3 was used as a biochemical marker of STS-induced apoptosis. Cultivation of vector control

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**FIGURE 7.** Establishing a stable clone of telomerase with reverse transcriptase activity (TERT)-overexpressing NSC-34 cells (NSC-34–hTERT cells). (A) Representative phase-contrast photomicrography showed the morphology of NSC-34 cells in culture; these cells express choline acetyl transferase (ChAT; red fluorescence). Nuclei are counterstained with DAPI (blue). Scale bar = 20 μm. (B) RT-PCR shows that the NSC-34 cells stably transfected with vector containing full-length human TERT complementary DNA (PLXIN-hTERT) (NSC-34–hTERT cells), but not the empty vector control cells (NSC-34–PLXIN cells), express hTERT mRNA. (C) Representative Western blotting results showed the higher level of hTERT protein expression in the NSC-34–hTERT cells versus the empty vector control NSC-34–PLXIN cells. (D) Telomeric repeat-amplification protocol (TRAP) assay shows a higher level of telomerase activity in NSC-34–hTERT cells versus NSC-34–PLXIN cells.
NSC-34–PLXIN cells in STS resulted in an increase in the expression level of cleaved caspase-3 in a dose- and time-dependent manner, with a maximum increase at 0.4 μmol/L by 4 hours (Figs. 8Aa, Ab). The increase in cleaved caspase-3 expression level was inhibited in the NSC-34–hTERT cells incubated with STS at the same doses and time intervals (Figs. 8Aa, Ab). Thus, overexpressing TERT in SMNs made the cells more resistant to apoptosis.

Cleavage of caspase-9 can initiate the downstream cleavage of caspase-3 to generate the apoptosis mediator cleaved caspase-3. Therefore, we measured the cleaved caspase-9 expression level in the NSC-34–hTERT cells and vector control NSC-34–PLXIN cells after treatment with 0.2 μmol/L STS for 4 hours. Western blot results showed a significant increase in cleaved caspase-9 expression in the vector control NSC-34–PLXIN cells after STS treatment (*, p < 0.05; **, p < 0.001) but not in the NSC-34–hTERT cells (Figs. 8Ba, Bb). The expression level of cleaved caspase-3 was significantly lower in the STS-treated NSC-34–hTERT cells than in vector control cells (**, p < 0.001) (Fig. 8Bc). The number of

**FIGURE 8.** Telomerase with reverse transcriptase activity (TERT)–overexpressing NSC-34 cells (NSC-34–hTERT cells) are more resistant to staurosporine (STS)-induced apoptosis. (A, B) Representative Western blotting shows that STS-induced apoptosis is dose-dependent (Aa) and time-dependent (Ab) in both NSC-34–hTERT cells and vector control NSC-34–PLXIN cells. At the same dose and time intervals, the expression of cleaved caspase-3 was dramatically inhibited in the NSC-34–hTERT cells compared with NSC-34–PLXIN cells. (Ba, Bb) After treating the cells with STS for 4 hours, the expression level of cleaved caspase-9 increased significantly in the NSC-34–PLXIN cells, but not in the NSC-34–hTERT cells (*, p < 0.05; **, p < 0.001). (Ba, Bc) In the NSC-34–hTERT cells after treatment with STS, the expression level of cleaved caspase-3 was significantly lower than in vector control cells (NSC-34–PLXIN cells) (**, p < 0.001). (C) TUNEL assay demonstrates apoptotic cells (Ca, green); nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. Staurosporine for 4 hours induced a weaker apoptotic response in the NSC-34–hTERT cells compared with the NSC-34–PLXIN cells (16.6% vs 5.8% apoptotic cells) (**, p < 0.001) (Cb).
TUNEL-positive NSC-34–hTERT cells was much lower than the number of vector control NSC-34–PLXIN cells after STS treatment for 4 hours (Fig. 8Ca). The percentage of apoptotic NSC-34–hTERT cells induced by STS decreased when compared with the vector control NSC-34–PLXIN cells (16.6% vs 5.8%; **, p < 0.001) (Fig. 8Cb).

**FIGURE 9.** Knockdown of telomerase with reverse transcriptase activity (TERT) expression increased the sensitivity of NSC-34 cells to staurosporine (STS)-induced apoptosis. (A, B) RT-PCR (A) and Western blotting (B) results show a decrease of endogenous TERT mRNA and protein expression levels in NSC-34 cells after transfection with short interfering TERT RNA (siTERT) for 24 hours. (C) Western blotting shows that, after the cells were treated with STS, the expression level of cleaved caspase-3 increased significantly in the NSC-34–siTERT cells versus vector control cells (NSC-34–siCON cells) (**, p < 0.001). (D) Apoptotic cells were detected by TUNEL assay (green) (Da). All nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. After the addition of STS, the number of apoptotic cells in NSC-34–siTERT group was higher than in the NSC-34–siCON group (20% vs 10% apoptotic cells at 4 hours (**, p < 0.001) (Db).
Knockdown of TERT Expression Increases the Sensitivity of NSC-34 Cells to STS-Induced Apoptosis

Knockdown of endogenous TERT gene expression by siRNA decreased TERT mRNA (Fig. 9A) and protein expression (Fig. 9B) in NSC-34 cells. During a 4-hour treatment of 0.2 μmol/L STS, the expression level of cleaved caspase-3 in NSC-34–siTERT cells was significantly higher than in the vector control NSC-34–siCON cells (**, p < 0.001) (Fig. 9C). In addition, there were more TUNEL-positive NSC-34–siTERT cells than NSC-34–siCON cells after 4 hours of STS (Fig. 9D). STS induced a higher percentage of apoptotic NSC-34–siTERT cells than NSC-34–siCON cells (20% vs 10%; **, p < 0.001) (Fig. 9Db).

The Antiapoptotic Function of TERT in NSC-34 Cells Was Mediated Through the Upregulation of Bcl-2 and the Downregulation of Bax and p53 and the Maintenance of Mitochondrial Membrane Potential

STS decreases the expression of prosurvival protein Bcl-2 and increases the expression of proapoptotic protein Bax to trigger a mitochondria-dependent apoptotic pathway in numerous cell types including neurons (40–42). The depolarization of mitochondria leads to the release of cytochrome c to activate the downstream-cleaved caspase-3 to initiate apoptosis. STS treatment decreased Bcl-2 expression (Fig. 10A) and increased Bax expression (Fig. 10B) in vector control NSC-34 cells (NSC-34–PLXIN cells). However, after STS treatment of the TERT-overexpressing NSC-34 cells (NSC-34–hTERT cells), Bcl-2 expression was significantly increased and Bax expression was significantly decreased versus vector control NSC-34–PLXIN cells (Figs. 10A, B). Knockdown of endogenous telomerase gene expression in the NSC-34 cells increased Bax expression and decreased Bcl-2 expression in the absence of STS (Fig. 10C). Tumor suppressor gene p53 is involved in the neuronal apoptosis pathway (43). p53 can induce cell apoptosis both by regulating downstream target genes at transcriptional level and by direct protein-protein interaction at the posttranscriptional level (44, 45). In TERT-overexpressing NSC-34–hTERT cells, the p53 expression level was significantly lower than that in the vector control NSC-34–PLXIN cells. After STS treatment, p53 level in the NSC-34–hTERT cells remained significantly lower versus the vector control (**, p < 0.001) (Fig. 10D).

Overloading of mitochondrial Ca2+ and loss of mitochondrial membrane potential (ΔΨm) resulted in interruption of ATP synthesis and eventual neuronal death. Mitochondrial membrane potential in the NSC-34–PLXIN or NSC-34–hTERT cells after STS treatment was analyzed using JC-1 immunostaining and fluorescence measurement. In the presence of STS, most NSC-34–PLXIN cells emitted green fluorescence, indicating that they were undergoing apoptosis (Fig. 10E). In contrast, the proportion of NSC-34–hTERT cells emitting red fluorescence increased in the presence of STS and the proportion of green fluorescent cells decreased, indicating that NSC-34–hTERT cells are more resistant to STS toxicity (Fig. 10E). The red-green optical density ratio dropped significantly in the NSC-34–PLXIN cells when compared with the normal control and also with the NSC-34–hTERT cells after STS treatment (**, p < 0.001) (Fig. 10F). By contrast, there was no significant decrease of the red-green optical density ratio in the NSC-34–hTERT cells after the addition of STS (Fig. 10F). These data indicate that TERT overexpression in NSC-34 cells could maintain the mitochondrial membrane potential, thus inhibiting the initiation of the mitochondria-dependent apoptotic pathway induced by STS.

DISCUSSION

Both primary SMNs prepared from the ventral spinal cords of E14.5 rat embryos and the SMN cell line NSC-34 were used to investigate the protective role of telomerase against neuronal cell death. Our characterization of the primary SMN cultures indicated that the methods used provided a reliable and efficient way to isolate a large population of SMNs. We tried different dosages of BDNF in the primary SMNs to identify a BDNF concentration that could induce the highest level of telomerase and also protect the maximum number of neurons after glutamate treatment; maximal survival effects with BDNF were observed at 100 ng/ml (data not shown). This concentration of BDNF was used in our experiments.

Quantitative PCR and Western blotting results showed that BDNF significantly increased telomerase mRNA and protein expression levels in the SMNs at 6 hours, and the levels kept increasing until 24 hours after the addition of BDNF. In addition, the TRAP assay showed an increase in telomerase activity in BDNF-treated SMNs. These data indicate that BDNF could upregulate telomerase expression and activity in the SMNs and suggest that telomerase might be a downstream target of BDNF/TrkB survival signaling pathways. To examine whether the activation of BDNF/TrkB signaling was specific for the activation of telomerase, we assessed the effects of NGF and GDNF on the telomerase activation pathways in the SMNs and suggest that telomerase might be a downstream target of BDNF/TrkB survival signaling pathways. To examine whether the activation of BDNF/TrkB signaling was specific for the activation of telomerase, we assessed the effects of NGF and GDNF on the telomerase activity. Nerve growth factor binds with its preferred high-affinity receptor TrkA to activate the downstream signaling pathway (46, 47). The membrane receptor for GDNF is GFRα1. Glial-derived neurotrophic factor has a protective function for SMNs both in vitro and in vivo (48, 49). We found, however, that NGF had only a weak effect in increasing telomerase activity, and GDNF had almost no effect on telomerase activity in the SMNs. This suggests that activation of TrkB receptors by BDNF was specific for telomerase activation in the SMNs. Because SMNs are a distinct neuron type that is responsive to BDNF, activation of telomerase by BDNF in these cells might be a novel mechanism for promoting neuronal survival after injury.

Telomerase can be regulated at the transcriptional level through the binding between its promoter and transcription factors. There are binding sites in the telomerase promoters for NF-κB (33), c-Myc (34–36), Sp1 (37), hypoxia-inducible factor (50, 51), activator protein 2 (52), estrogen receptor (53, 54), and E26 (Ets) (26). Among them, NF-κB, c-Myc, Sp1, and Ets could be downstream targets of PI3K/Akt and MAPK/ERK1/2 signaling pathways, that is, important
signaling pathways downstream of BDNF/TrkB that can mediate neuronal survival (20–22). Evidence from recent work in cancer biology and immunology indicates that the PI3K/Akt signaling pathway can regulate telomerase expression and activity at the transcriptional level through the activation of NF-κB. It can also phosphorylate telomerase to increase its activity depending on the posttranscriptional mechanism (23, 24). In addition to binding to the telomerase promoter directly, NF-κB also can regulate c-Myc and Sp1 to exert the same function (38, 39). The regulation of telomerase by the MAPK/ERK1/2 signaling pathway has also been investigated in nonneuronal cells (55). Extracellular signal–regulated kinases 1 and 2 could regulate telomerase through the transcription factors c-Myc and Sp1 (25). The other transcription factor Ets is also the downstream target of ERK1/2 signaling, the mechanism by which Ets regulates telomerase, including binding to the telomerase promoter directly or through c-Myc (56). Taken together, the transcriptional regulation of telomerase is complex, and there is crosstalk and networking among the functions of these upstream signaling pathways and their downstream transcription factors.

We found that the addition of BDNF could increase the phosphorylation levels of both Akt and ERK1/2 in SMNs, and blocking the TrkB receptor by K252a and inactivating PI3K/Akt and MAPK/ERK1/2 signaling pathways by LY294002 and PD98059, respectively, reversed the increase in telomerase expression induced by BDNF. These results indicate that, in SMNs, BDNF increased telomerase expression through the TrkB receptor–mediated activation of PI3K/Akt and MAPK/ERK1/2 signaling pathways. Luciferase reporter assay results showed that exogenous BDNF increased TERT promoter activity in the SMNs and that this effect could be abolished by the inhibition of TrkB. This result suggests that BDNF targeted the TERT core promoter and could regulate telomerase at the transcriptional level through binding with TrkB receptor to activate downstream signaling pathways. Interestingly, we observed activation of the NF-κB signaling pathway and upregulation of the transcription factors c-Myc and Sp1 in the BDNF-treated SMNs. Inhibition of PI3K/Akt and MAPK/ERK1/2 signaling pathways abolished the increases in phosphorylated IκB, c-Myc, and Sp1 expression. In addition, the NF-κB signaling pathway inhibitor Bay11-7082 reversed the increase in c-Myc and Sp1 expression induced by BDNF. These data indicate that there is a network within the PI3K/Akt and MAPK/ERK1/2 signaling pathways that could directly regulate NF-κB, c-Myc, and Sp1 and indirectly regulate c-Myc and Sp1 through the activation of the NF-κB signaling pathway in SMNs. The luciferase reporter assay results showed that inhibition of the NF-κB signaling pathway could totally abolish the increase in telomerase promoter activity, suggesting that NF-κB might be a more important downstream target of PI3K/Akt and ERK1/2 signaling pathways regulating telomerase in SMNs. The redundancy in the PI3K/Akt and MAPK/ERK1/2 signaling pathways in telomerase regulation in SMNs could amplify the effect of BDNF on the upregulation of telomerase expression and activity in SMNs. Telomerase might therefore be a critical downstream target of BDNF/TrkB signaling that promotes SMN survival.

Apoptosis of SMNs is an important pathologic process in motor neuron degenerative diseases including amyotrophic lateral sclerosis (57) and spinal muscular atrophy (58). Motor neuron death in these disorders is related to mitochondrial dysfunction, which may activate the mitochondria-dependent apoptosis pathway (10, 58, 59). In this pathway, there is release of cytochrome c, upregulation of Bax, downregulation of Bcl-2, activation of caspase-9, and finally activation of the so-called apoptosis executioner caspase-3. Glutamate-induced excitotoxicity through the upregulation of Bax and activation of caspase-3 has been reported (60). In addition, STS as a protein kinase inhibitor can also induce caspase-3-dependent apoptosis in neurons (40–42). Thus, we used glutamate and STS to induce SMNs to undergo mitochondria-dependent apoptosis and investigated the antia apoptotic function of telomerase. We found that the BDNF-treated SMNs with higher telomerase expression and activity became more resistant to glutamate toxicity compared with controls; in particular, there was a lower expression of cleaved caspase-3. This suggests that inhibiting the expression of cleaved caspase-3 might be the explanation for the antia apoptotic effect of BDNF on the SMNs treated with glutamate or STS. In addition, as a downstream target of BDNF, telomerase might be the antiapoptotic effector of the BDNF/TrkB survival signaling pathway in SMNs. Our results support previous observations by Fu et al (15) who demonstrated that telomerase could

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**FIGURE 10.** The antiapoptotic function of telomerase with reverse transcriptase activity (TERT) in NSC-34 cells is mediated through upregulation of Bcl-2, downregulation of p33 and Bax, and maintenance of mitochondrial integrity. (A) Staurosporine (STS) treatment decreased Bcl-2 expression in vector control NSC-34–PLXIN cells. In contrast, the expression of Bcl-2 increased significantly in TERT-overexpressing NSC-34–hTERT cells (*, p < 0.05; **, p < 0.001). (B) STS treatment increased Bax expression in the vector control NSC-34–PLXIN cells. In contrast, after the STS insult, the expression level of Bax decreased significantly in the TERT-overexpressing NSC-34–hTERT cells (*, p < 0.05; **, p < 0.001). (C) Knockdown of endogenous telomerase gene expression in NSC-34 cells by siRNA increased the expression level of Bax and decreased the expression level of Bcl-2 in the absence of STS. (D) In TERT-overexpressing NSC-34–hTERT cells, the expression level of p33 was significantly lower than that in vector control NSC-34–PLXIN cells. After STS treatment, p33 level in the NSC-34–hTERT cells remained significantly lower versus the vector control (**, p < 0.001). (E) JC-1 immunostaining showed that most NSC-34–hTERT cells and NSC-34–PLXIN cells were healthy (red fluorescence, red arrows) in the absence of STS. After the addition of STS, most NSC-34–hTERT cells could still maintain normal mitochondrial membrane potential (red arrow); only a small proportion of the cells underwent apoptosis (green fluorescence, green arrow). Most NSC-34–PLXIN cells underwent apoptosis after treatment with STS (green arrow). (F) JC-1 fluorescent measurement result showed that the red-green fluorescent ratio dropped significantly in the NSC-34–PLXIN cells after the treatment of STS when compared with both the NSC-34–PLXIN cells in the absence of STS and the STS-treated NSC-34–hTERT cells (**, p < 0.001).
mediate the neuroprotective function of BDNF in primary hippocampal neurons. Furthermore, the antiapoptotic effects observed in the stable clone of the TERT-overexpressing SMN cell line NSC-34 further confirmed that neurons with high telomerase levels had a better ability to withstand apoptotic insults than did the vector control neurons. These results are consistent with previous studies in nonneuronal cells and neurons. For example, transient overexpression of hTERT-HA in 293T cells decreased STS-induced apoptosis; the protective effect by overexpressing hTERT-HA was comparable to the antiapoptotic effect observed in the cells transfected with X-linked inhibitor of apoptosis (61). In addition, mouse embryonic fibroblasts (MEFs) from TERT-deficient mice were found to be more sensitive to STS than MEFs from wild type mice, whereas MEFs from hTERT transgenic mice were less sensitive to STS (17). Overexpression of TERT in neuronal cell line PC12 cells protected these cells against various kinds of apoptotic insults (14), and transgenic overexpression of mTERT attenuated neuronal apoptosis induced by excitotoxicity both in vivo and in vitro (16). In mTERT-overexpressing neonatal mice, the number of apoptotic SMNs induced by sciatic nerve axotomy was much lower than in wild type animals (17). Taken together, our data suggest that telomerase might mediate the antiapoptotic function of BDNF in SMNs and that high levels of telomerase expression and activity could protect SMNs against apoptotic insults.

The mechanisms underlying the antiapoptotic functions of telomerase are not fully understood. The results from nonneuronal cells showed a direct interaction between TERT, p53, and poly (ADP-ribose) polymerase in the breast cancer cell line PMC-42 (62). TERT can suppress p53-dependent apoptosis in the Burkitt lymphoma cell line BL41 (63). These results indicate that telomerase might exert its antiapoptotic function through the interaction with other proteins that induce apoptosis. Zhu et al (64) investigated the antiapoptotic function of telomerase in cultured neurons and found that telomerase could inhibit mitochondrial dysfunction and caspase activation. This study raised the possibility that telomerase could alter the sensitivity of mitochondria to apoptotic insults. There is also the possibility of translocation of telomerase between nucleus and cytoplasm because of the nuclear export signal-like motif of telomerase (65). Thus, the cytoplasmic localization of telomerase might be another mechanism for the antiapoptotic function of telomerase. Furthermore, ectopic expression of telomerase in human mammary epithelial cells could increase the expression levels of genes that promote cell growth, such as epidermal growth factor receptor and fibroblast growth factor, but decrease the expression levels of the proapoptotic genes such as the tumor necrosis factor-related apoptosis-inducing ligand (66).

We found that overexpressing TERT in NSC-34 cells can inhibit Bax expression but increase the expression level of Bel-2 after induction of STS when compared with the empty vector control. Knockdown of endogenous telomerase expression in NSC-34 cells by siRNA increased Bax expression but decreased Bel-2 expression. Overexpressing TERT in NSC-34 cells also downregulated the expression level of p53. p53 can activate the expression of several proapoptotic genes such as PUMA, Noxa, and Bax to induce neuronal death in the absence of apoptotic insults (45). In addition, p53 can also accumulate in the cytoplasm and induce neuronal apoptosis through direct protein-protein interaction at the posttranscriptional level (44). Although p53 has been found to be upregulated in injured neurons, STS treatment did not induce a significant elevation of p53 expression in SMNs in this study. There are 2 possible explanations for this: the treatment time for apoptotic toxicity was not long enough for inducing upregulation of p53 expression or the translocation of p53 to the cytoplasm is more important than the total p53 expression level in inducing apoptotic signaling in the SMNs. There is evidence to show that STS can promote p53 cytoplasmic accumulation in neural precursor cells to induce apoptosis (44). Taken together, our results provide evidence that telomerase might influence the expression of p53, Bax, and Bel-2 directly. As downstream targets of p53, the expression levels of Bel-2, the mitochondrial membrane potential, were much higher, but the expression level of Bax was much lower in TERT-overexpressing NSC-34 cells than in vector control cells after apoptotic insult. All of these data suggest that telomerase inhibits the initiation of mitochondria-dependent apoptosis in the SMNs. Although our results did not provide evidence for the cytoplasmic localization of telomerase in the SMNs, it may be translocated to the cytoplasm to interact with proapoptotic as well as prosurvival molecules and also with mitochondria. As a consequence of the integrity of the mitochondrial membrane being maintained, activation of caspase-9 and caspase-3 was blocked in TERT-overexpressing SMNs, resulting in the protection from apoptotic injury. Thus, our data strongly suggest that the underlying mechanism of the antiapoptotic function of telomerase is to inhibit the initiation of the mitochondria-dependent apoptotic pathway.

Taken together, we establish the neuroprotective relationship between BDNF and telomerase in SMNs and suggest that telomerase might be a mediator of the BDNF/TrkB signaling pathway in promoting neuronal survival. In addition, high levels of telomerase expression and activity could inhibit the initiation of the mitochondrial apoptosis pathway in SMNs. Thus, we describe a novel mechanism for the neuroprotective function of BDNF in SMNs and raise a new direction for future therapeutic strategies to protect motor neurons after spinal cord injury and in neurodegenerative diseases.

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