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Research Article

The Neuroprotective Role of TERT Influences the Expression of SOD1 in Motor Neurons and Mouse Brain: Implications for fALS

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Abstract

Amyotrophic lateral sclerosis (ALS) disease is characterized by degeneration of motor neurons and elevation of brain oxidative stress. Previous studies demonstrated the neuroprotective effects of Telomerase reverse transcriptase (TERT) from oxidative stress. We showed that increasing TERT expression in the brain of the Tg hSOD1^{G93A} mouse ALS model attenuated the disease pathology and increased the survival of motor neurons exposed to oxidative stress. How TERT increased the survival of motor neurons exposed to oxidative stress is not yet clear. Here we investigated the consequence of TERT depletion in motor neuron cells under normal and oxidative stress conditions and in mouse brains of TERT knockout mice, on the expression and activity of SOD1 and catalase enzymes. Depletion of mouse TERT caused mitochondrial dysfunction and impaired catalase and SOD1 activity. Compensation with hTERT restored the activity of SOD1. SOD1 expression increased in the brain of TERT KO and in ALS mice and decreased in the brain of WT mice treated with telomerase-increasing compounds. We suggest that the ability of TERT to protect neurons from oxidative stress affects the expression and activity of SOD1, in a TERT-dependent manner, and supports the notion of TERT as a therapeutic target for neurodegenerative diseases like ALS.

More Information

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Introduction

Amyotrophic Lateral Sclerosis (ALS) known also as Lou Gehrig`s disease is a paralytic disorder that is characterized by degeneration of the upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord that causes progressive denervation of voluntary muscles. This is a progressive and lethal disease with a low life expectancy of two to five years. The cause of sporadic ALS is yet unknown. The mechanisms involved in motor neuron degeneration are multifactorial and complex. The heterogeneity of ALS suggests that different mechanisms may play prominent roles in individual patients. ALS is a complex disease in 10% - 21% of patients it demonstrates a monogenic while in the majority of affected individuals, an interaction of multiple genetic and environmental risk factors was detected [1]. Accumulating evidence suggests oxidative stress as potential pathogenesis of the disease: 1. Increased oxidative damage is found in vast regions in the brains of ALS patients and in mouse disease models [1-3], including neurons and glia cells [4]. 2. Reactive Oxygen Species (ROS) was demonstrated to mediate the damage of glutamate excitotoxicity, mitochondrial dysfunction, mutant SOD1 protein aggregation, and abnormal accumulation of neurofilaments [5-8]; all of them were suggested to be ALS pathogenesis factors. In addition, human Telomerase Reverse Transcriptase (hTERT) expression level was significantly lower in ALS patients and was correlated either to p53 mRNA expression or p21 expression, suggesting that a decrease in telomerase could be a pathogenic contributor to neurodegeneration in ALS [9].

Telomerase is a ribonucleoprotein complex, which functions in telomeres maintenance. The active enzyme consists of Telomerase Reverse Transcriptase (TERT), an

RNA subunit (TERC), and several additional proteins that are important for telomerase assembly, stability, and trafficking into the telomere [10-12]. TERT is mainly active in highly proliferated cells (e.g., antigen-stimulated lymphocytes, germ cells, embryonic cells, and most cancers). In somatic cells TERT expression is tightly regulated therefore, somatic cells usually do not demonstrate telomerase activity (mechanisms of TERT regulation are summarized in [13]. Emerging evidence indicates that low levels of TERT expression are observed in somatic cells such as Purkinje cells in the brain [14], bone marrow mesenchymal stromal cells [15], breast tissues [16], epithelial cells [17] and fibroblasts [18]. In the last decade, we and others demonstrated the neuroprotective effects of TERT in mouse brain [19-23]. In addition, accumulating evidence indicates that TERT possesses additional non-telomeric activities. Under oxidative stress, TERT migrates from the nucleus into the mitochondria. This migration is followed by an improved coupling of the Mitochondrial Membrane Potential (MMP) with ATP production and by inhibition of the mitochondrial apoptotic pathway [24,25]. The mechanism for these activities of TERT is not completely understood. It has been shown that TERT lacking reverse transcriptase activity can still protect mouse and human cells, thus the protective effect of TERT is not necessarily related to its ability to maintain telomeres [26]. In addition, TERT did not improve base excision repair in the mitochondria under oxidative stress but increased the level of MnSOD protein [27]. It was previously shown in our lab that overexpression of human TERT (hTERT) reduced the sensitivity of NSC-34 cells, which are a mouse motor neuron-like cell line, to oxidative damage [14], indicating hTERT ability to function in mouse cells. We also showed that increasing telomerase activity and expression in the brain by specific compounds (AGS) designed by our laboratory resulted in attenuation of the ALS disease pathology in the Tg $SOD1^{G93A}$ mouse model [21]. We also showed that increasing telomerase expression and activity by AGS compounds protect hippocampal neurons from amyloid beta toxicity by enhancing the expression of neurotrophins and plasticity-related genes [19].

One may speculate that the therapeutic benefits that telomerase confers for ALS disease are mediated by its ability to protect motor neurons from oxidative stress. Here we examined this hypothesis and the way in which TERT mediates this effect in motor neurons like cells (NSC-34) that were depleted from the mouse TERT and in some experiments were compensated with hTERT following the depletion of mouse TERT. In addition, we also examined the effect of TERT on the expression of oxidative stress enzymes (SOD1 and catalase) *in vivo* in the brains of WT and TERT KO mice. The expression of these enzymes was also examined in the brain of hSOD1 Tg mice the model for ALS.

These results indicate that the ability of mouse TERT to protect mouse motor neuron cells from oxidative stress is in part mediated by its effect on the expression and activity of antioxidant enzymes such as SOD1 and catalase and that human TERT can partially compensate for mouse TERT depletion.

Materials and methods

Cell cultures

Mouse NSC-34 cell line: Mouse NSC-34 cells, a motor neuron-like cell line [28], was kindly received from Dr. Daniel Offen, Tel Aviv University, Israel. The cells were grown in medium DMEM (4.5 gm/l D-Glucose) supplemented with 1% L-Glutamine, 1% penicillin-streptomycin (Biological Industries, Beit Haemek, Israel), and 10% FCS (Gibco, Carlsbad, CA, USA) at 37 °C and 5% CO_2.

Plasmids

For TERT manipulation in NSC-34: TRCN0000070968-72 Vectors: pLKO.1-puro containing mTERT shRNA (Sigma, Rehovot, Israel) was screened for the efficacy of mTERT depletion. Then, a combination of both TRCN0000070971 and TRCN0000070969 was used for the depletion of mTERT in the NSC-34 cells. For hTERT over-expression we used a pWZL-Blasticidin vector containing flag-HA- hTERT and Scrambled shRNA (Addgene, Cambridge, MA, USA)

Lentivirus production

Packaging of the mTERT shRNAs TRCN0000070971 and TRCN0000070969 vectors was performed in GP2-293 packaging cells (Clontech, Mountain View, CA, USA) as previously described [29] using pCMVVSV-G, pMDL.gp.RRE and pRSV-Rev plasmids kindly provided by Dr. A. Chen (Weizmann Institute of Science, Rehovot, Israel).

NSC-34 gene manipulation

For mTERT down-regulation in the NSC-34 cell line, cells (10^6) were grown in a T-25 flask and were incubated with lentivirus containing both mTERT shRNAs TRCN0000070971 and TRCN0000070969 for 48h, following growth in medium containing 10 µg/ml puromycin (Sigma, Saint Louis, MO, USA) for additional 3 - 5 days. For hTERT overexpression, the $NSC\text{-}34^{mTERT\text{-}shRNA}$ cells, which were confirmed for mTERT down-regulation, were transfected with pWZL-Blasticidin containing flag- HA-hTERT using the jetPrime Polyplus Transfection reagent (POLYPLUS- TRANSFECTION SA, Cedex, France) according to manufacturer's protocol for 24h.

Cells were then grown in a medium containing 1 – 5 µg/ml Blasticidin (Sigma, Saint Louis, MO, USA) for an additional\3 - 5 day.

Preparation of protein extract

Cells were washed with PBS to remove the medium. Then, the cells were incubated with CHAPS buffer lysis, containing 10mM TRIS HCl pH-7, 1 mM MgCl2, 1mM EDTA, 0.1 mM PMSF (phenyl-methylsulfonyl), 0.5% CHAPS (3[(3 Cholamidopropyl)

dimethylammonio]-propanesulfonic acid) and 10% Glycerol for 30 minutes in 4 ºC. Then, the cells were centrifuged for 30 minutes at 13000 RPM at 4 ºC. The supernatant was collected, and the total protein concentration was determined by BIO--Rad protein assay kit (Bio-Rad Laboratories).

Telomerase activity

TRAP assay: was performed as previously described [30]. In brief, protein extract (at the indicated amount) was incubated with TS primer (5`AATCCGTCGAGCAGAGTT-3') for 45 min at 30 ºC followed by PCR assay with [α-P^{32}] dCTP using ACX primer: (5'- GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3'). Internal standard primers used as a control: IS primer (5'AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT-3') and ISR primer (5'-ATC GCT TCT CGG CCT TTT-3'). For the detection of telomerase products of hMSC 32 PCR cycles were used, and the internal standard primers were diluted to a concentration of 5 x 10^{-15}M. The PCR products were labeled by [α-P^{32}] dCTP and separated on 12.5% polyacrylamide gel. The radioactive labeled products were detected by a Phosphoimager (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

Antibodies: SOD1 was measured using anti-SOD1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin was measured using an anti-β-actin antibody (ICN, Irvine, CA, USA). Protein extract was prepared and analyzed by Western blot assay as previously described [31,32] using the various antibodies. The immune complexes were detected by enhanced chemiluminescence (ECL) (Biological Industries., BeitHaemek, Israel). Densitometric analysis was performed using the EZ-Quant software (EZ-Quant, Rehovot, Israel).

Cell survival under oxidative stress

Cells were plated as triplicate in 96-well plates at a density of 20000 cells/well and were exposed to various concentrations of H_2O_2 (10 - 100 nM) for 1h, in serum-free medium. The medium was replaced with fresh serum-containing medium for 24h. Cell cytotoxicity was measured by the Neutral Red assay.

Detection of antioxidant properties and cellular ROS accumulation

Intracellular ROS formation and stabilization due to exposure of cells to H_2O_2 was detected using the reagent 2'-7'-Dichlorodihydrofluorescein diacetate (Sigma, Saint Louis, MO, USA), as previously described [33,34]. Briefly, Cells were cultured overnight in triplicate in a 96-well plate at a density of 20000 cells/well). The following day, the cells were incubated with 0, 20, and 40 μM of H_2O_2 for 30 minutes at 37 ºC and 5% CO_2. The medium was removed, cells were washed with PBS and a normal growth medium containing DCFH-DA was added. Using TECAN Infinite® 200 PRO microplate readers the cells were measured every minute for 45 minuts long at excitation 485 nM, emission 520 nM.

Measurement of mitochondrial membrane potential (MMP)

The different NSC-34 cells were cultured as triplicates in 96-well plates at a density of 20000 cells/well overnight. Day after, cells were incubated with Tetra-Methyl- Rhodamine-Methyl-Ester (TMRM) (Sigma, Saint Louis, MO, USA) at 50 μM for 45 min in medium DMEM without phenol red (Biological Industries, Beit Haemek, Israel). Then, cells were exposed to 0 and 20 μM H_2O_2 for 30 minutes in a serum-free medium. TMRM emission at 573 nm was measured using Eliza (Bio-Rad Laboratories, Hercules, CA, USA).

Measuring the proton motive force

Measurement of proton motive force was performed as previously described [35]. Briefly, the different NSC-34 cells were cultured (3 x 10^5 cells) in fluoro-dish (NBT, Jerusalem, Israel) overnight. Day after, the cells were incubated with TMRM [50 μM] supplemented to medium DMEM without phenol red for 45 min (Biological Industries, Beit Haemek, Israel). At the indicated time, 1 μM of Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) was added to the cells and the rate of decay in TMRM (fluorescent intensity reduction/min, illumination at 514 nm and detection at 570 nm) was visualized and analyzed by the confocal microscopy system FV1000 (Olympus, Tokyo, Japan).

Measurement of ATP concentration

ATP concentration in the different NSC-34 cells was measured with the "ATP Bioluminescence Assay Kit HSII" (Roche, Basel, Switzerland), according to the manufacturer's recommended protocol, using the Glomax 20/20 luminometer (Promega, Fitchburg, Wisconsin, USA).

Quantification of gene expression qRT-PCR

Total RNA from the different NSC-34 cells was prepared as previously described [36] and then was transcribed to cDNA with the "Revert Aid First Strand Synthesis Kit" (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Then, quantitative Real-Time PCR was performed using "Fast Start Universal SYBR Green Master" (Roche, Basel, Switzerland) with the 7500 Real-time PCR System (Applied BioSystems, Carlsbad, California, USA) for the following primers:

β-actin	Fw: 5'-GCA GCT CAG TAA CAG TCC GCC T-3' Rv: 5'-TGG CCT CAC TGT CCA CCT TCC A-3'
SOD1	Fw: 5'-TCC GTC GGC TTC TCG TCT TG-3' Rv: 5'-TCA CCG CTT GCC TTC TGC TC-3'
Catalase	Fw: 5'-CTT CAG GGC CGC CTT TTT GC-3' Rv: 5'-ATA GTT GGG GGC ACC CT-3'
mTERT	Fw: 5'-GAA AGT AGA GGA TTG CCA CTG GC-3' Rv: 5'-CGT ATG TGT CCA TCA GCC AGA AC-3'
hTERT	Fw: 5'-GCC GAT TGT GAA CAT GGA CTA CG-3' Rv: 5'-GCT CGT AGT TGA GCA CGC TGA A-3'

Antioxidants enzymatic activity

Superoxide dismutase1 (SOD1): Enzymatic activity was evaluated indirectly using the nitro blue tetrazolium assay as previously described [37]. Briefly, the reaction medium and the required amount of protein extract (10 µg – 15 µg) were illuminated for 10 minutes at 25 °C, and absorbance at 560 nm was subsequently measured by microplate reader 680 (Bio-Rad Laboratories, Hercules, CA, USA).

Catalase: Reaction was initiated by the addition of 60 mM H_2O_2 to protein extract (10 µg - 15µg) in phosphate buffer. At fixed time intervals, aliquots were removed and quenched by addition into a mixture of 0.6 N H_2SO_4: 10mM FeSO (4:1, v/v). After all samples had been collected, color was developed by the addition of 2.5 M KSCN. Color intensity was measured at 490 nm by microplate reader model 680 (Bio-Rad, Hercules, CA, USA).

In vivo studies

Mice: All animal experiments were approved by the animal ethics committee at Ben-Gurion University (IL-07-06-14 and IL-11-09-2018C.) Tert heterozygous (B6.129S-Tert^{tm1Yjc}/J) and their WT counterparts, male and female mice were purchased from Jackson Laboratory. TERT KO mice were established and identified by genotyping and the lack of TERT expression and activity in the brain and other organs. WT, TERT KO mice (generation 2 and generation 3) and ALS mice (B6SJL- Tg (SOD1*G93A)1Gur/J) at the age of 3 - 4 months were sacrificed and the brain was removed as previously described by us [14,19,21].

AGS treatment: WT Mice (C57BL/6) (3 months old) were subcutaneously injected with AGS-534 (6 mg/kg) or vehicle (DMSO) as we previously described [13,18,19]. Twelve hours post treatment mice were sacrificed and the brain was removed and subjected to RNA extraction and gene expression procedures using the appropriate DNA primers as we previously described [14,19,21].

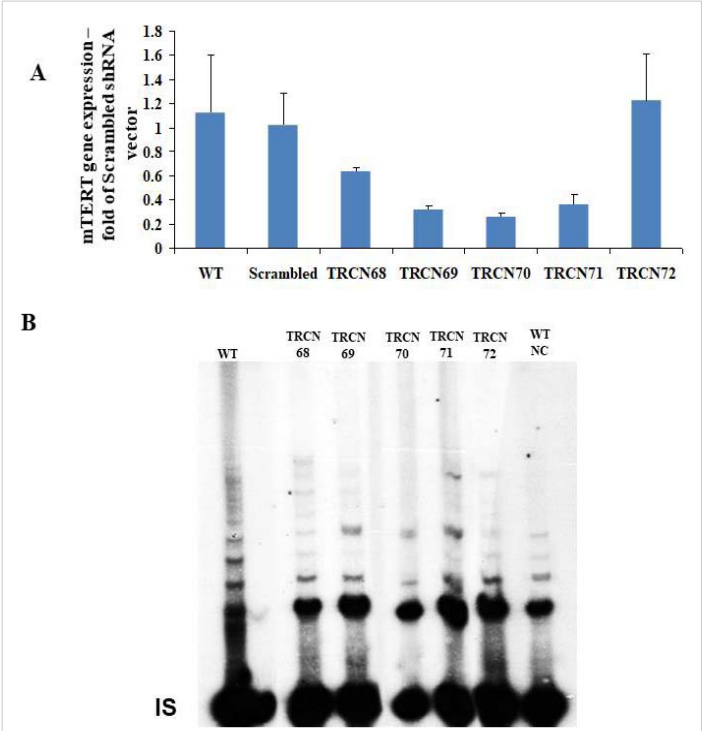
Statistical analysis

quantitative analysis results are the mean of at least 3 independent experiments ± standard deviation. The significance of the results was performed using the student's T-test or One-Way ANOVA by the GraphPad InStat software (GraphPad Software, La Jolla, CA, USA).

Results

NSC-34 cells were depleted of mTERT and compensated with hTERT

To deplete mTERT in the NSC-34 wild-type cells (NSC34^{WT}) the effect of a series of putative mTERT shRNA vectors TRCN68 - 72 on mTERT expression and telomerase activity was examined. TRCN69, 70 and 71 vectors efficiently reduced mTERT expression and telomerase activity (Supplemental 1).



Suplemental Figure 1: Screening of mTERT shRNA vectors for the depletion of TERT expression in NSC-34 cells.
A. The effect of mTERT shRNA TRCN vectors (purchase from Sigma) on mTERT gene expression relative to β-actin in NSC-34 cells. Gene expression was measured using qRT-PCR and is represented in the graph as the mean of 3 different measurements ± SD. The results are fold of Scrambled shRNA vector.
B. The effect of transfected mTERT shRNA TRCN vectors to WT NSC-34 cells on telomerase activity using the radioactive TRAP assay. Symbols: WT – Wild Type, NC – Negative Control by boiling the WT protein extract, IS- internal standard

Then, a combination of TRCN 69 and TRCN71 vectors was employed to generate mTERT knocked-down cells (NSC34^{mTERTshRNA}) and down regulation of mTERT was observed by detecting telomerase activity (Fig. 1A, compare lane 2 to lane 3) and by measuring mTERT gene expression (Figure 1B). Next, hTERT was introduced to the NSC34$^{mTERT-shRNA}$ (NSC34$^{mTERT-shRNA\ hTERT}$), which result in a restoration of telomerase activity, although in a different pattern (Figure 1A, compare lane 5 to lane 2 and 3), and was confirmed by real time PCR with human TERT specific primers (Figure 1C). Impaired growth and proliferation capacity were observed in the NSC34$^{mTERT-shRNA}$ compared to the NSC34^{WT} cells (Figure 1D). Interestingly, no improvement in proliferation and growth capacity was observed in the NSC34$^{mTERT-shRNA\ hTERT}$ cells compared to the NSC34$^{mTERT-shRNA}$ cells (Figure 1D), suggesting that hTERT cannot replace mTERT for this purpose.

TERT protects NSC-34 from the insults of H_2O_2 oxidative stress

To examine the possible TERT protective effect from oxidative stress in NSC-34 cells, the various NSC-34 transfected cells (NSC-34^{WT}, NSC-34$^{mTERT\ shRNA}$, and NSC- 34$^{mTERT\ shRNA\ hTERT}$) were treated with increasing doses of H_2O_2 (0 nM – 100 nM) and the CC_{50} (the dose that kills 50% of the cells) was determined (Figure 2A). The H_2O_2 CC_{50} in NSC-34WT,

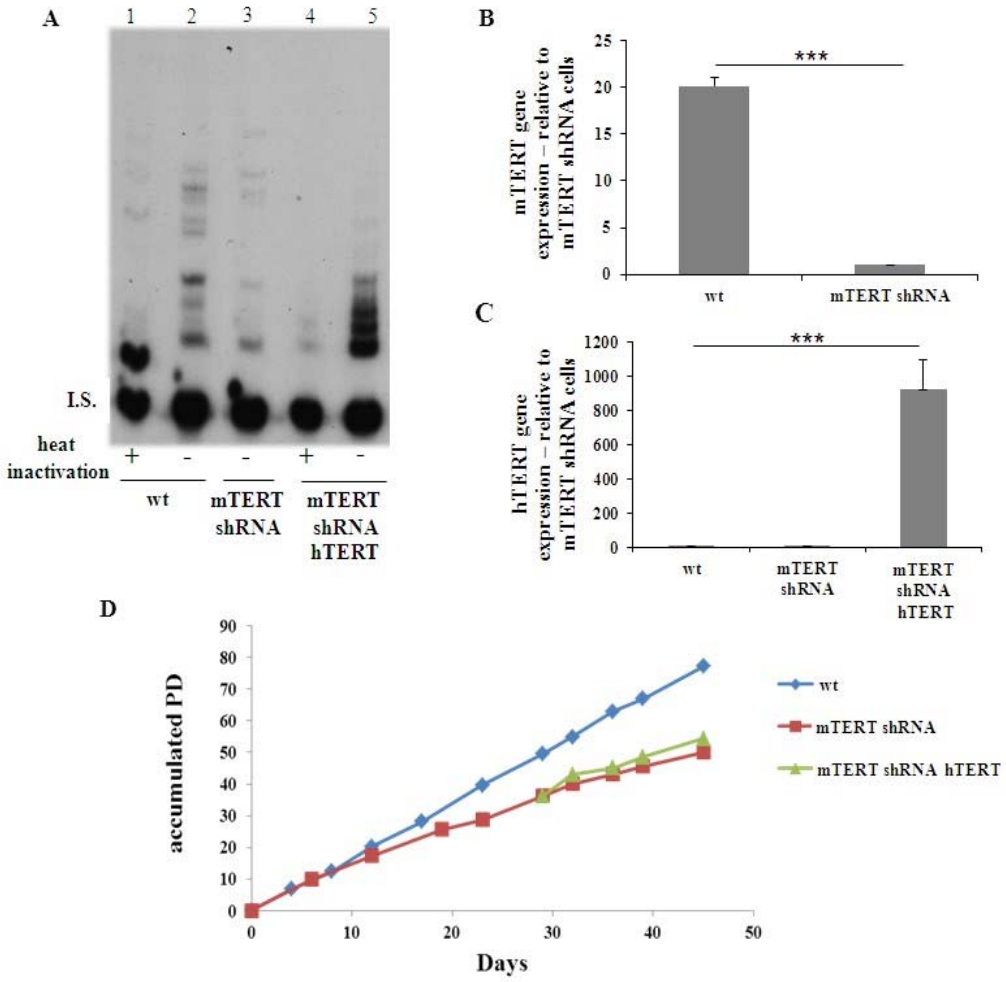


Figure 1: Depletion of mTERT expression and activity in NSC-34 cells and restoration of the activity and expression by hTERT. NSC-34 cells (NSC-34^{WT}) were depleted of mTERT (NSC-34$^{mTERT\ shRNA}$), and samples were transfected with hTERT expressing vector (NSC-34$^{mTERT\ shRNA\ hTERT}$). (A). The activity of telomerase detected by TRAP assay in the NSC-34^{WT} cells (lanes 1 and 2), NSC-34$^{mTERT\ shRNA}$ (lane 3), and NSC-34$^{mTERT\ shRNA\ hTERT}$ (lanes 4 and 5). I.S. – Internal standard Negative control – heat inactivation of the protein extract. B and C. Quantitative gene expression analysis by TR-PCR of mTERT (B) in the NSC-34^{WT} and NSC-34$^{mTERT\ shRNA}$ cells (n = 3 independent experiments); and of hTERT (C) in NSC-34WT, NSC-34mTERT shRNA, and NSC-34mTERT shRNA hTERT (n = 3 independent experiments). The results are normalized to β-actin gene expression. Significance – Student's t-test. ***p < 0.001. D. A graph representing the accumulated PD the cells undergo with time (n = 2 independent experiments).

NSC-34mTERT-shRNA hTERT, and NSC-34mTERT-shRNA cells was 34 μM, 25 μM, and 18 μM, respectively (Figure 2A), indicating that TERT depletion increased the sensitivity of NSC-34 cells to H_2O_2, and restoration of telomerase expression by hTERT partially protected the cells from oxidative stress.

The accumulation of intracellular ROS was measured using the DCFH-DA reagent. In the absence of H_2O_2, no differences in the levels of intracellular ROS were observed between the cells (Figure 2B). However, in the presence of 20 μM or 40 μM H_2O_2 the levels of intracellular ROS were higher in the NSC-34$^{mTERT\text{-}shRNA}$ cells, while in the NSC34$^{mTERT\text{-}shRNA\ hTERT}$ cells ROS levels decreased to the levels observed in the NSC-34^{WT} cells (Figure 2C-D).

Depletion of mTERT affected Mitochondrial Membrane Potential (MMP) and intracellular ATP levels

The by-product of the ATP generation process in the mitochondria is the formation of ROS. Therefore, we investigated the effect of TERT on the mitochondrion's functionality under H_2O_2-induced oxidative stress.

The MMP is decreased following exposure to H_2O_2 [38]. Using TMRM reagent, the effect of TERT on intracellular MMP was determined. In the absence of H_2O_2, the NSC-34$^{mTERT\text{-}shRNA,}$ and NSC-34$^{mTERT\text{-}shRNA\ hTERT}$ cells demonstrated a higher MMP compared to NSC-34^{WT} cells (1.61 ± 0.008 fold of WT, p < 0.001 and 1.68 ± 0.11 fold of WT, p < 0.001, respectively; Figure 3A). In the presence of 20 μM H_2O_2, the MMP in the NSC-34^{WT} cells increased by 1.31 ± 0.06 fold, while in the NSC-34$^{mTERT\text{-}shRNA}$ and NSC-34$^{mTERT\text{-}shRNA\ hTERT}$ cells, it decreased to the level observed in the NSC-34^{WT} cells (1.23 ± 0.14 fold, a reduction of 23%, p < 0.05, and 1.17 ± 0.12 fold, a reduction of 30%, p < 0.05, respectively). These results are not compatible with a previous report showing that in human fibroblasts, in non-oxidative stress conditions, the over-expression of hTERT resulted in an

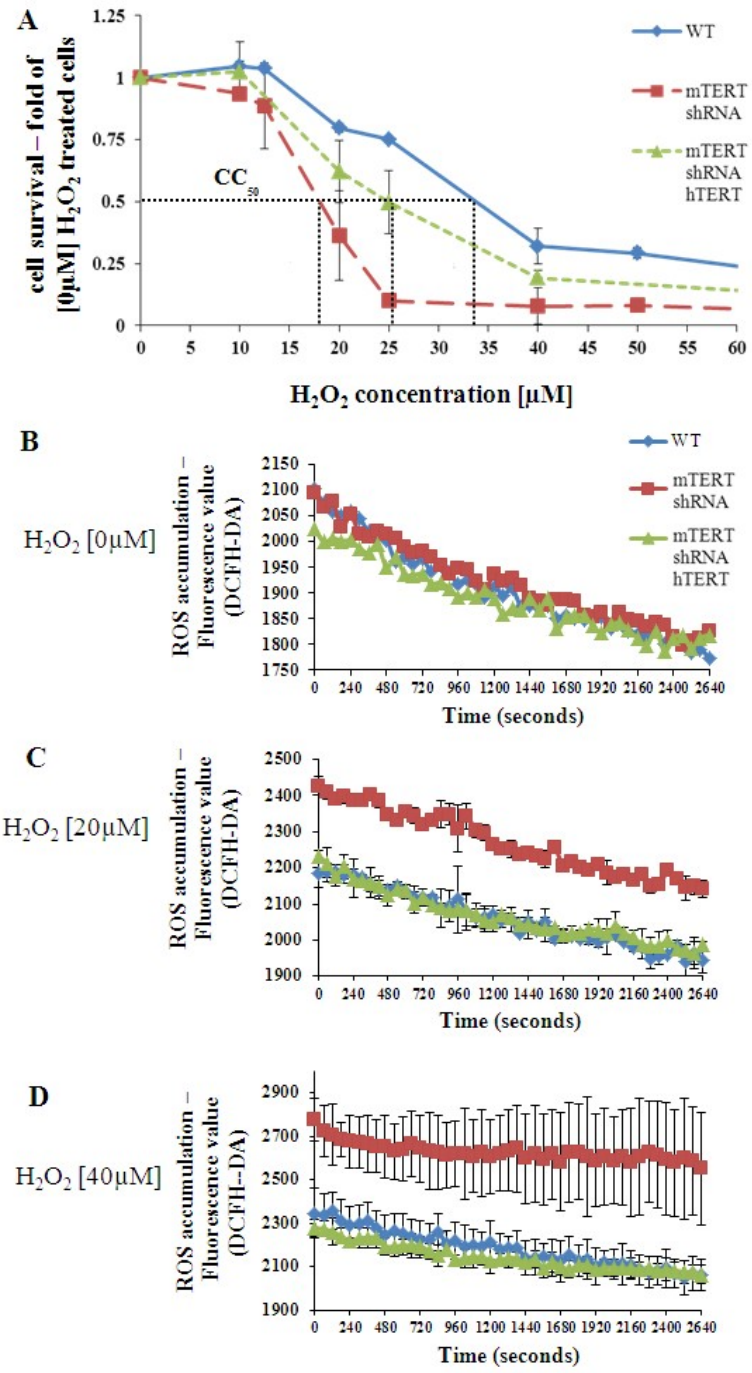


Figure 2: TERT protects NSC-34 from the H_2O_2–induced oxidative stress.
(A). A graph representing the cytotoxic effect of gradually increasing doses of H_2O_2 on survival of NSC-34WT, NSC-34mTERT shRNA, and NSC-34mTERT shRNA hTERT cells. CC_{50} = H_2O_2 concentration is required for killing 50% of cells. B-D. Measurement of intracellular ROS accumulated in the NSC-34^{WT}, NSC-$34^{mTERT\ shRNA}$, and NSC- $34^{mTERT\ shRNA\ hTERT}$ cells exposed to H_2O_2 0 μM (B), 20 μM (C), and 40 μM (D). The results are means of at least 3 different experiments.

increased MMP compared to their control cells [24]. Therefore, the Proton Motive Force (PMF) was evaluated by measuring the rate of MMP depolarization using the protonophore Carbonyl-cyanide-4- (trifluoromethoxy)-phenylhydrazone (FCCP). In the NSC-$34^{mTERT\text{-}shRNA}$ and NSC-$34^{mTERT\text{-}shRNA\ hTERT}$, the rate of MMP depolarization was significantly faster than in the NSC-34^{WT} cells (1.31 ± 0.06-fold, $p < 0.05$, and 1.51 ± 0.1, $p < 0.01$, respectively; Figure 3B, indicating higher PMF in the mTERT-depleted cells compared to the WT cells. PMF is used for the generation of ATP thus we examined the ATP levels. NSC- $34^{mTERT\ shRNA}$ and NSC-$34^{mTERT\ shRNA\ hTERT}$ have decreased ATP levels compared to NSC-34^{WT} cells (0.58 ± 0.25-fold, $p < 0.05$, and 0.32 ± 0.24-fold, $p < 0.01$ respectively) (Figure 3C). These results demonstrate significant changes in mitochondria functionality and cell energy levels due to depletion of mTERT. However, the introduction of hTERT did not compensate for the depletion of mTERT, suggesting the species-dependent function of TERT in the mitochondria.

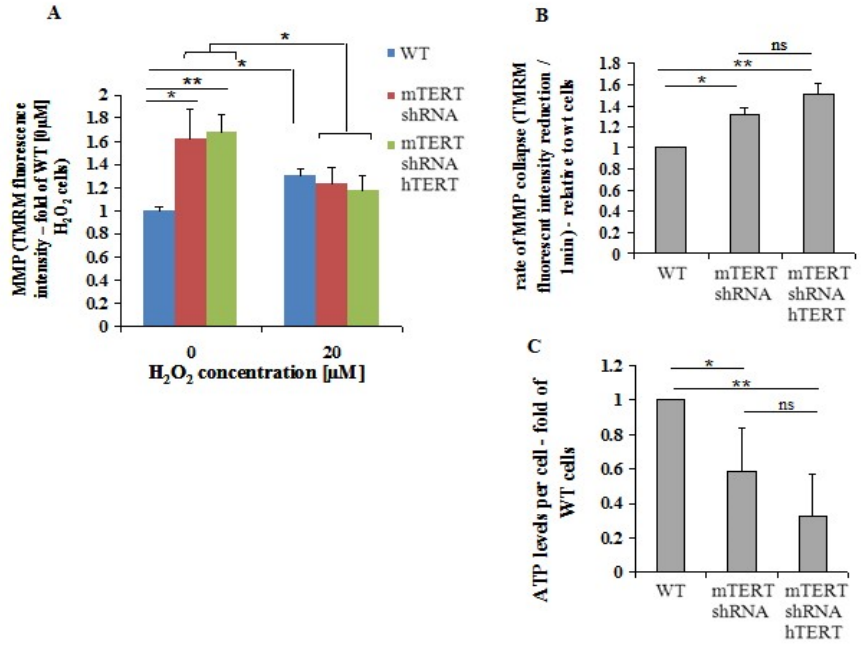


Figure 3: TERT affects MMP and ATP levels.
A. The MMP was measured in the presence of 0μM and 20μM H_2O_2 in the NSC- 34^{WT}, NSC-$34^{mTERT\ shRNA}$, and NSC-$34^{mTERT\ shRNA\ hTERT}$ using TMRM reagent. B. The PMF in the NSC-34WT, NSC-34mTERT shRNA, and NSC-34mTERT shRNA hTERT cells was demonstrated by measuring the MMP depolarization rate, by quantifying the reduction rate of TMRM fluorescent intensity due to the addition of the protonophore FCCP. C. ATP levels in the NSC-34^{WT}, NSC-$34^{mTERT\ shRNA}$, and NSC-$34^{mTERT\ shRNA\ hTERT}$ quantified using the ATP Bioluminescence Assay Kit HSII (Roche, Basel, Switzerland). The results were normalized to a number of cells.
All the results are represented as folds of the NSC-34^{WT} cells. The results are means of $n = 3$ different experiments, significance by one-way ANOVA and Tukey's post hoc analysis. *$p < 0.05$, **$p < 0.01$.

TERT affects the expression and activity of Catalase and Superoxide dismutase 1 under normal or oxidative stress conditions

To further investigate the way TERT expression protects motor neuron cells from oxidative stress we investigated the fate of antioxidant enzymes such as Superoxide Dismutase1 (SOD1) and Catalase, in TERT depleted and TERT knock-in cells.

In the absence of H_2O_2, the NSC-$34^{mTERT\ shRNA}$ exhibited an increased expression of SOD1 (1.36 ± 0.13-fold, $p < 0.01$) and Catalase (1.94 ± 0.37-fold, $p < 0.05$) genes relative to NSC-34^{WT} cells (Figure 4A). However, in the NSC-$34^{mTERT\ shRNA\ hTERT}$ cells, the expression of Catalase was restored to the levels observed in the NSC-34^{WT} cells (Figure 4A), while the expression of the SOD1 gene was even lower than the levels of the NSC34^{WT} cells (0.43 ± 0.24 fold of WT, $p < 0.001$; Figure 4A). This effect was confirmed by the examination of SOD1 protein levels in the different cells, revealing a reduction in SOD1 levels in the NSC-$34^{mTERT\ shRNA\ hTERT}$ cells relative to the NSC- 34^{WT} cells. Nevertheless, although an increase in SOD1 mRNA was detected in the NSC-$34^{mTERTshRNA}$ compared to the NSC-34^{WT} cells, no effect on the levels of SOD1 protein was observed (not shown). Exposures of cells to H_2O_2 [20 μM] resulted in an elevated expression of SOD1 and Catalase genes in the NSC-34^{WT} cells (1.3 ± 0.05- fold, $p < 0.01$; and 1.5 ± 0.17-fold, $p < 0.01$, respectively) and in the NSC-$34^{mTERTshRNA}$ cells (2.21 ± 0.15-fold, $p < 0.001$; and 3.41 ± 0.3-fold, $p < 0.001$, respectively) relative to the NSC-34^{WT} cells untreated with H_2O_2 (Figure 4B).

To examine whether the differences in the gene expression are compatible with the activity, whole-cell proteins were extracted and the activities of catalase and SOD1 in the presence and absence of H_2O_2 were measured. Without H_2O_2 exposure, the Catalase activity in the NSC-$34^{mTERT\ shRNA}$ and in the NSC-$34^{mTERT\ shRNA\ hTERT}$ cells was higher than in the NSC-34^{WT} cells (3.7 ± 1.1-fold, $p < 0.001$; and 1.5 ± 0.14-fold, $p < 0.05$, respectively; Figure 4C). Under H_2O_2 [20 μM] exposure, the catalase activity in the NSC-$34^{mTERT\ shRNA}$ and in the NSC-$34^{mTERT\ shRNA\ hTERT}$ was significantly reduced compared to that observed in the NSC-34^{WT} cells (0.43 ± 0.2-fold, $p < 0.01$; and 0.56 ± 0.07-fold, $p < 0.05$, respectively; Figure 4C). It was reported that sub-lethal doses of ROS increased cellular catalase activity as an intrinsic protective mechanism. Indeed, exposure of the cells to oxidative stress increased the Catalase activity in the NSC-34^{WT} cells (by 5.5 ± 2.4-fold, $p < 0.001$), while no significant induction was observed in the NSC34$^{mTERT\ shRNA}$ cells (Figure 4C). However, a partial compensation was observed in the NSC-$34^{mTERT\ shRNA\ hTERT}$ cells, as H_2O_2 exposure resulted in increased catalase activity by 2.1 ± 0.3-fold, $p < 0.05$, relative to cells untreated with H_2O_2 (Figure 4C).

The effect of TERT on the activity of SOD1 was examined, and under non-oxidative stress conditions, a significant

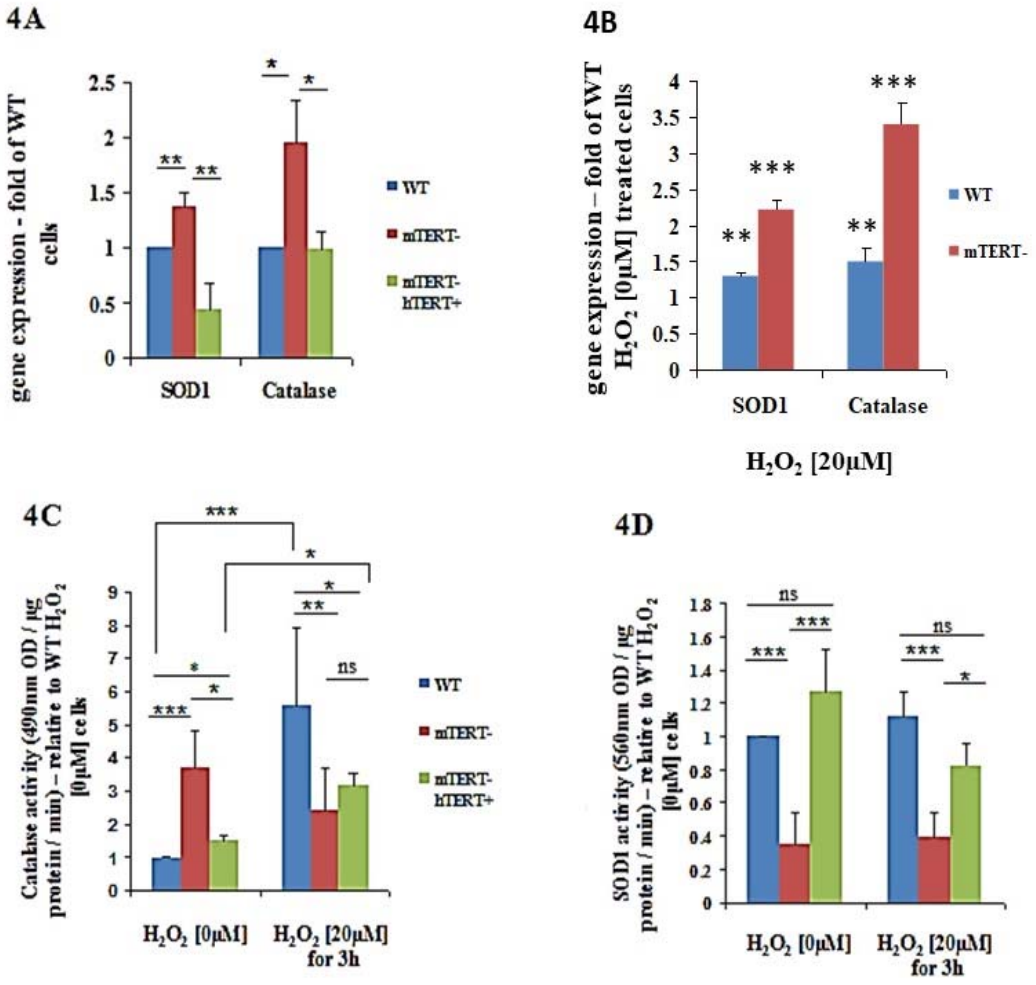


Figure 4: The effects of TERT on expression and activity of antioxidant genes SOD1 and Catalase in normal and oxidative stress conditions.
A. Quantification of the expression of SOD1 and Catalase genes normalized to β-actin in the NSC-34^{WT}, NSC-34$^{mTERT\ shRNA}$, and NSC-34$^{mTERT\ shRNA\ hTERT}$ performed by qRTPCR. The results are represented as fold of the NSC-34^{WT} cells (without H_2O_2) and are means of n = 5 different experiments. Significance by one-way ANOVA and Tukey's post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001. B. Quantification of the expression of SOD1 and Catalase genes normalized to β-actin in the NSC-34^{WT}, NSC34$^{mTERT\ shRNA}$ cells exposed to 20 μM H_2O_2. The results are represented as a fold of the NSC- 34^{WT} cells (without H_2O_2) are means of n=5 different experiments. C and D. Quantification of the antioxidant activity of Catalase (C) and SOD1 (D) in the NSC- 34^{WT}, NSC-34$^{mTERT\ shRNA}$, and NSC-34$^{mTERT\ shRNA\ hTERT}$ cells treated with or without 20 μM H_2O_2. All the results are represented as folds of the NSC-34^{WT} cells. The results are means of 6 different experiments. Significance by one-way ANOVA and Tukey's post hoc analysis. *p < 0.05, **p < 0.01, ***p < 0.001

reduction in SOD1 activity (0.34 ± 0.19 fold of WT, p < 0.001; Figure 4E) was observed in the NSC-34$^{mTERT\ shRNA}$ cells. In contrast, the SOD1 activity in NSC-34$^{mTERT-\ hTERT+}$ significantly increased compared to the NSC-34$^{mTERT\ shRNA}$ cells (3.7 ± 0.2 fold higher, p < 0.001; Figure 4D). Under oxidative stress conditions, a similar pattern of activity was detected: SOD1 activity in the NSC-34^{mTERT-} cells was decreased by 0.34 ± 0.15 fold of the NSC-34^{WT}, p < 0.001, and the activity in the NSC34$^{mTERT\ shRNA\ hTERT}$ was increased by 2.12 ± 0.14 fold of NSC-34$^{mTERT\ shRNA}$ cells, p < 0.05 (Figure 4D).

SOD1 and catalase gene expressions in mouse brains decreased following an increase in TERT expression by AGS compound and increase in TERT knockout mouse brain The endogenous SOD1 expression increased in the brain of ALS mice and TERT KO mice and decreased in AGS-treated WT mice.

TERT influences the expression of catalase and SOD1 in mouse brain

To examine the effect of increased TERT expression *in vivo* in mouse brain we treated the WT mice with a single injection of AGS -534, a TERT increasing compound [15,18,19]. RNA brain extracts were prepared 12 hr. after AGS treatment and SOD1 and Catalase gene expressions were detected by Real-Time PCR using the appropriate primers. As seen in Figure 5A Increasing TERT expression in the brain by pharmaceutical compounds significantly decreased the expression of SOD1 to 78 ± 7.09%, p = 0.001, and catalase to 67 ± 8.3% p = 0.004. To determine the effect of TERT knockout on the expression of SOD1 in the mouse brain we prepared RNA extracts from the brain derived from TERT KO generation 2 and generation 3 mice and from their WT counterpart and examined the expression of SOD1 gene. As can be seen in Figure 5B the expression of SOD1 significantly increased in the brain of

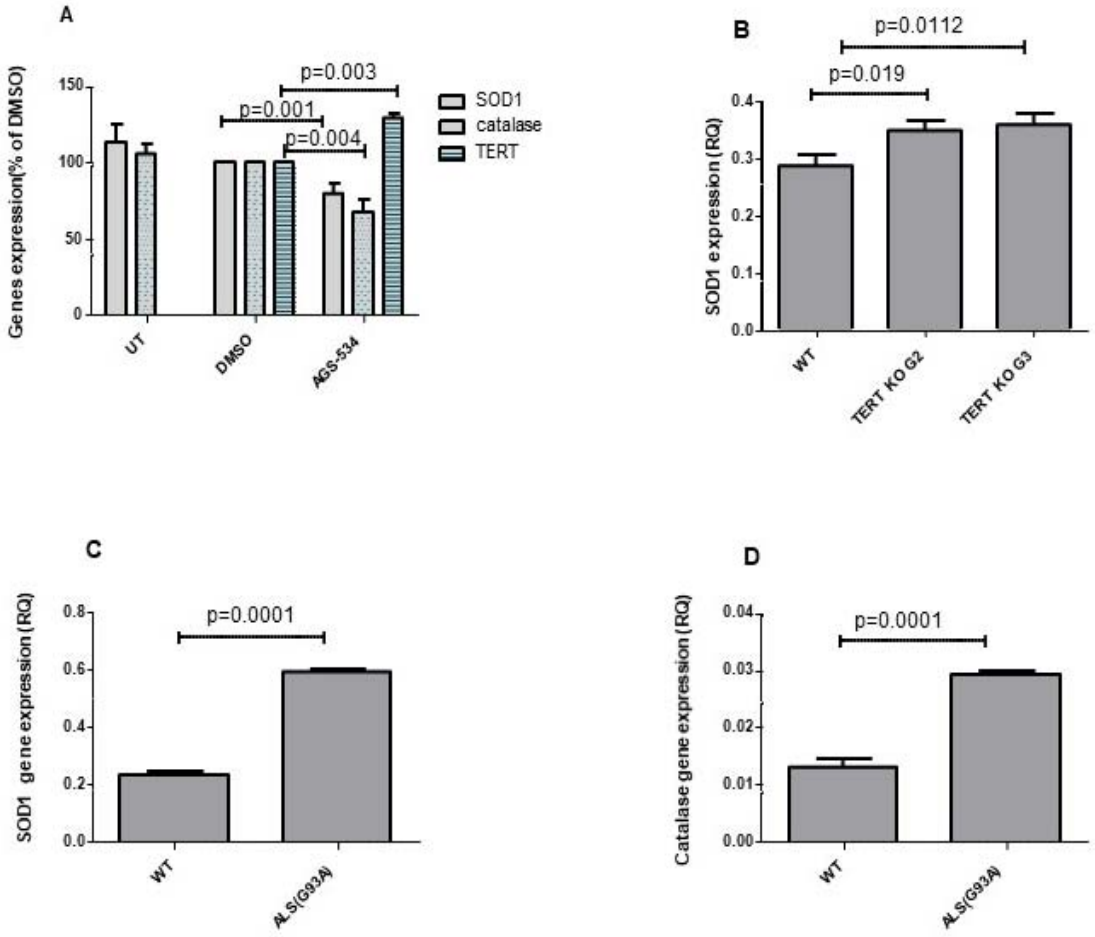


Figure 5: SOD1 and catalase expression increased in the brain of TERT KO mice and decreased in WT brain treated with AGS
(A). ICR WT mice were S.C. injected with a single dose of AGS compound (6 mg/Kg) or with vehicle (0.5% DMSO) and sacrificed 12 hrs. later. SOD1, Catalase, and mTERT expression were measured in the brain RNA extracts by Real-time PCR with the appropriate primers. The results are % of vehicle and mean ±SEM of 3 - 5 mice per group (A) *p* values were calculated by one-way ANOVA test.
SOD1 expression in the brains of TERT KO G2 and G3 mice and their WT counterparts was examined using real-time PCR with the appropriate primers. The results are manse of 5 -10 mice per group. *p* values were calculated by the student T-test (C and D) The brain from hSOD1 Tg mice (ALS) and their WT counterparts were subjected to RNA extraction and the level of the endogenous SOD1, and catalase expression was evaluated by real-time PCR with the appropriate primers. The results are presented as relative quantity and are mean ±SEM of 3 hSOD1 Tg mice and 5 WT mice. *p* values were calculated by the Student T test.

TERT KO mice to 121.1% ± 0.01%, $p = 0.0012$ for G2, and to 122 ± 0.02%, $p = 0.00192$ for G3 compared to that found in the WT counterpart. The results obtained in the mouse brain are compatible with the motor neuron cell culture data showing the impact of TERT on SOD1 and catalase gene expressions. Finally, since elevation of oxidative stress in the brain and spinal cord, was suggested as one of the possible factors for motor neuron degeneration in ALS disease, we examined the expression of SOD1 and catalase in the brain of ALS mouse model ($hSOD1^{G93A}$ Tg mice) at the symptomatic stage. The results revealed a significant increase in SOD1 by 2.4-fold (Figure 5C) and catalase by 3-fold (Figure 5D) compared to the WT counterpart suggesting a high oxidative stress situation in the brain of ALS mice.

Discussion

Several studies illustrated the therapeutic benefits of the telomerase-based approach in ALS and other neurodegenerative diseases particularly the neuroprotective effects of TERT in the brain [19,20-23,39]. This can be attributed to two distinct capabilities of TERT: Its ability to elongate telomeres, thus supporting cell division and cellular life span, which is less relevant to long-lived postmitotic cells as neurons but may affect the disease through neuroinflammation and other glia-related functions; and its protective effect from oxidative stress which was demonstrated in several independent groups at various cell types and animal models including embryonic hippocampal and motor neuron primary culture [14,19,22,36].

These two capabilities are related since it was shown that oxidative stress interferes with telomere maintenance at two levels: it increases the rate of telomeres shortening [40], and it prevents telomerase from counteracting telomere shortening by inducing its export from the nucleus into the mitochondria, where it protects mitochondrial DNA from oxidative damage and inhibits cellular apoptosis [24,41].

Since Motor neurons are non-dividing cells, their telomeres will get shorter mostly due to DNA damage. In addition, ALS is characterized by severe oxidative damage [5]. Therefore, it is reasonable to suggest that the neuroprotective effect of TERT is mostly mediated by reducing oxidative stress rather than maintaining telomeres length. We showed that hTERT confers an anti-apoptotic effect from oxidative stress on human and mouse cells as well [21,36], suggesting that TERT possesses an intrinsic protective mechanism which is not dependent on the external environment. Hence, we investigated the mechanism by which TERT, regardless of mouse or human origin, mediates protection from oxidative stress in a mouse motor neuron-like cell line, NSC-34.

To this end, NSC-34 cells were manipulated by knockdown of mTERT and then knocked-in of hTERT. The downregulation of mTERT resulted in an impaired growth and proliferation capacity, which were not restored by the knocked-in of hTERT. This implies that, although hTERT can interact with the mTR to possess telomerase activity in-vitro, it does not support telomere maintenance in-vivo. These results are in agreement with a previous report [42,43] and highlight the complex regulation of telomerase ribonucleoprotein complex assembly and trafficking to telomeres.

We used an early generation of NSC34$^{mTERT\text{-}shRNA}$ cells that did not demonstrate an impaired growth capacity, to examine the cells' sensitivity to H_2O_2 insult. The mTERT knocked-down cells had a significantly higher sensitivity to H_2O_2. hTERT Knocked-in only partially compensated for the loss of mTERT. The results suggest that mTERT can protect mouse motor neurons from oxidative damage and that hTERT cannot fully replace mTERT loss of function.

We were interested in the mechanism of mTERT cellular protection. H_2O_2 pathology is mediated by ROS, both by itself and by inducing mitochondrial ROS production. Under normal conditions, the ROS levels were similar in the WT and the knocked-down cells. However, in the presence of TERT (in WT and TERT knock-in NSC34 cells), following H_2O_2 treatment ROS levels were lower compared to their level in mTERT knocked-down cells. This implies that the effect of TERT on ROS reduction is reflected and more prominent under oxidative stress conditions.

Various reports indicate that the presence of TERT in the mitochondria ameliorates mitochondrial function [24,25,42,43]. Therefore, we examined if the increase in cellular ROS levels, following knocking-down of mTERT, is due to mitochondrial dysfunction.

In the absence of H_2O_2, mTERT knocked down resulted in a significant increase in the MMP is relative to the WT cells. However, under the H_2O_2-induced stress condition, the MMP was significantly reduced in the mTERT knocked-down cells, while no significant change was observed in the WT cells. Knocking-in hTERT in the mouse motor neurons did not improve the altered MMP caused by the downregulation of mTERT. To confirm these results, we measured the rate of MMP collapse using the protonophore FCCP. The rate of MMP collapse is in direct correlation with the PMF generated in the mitochondrial intermembrane space by the respiratory chain [44]. Compatibly, relative to the WT cells, the mTERT knocked-down cells showed higher PMF, regardless of the presence of hTERT. Higher PMF is generated by an increased activity rate of the respiratory chain, which may lead to an increased ROS production unless the respiratory chain is better coupled to ATP generation [44]. Examining the intracellular ATP levels revealed that downregulation of mTERT reduced ATP levels, which were not compensated by hTERT. Abnormality in any of these processes can be designated as mitochondrial dysfunction [44]. The reduction in ATP levels can explain the increased sensitivity of the NSC-34$^{mTERT\text{-}shRNA}$ cells to the insult of H_2O_2, as many of the cellular repair mechanisms are dependent on energy consumption. In addition, this reduction is compatible with the impaired growth capacity of the NSC-34$^{mTERT\text{-}shRNA}$ and NSC34$^{mTERT\text{-}shRNA\ hTERT}$ cells compared to the NSC34^{WT} cells.

The ability of TERT to improve coupling of the MMP and ATP generation, which reduced ROS formation and thus enabled the cells to survive under oxidative stress was previously reported in human fibroblast and endothelial cells [24]. Our results show that mTERT possesses a similar function and it requires species commutability between the TERT and the mitochondria. This notion is supported by a previous study reporting that mouse and human mitochondrial fusion do not survive on selective medium and lose mitochondrial activity due to incompatibilities between the human mtDNA sequence and the nuclear-encoded mouse mitochondrial proteins [45].

Changes in cellular ROS levels can be attributed to either alteration in ROS production or in ROS degradation. We examined the effect of TERT expression on the expression and activity of selected cytosolic antioxidant enzymes (Catalase, SOD1). Relative to the WT cells, down-regulation of mTERT significantly increased the expression of SOD1 and catalase. However, hTERT knocked-in cells prevented the increase in catalase expression and even demonstrated a significant decrease in SOD1 mRNA and protein expression compared to the WT cells. We assume that the increased expression levels of these antioxidant genes represent a deterioration of the cellular redox state.

This is supported by several reports demonstrating an increase in these genes' expression levels due to H_2O_2 exposure [46-48].

The changes in enzyme expression levels are not necessarily correlated with changes in its activity. Since an active enzyme is needed for the cellular adaptive response to any environmental challenge, we examined the effect of TERT on the activities of catalase and SOD1 under oxidative and

non-oxidative stress conditions. Measuring Catalase activity in the NSC-34 cells revealed that under non-oxidative stress conditions knocking down of mTERT resulted in a significant increase in catalase activity compared to the WT cells or the hTERT knocked-in cells. This shows that TERT is necessary for the cells to cope with the normal ROS levels but in its absence, the cell increases the activity of antioxidant enzymes such as catalase and SOD1.

H_2O_2 increases cellular Catalase activity as an intrinsic protective mechanism [48,49]. Examining the effect of 20 μM H_2O_2 on Catalase activity revealed an increase in the mTERT knocked-down, hTERT knocked-in, and WT H_2O_2 treated cells, compared to the untreated cells. However, the extent of the increase differed between the cells. This may imply that TERT does not regulate the oxidative stress-induced increase in catalase activity.

Examination of the effect of TERT on the activity of another cytosolic oxidative stress enzyme, SOD1, indicates that under normal conditions or oxidative stress conditions, the activity was significantly reduced in mTERT knocked-down cells, but restored when hTERT was knocked-in. This suggests that the activity of SOD1 is regulated by TERT independent of ROS formation. Interestingly, SOD1 gene expression was increased in mTERT-depleted cells while SOD1 activity decreased independently on oxidative stress exposure. This suggests that in the presence of TERT SOD1 undergoes post-translational modifications that reduced its activity. ROS toxicity is partly achieved by the induction of DNA damage. We have previously found that the expression of hTERT in human Mesenchymal Stem cells (hMSC) and of mTERT in mouse Embryonic Stem cells (mESC) significantly reduced the H_2O_2 induced- DNA γH2AX foci, that mark DNA lesions [21,36].

To confirm the effect of TERT on SOD1 and catalase expression *in vivo* in mouse brain we used the AGS compounds which we previously showed their ability to increase TERT expression and activity in various regions of the mouse brain [21] and to protect neurons from oxidative stress in a TERT dependent manner [19,21]. Elevation of TERT in the mouse brain indeed decreased the expression of catalase and SOD1. This is compatible with the *in vitro* data showing that transfection of TERT-depleted neuronal cells with hTERT decreased the expression of these enzymes. Moreover, examination of SOD1 expression in the TERT KO mouse brain revealed an increase in its expression compared to WT mice. These data are also compatible with the *in vitro* results in motor neurons depleted TERT. The neuroprotective effect of TERT from oxidative stress reduced the amount of ROS and therefore the level of the intrinsic anti-oxidative enzyme is reduced, as can be seen *in vitro* and *in vivo*, while depletion of TERT increased ROS level and therefore induced the expression of the intrinsic antioxidant enzymes.

Altogether, our results show that the TERT protective effect is pleiotropic: 1. Reduced ROS generation by improving mitochondrial functionality. 2. Increased ATP levels that enable better defense mechanisms activation. 3. Regulation of the expression of antioxidative enzymes, probably not directly but rather due to the reduction in the amount of ROS.

hTERT only slightly affects catalase activity in mTERT knocked-down cells, suggesting that the effect of TERT on catalase is species-specific. In contrast, SOD1 activity was regulated by both mTERT and hTERT.

The ability of TERT to moderate cellular oxidative stress and to affect SOD1 activity and expression is particularly important for motor neurons considering the distinctive features of these cells. (1) The motor neurons are exceptionally sensitive to SOD1 mutations, which may lead to familial ALS disease [50]. SOD1 depletion cause defects in motor neuron specifically (2) Motor neuron are among the largest cells in the body that deliver messages through long axons, which require a high level of mitochondria and energy. The ability of mTERT to improve mitochondrial functionality and to increase ATP level may be important for the function of these cells and their survival ;(3) We previously demonstrated that motor neurons are significantly more sensitive to oxidative stress compared to other cells such as hMSC or mESC. Comparing the dose of H_2O_2 required for substantial cytotoxic effect in the NSC-34, to human Mesenchymal stem cells (hMSC) or mouse embryonic stem cells (mESC) revealed that the H_2O_2 CC_{50} for NSC-34 was 34 μM H_2O_2 for 1h, while 40% cell death occurred in hMSC and mESC after treatment of H_2O_2 in a concentration of 300 μM for 4h or 200 μM for 2h, respectively [21,36].

Conclusion

Few studies demonstrated the benefits of telomerase as a therapeutic approach for neurodegenerative diseases based on its functions in telomere maintenance and oxidative stress. In humans, telomerase expression is significantly reduced in the spinal cord of ALS patients. Indeed, we demonstrated the beneficial effects of increasing telomerase by AGS compounds on the onset and progression of ALS in SOD1 Tg mice and the survival of motor neurons. Here we show for the first time the involvement of TERT in the regulation of SOD1 in motor neuron-like cells and *in vivo* in the mouse brain which strengthens the notion that increasing TERT may improve the ability of motor neurons to cope with oxidative stress and increase their survival in neurodegenerative disease like ALS.

Author contribution

Conceptualization, A.T., E.E., and E.P.; Performed the experiments A.T., E.E., and S.T.; Supervision, E.P., and E.BY.; Writing—original draft, A.E., E.E., and E.P.; Writing— review & editing, E.P., E.BY and E.E. All authors have read and agreed to the published version of the manuscript.

E.P.- filed patents on AGS compounds.

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