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hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells

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ABSTRACT

The human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the telomerase holoenzyme. Evidence is accumulating to link hTERT to activities other than telomere maintenance and immortalization. Here we show that hTERT overexpression not only reduces the basal cellular ROS levels but also inhibits endogenous ROS production in response to stimuli that induce intracellular ROS generation. Conversely, siRNA-mediated gene silencing of hTERT potentiated the increase in cellular ROS levels following exposure to oxidative stress. This anti-oxidant effect of hTERT is mediated via a significant increase in the ratio of reduced to oxidized glutathione (GSH:GSSG) as well as efficient recovery of the oxidized peroxiredoxin to its non-oxidized form. Our data also provide evidence for mitochondrial localization of hTERT, and a significantly higher activity of cytochrome c oxidase, the rate-limiting enzyme in the mitochondrial electron transport chain, in hTERT overexpressing cells. To ascertain whether the improved mitochondrial function and anti-oxidant effect of hTERT could provide cancer cells with a survival advantage, the effect of oxidative stress on mitochondrial apoptosis was evaluated. Indeed, hTERT overexpressing cells inhibited cytosolic acidification, translocation of Bax, the drop in mitochondrial transmembrane potential, the release of cytochrome C to the cytosol, and cell death. Taken together, these data demonstrate a hitherto undefined role of hTERT in alleviating cellular ROS levels by way of potentiating the cellular anti-oxidant defense systems, and in doing so endowing cancer cells with the ability to evade death stimuli.

INTRODUCTION

Telomeres are specialized structures that cap and preserve chromosomal integrity by protecting chromosomal ends from degradation, end to end fusions, and rearrangement (1). Telomerase, a ribonucleoprotein, is essential for the maintenance of telomeres. Telomerase consists of a catalytic protein subunit, human telomerase reverse transcriptase (hTERT), the RNA component of the telomerase (hTR or hTERC) that is used as the template by the reverse transcriptase to elongate the telomeric ends, and telomerase associated proteins such as TEP1, POT1 and TPP1 that play a primary role in maintaining the integrity of the telomeres in normal cells (2, 3). In recent years, roles deviating from the canonical activity of hTERT, namely cellular immortalization, telomerase activity and overcoming cellular senescence, have been demonstrated with experimental models (4, 5). Towards that end, hTERT has been implicated in telomerase-mediated DNA repair (5), control of cell growth, mitochondrial function (4), and modulation of cells' response to apoptotic stimuli (6, 7).

There is accumulating evidence that cancer cells' response to apoptotic insults is regulated by the cellular redox status. In this regard, we previously demonstrated divergent effects of the two major ROS species, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), on cell death signaling in cancer cells, whereby a slight tilt in favor of O_2^- inhibited death execution and provided a survival advantage while an increase in intracellular H_2O_2 created a permissive milieu for death execution (8-10). Interestingly, intracellular ROS has been shown to regulate hTERT at many levels, such as regulation of its expression and activity as well as modulating its localization and in doing so altering its functions (4, 11, 12). Despite convincing evidence that cellular redox status impacts hTERT biology, the effect of hTERT on cancer cell redox milieu remains less well defined. In this regard, studies have suggested a protective cellular response against oxidative stress in hTERT overexpressing cells (13-15). In addition, reports

have also suggested that hTERT expression positively impacts mitochondrial function by improving its calcium buffering capacity and reducing O_2^- production (16). More recently, it was shown that mitochondrial hTERT exerted a novel protective function by binding to mitochondrial DNA, increasing respiratory chain activity, and protecting against oxidative stress-induced damage (17).

Here we investigated the effect of hTERT expression on intracellular redox status, as well as, the cellular response to ROS-induced apoptotic stimuli. In this regard, the effect on cells anti-oxidant defenses and the activity of cytochrome C oxidase (COX; complex IV), the rate limiting step in the mitochondrial electron transport and a reliable endogenous marker of oxidative metabolism (18), were assessed. Results indicate that hTERT expression antagonizes ROS-induced ROS production, thereby preventing toxic increase in intracellular ROS and bestowing survival advantage upon cancer cells.

MATERIALS AND METHODS

Cell Lines, culture conditions and plasmids:

The human cervical cancer cell line HeLa (CCL-2™) and the human neuroblastoma cell line SH-SY5Y (CRL-2266™) were purchased from *American Type Culture Collection* (ATCC; Rockville MD, USA). Both the cell lines were characterized and authenticated by the source cell bank ATCC. Normal fetal lung fibroblast cell line MRC-5 (Cat #AG05965) was obtained from Coriell Cell Repositories, Camden, NJ, USA. The cell lines used throughout this study were not used for more than 3 months and/or 20 passages, following purchase from the authentic repositories. MRC-5-hTERT cells were generously provided by Dr. Hidetoshi Tahara, Hiroshima University, Hiroshima, Japan. HeLa cells and SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium and RPMI-1640, respectively, supplemented with 1% L-glutamine, 1% streptomycin-penicillin (Gemini Bio-Products) (v/v) and 10% FBS. HeLa cells stably transfected with pBabe-Neo (Neo cells) or pBabe-Neo-hTERT (HT1 cells) plasmids were maintained in 500μg/ml of G418 disulphate salt solution (Roche Diagnostics Corp., Indianapolis, IN, USA). Both MRC-5 and MRC-5-hTERT cell lines were grown in DMEM-F12 supplemented with 1% L-glutamine, 1% penicillin/streptomycin 1mM sodium pyruvate solution and 1X non essential amino acids. All tissue culture related reagents were purchased from Hyclone, Logan, Utah, USA, unless otherwise stated. For cellular assays, cells were plated in G418-free complete medium over 2 days before treatment or analysis. The plasmids pBabe-Neo and pBabe-Neo-hTERT were a generous gift from Dr. Fabrizio d'Adda di Fagagna, IFOM-IEO, Milan, Italy.

Flow cytometric analysis of intracellular ROS:

Cells were loaded with 5-(and-6)-chloromethyl-2-,7-dichlorofluorescin diacetate (DCHF-DA) for cellular ROS measurement or MitoSox Red (Molecular Probes, Invitrogen Corp.,

Carlsbad, CA, USA) for intra-mitochondrial O_2^- assessment as described previously (19). Briefly, cells were harvested and washed with 1x PBS, followed by incubation with 5μM DCHF-DA or 10μM MitoSox Red for 15 min at 37 °C in the dark. Cells were then washed in 1 x PBS and resuspended in plain DMEM and analyzed by flow cytometry (Coulter EPICS Elite ESP). At least 10,000 events were analyzed.

Measurement of mitochondrial membrane potential using $DiOC_6$:

Cells were washed and loaded with 5,3,3'-dihexyloxacarbocyanine iodide [$DIOC_6(3)$] (Sigma Aldrich Co, St Louis, MO) as described above and previously (20) and analyzed by flow cytometry with excitation and emission wavelengths at 488 and 525 nm, respectively.

Measurement of Intracellular pH with 29,79-Bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF):

Intracellular pH (pHi) was measured by loading cells with membrane-impermeant dye BCECF-AM (Sigma Aldrich Co, St. Louis, MO) as described elsewhere (21). Briefly, cells were loaded with 40nM BCECF-AM for 15 min at 37 °C in the dark, washed, resuspended in plain DMEM, and analyzed by flow cytometry. The fluorescence ratio of 525:610 nm was used to derive cytosolic pH using a standard pH calibration curve.

Assessment of intracellular GSH:GSSG levels:

The glutathione assay was optimized from the protocol by Hissin and Hilf *et al*. (22). Reduced and oxidized glutathione (GSH and GSSG) and N-ethylamide (NEM) solution were obtained from Sigma Aldrich Co (St Louis, MO). Cells (0.2×10^6) were seeded over 2 days in a 6 well plate before treatment. Following H_2O_2 treatment, the cells were washed once with chilled phosphate buffered saline (PBS). Three hundred sixty microlitres of chilled TCA (6.5%) was added to each well and the plates were left at 4°C for 10 min. *o*–Phthaladehyde

(OPT) (Sigma Aldrich Co, St Louis, MO) solution was freshly prepared in reagent-grade absolute methanol (1mg OPT/ml methanol) (Merck Inc, White house Station, NJ). After 20 min of incubation in the dark, the plates were excited at 350 nm and fluorescence emission was detected at 420 nm with POLARstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany).

Glutathione Peroxidase and Reductase Assays:

The glutathione peroxidase assay was optimized from the protocol provided by Sigma and cumene hydroperoxide was used as the substrate to measure the total GPx activity. NADPH (β-Nicotinamide Adenine Dinulcleotide Phosphate, Reduced) and cumene hydroperoxide were obtained from Sigma Aldrich Co, (St Louis, MO). The activity was measured by reading the absorbance at 340nm once every minute for 15 min in a microplate reader (Tecan Spectrofluoroplus, Tecan Ltd, Maennedorf, Switzerland). The Glutathione Reductase assay was performed as described by the Cayman Glutathione Reductase assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA).

Isolation of mitochondrial and cytosolic fractions and assessment of Cytochrome c Oxidase Activity:

Mitochondrial and cytosolic fractions were obtained as described elsewhere (23). Cytochrome c oxidase activity was assessed as described previously (24). For details refer to the Supplementary Methods.

Statistical Analysis:

All experiments were performed at least three times for statistical significance. Numerical data were expressed as mean $\pm$ SD. Statistical analysis was performed using the paired

Student's t-test considering the variances unequal. P values < 0.05 were considered significant.

RESULTS

hTERT expression alleviates basal intracellular ROS levels:

To address the effect of hTERT expression on ROS, hTERT was stably expressed in HeLa cells, which have a moderate background expression of hTERT (Figure 1A). The cells stably transfected with pBabe-neo (Neo) and pBabe-neo-hTERT (HT1 and HT2) were then analyzed for basal intracellular ROS levels. Flow cytometric analysis revealed that HeLa cells overexpressing hTERT displayed significantly lower basal and mitochondrial ROS levels. The G-mean values of DCF fluorescence obtained from 6 independent experiments were used to calculate the fold difference in ROS levels between Neo, HT1 and HT2 cells (Figure 1B). In addition, the assessment of mitochondrial $O_2^{\cdot -}$ levels using MitoSox Red also displayed a 10% to 15% decrease in mitochondrial $O_2^{\cdot -}$ in HT1 and HT2 cells (Figure 1C). These findings were strengthened by transient hTERT overexpression in HeLa cells (Figure S1A, S1B) or upon siRNA mediated gene silencing of hTERT in SH-SY5Y cells that normally express higher levels of hTERT (Figure S1C); hTERT silencing increased basal levels of intracellular ROS. Similarly, intracellular ROS levels were significantly lower in normal human lung fibroblasts (MRC-5) immortalized with hTERT (MRC-5-hTERT), compared to the control MRC-5 cells (Figure S1D).

hTERT overexpression blocks ROS-induced ROS production:

Since transient hTERT expression resulted in a decrease in basal levels of intracellular ROS, it was of interest to investigate if hTERT overexpressing cells were better able to cope with stimuli that increased ROS production. To assess that, the response of hTERT overexpressing

cells to the exogenous addition of H_2O_2 or upon exposure to a small molecule compound C1 that we previously showed as a strong stimulus for intracellular ROS production(23, 25), was evaluated. Results showed that hTERT overexpressing HT1 cells displayed a significant decrease in intracellular ROS levels following treatment with H_2O_2 or C1 (Figure 1D). Of note, the response of HT1 cells to C1 was significantly more pronounced than exogenously added H_2O_2, thus indicating that the hTERT overexpressing cells were not only resistant to exogenous H_2O_2 but were also able to resist drug-induced intracellular ROS production. Corroborating these data, an improved resistance to oxidative stress was also observed in HeLa cells transiently transfected with hTERT or MRC-5-hTERT cells following treatment with H_2O_2 or C1 (Figure S2A, S2B and S2D). Conversely, hTERT silencing in SH-SY5Y cells resulted in a significant increase in intracellular ROS levels in response to 600 μM H_2O_2 for 4 hrs (Figure S2C).

hTERT overexpressing cells display enhanced glutathione antioxidant defense capacity:

Intrigued by the resistance of hTERT expressing cells to oxidative stress, we next investigated the mechanism of cellular adaptation/resistance to oxidative stress in this model system. Thus the GSH/GSSG couple was investigated to determine if hTERT overexpression influenced this redox couple, thus altering the cellular capacity to resist oxidative stress. As such, Neo and HT1 cells were exposed to H_2O_2 from 0 to 8 hrs and assayed for their intracellular ratios of GSH to GSSG as described in Materials and Methods. Indeed, hTERT overexpressing cells maintained a higher GSH/GSSG ratio following treatment with H_2O_2, compared to mock transfected cells (Figure 2A). These results were corroborated in MRC-5-hTERT cells (Figure S3Ai). Furthermore, silencing hTERT in SH-SY5Y cells resulted in a rapid decline in the GSH/GSSG ratio upon the exogenous addition of H_2O_2 (Figure S3Aii).

These data provide evidence to implicate the glutathione metabolism in the protective effect of hTERT against oxidative insults.

Approximately 10% of cellular GSH is found in the mitochondria. Since mitochondria do not contain catalase, they depend upon GSH peroxidase and non-enzymatic reaction with GSH to protect against ROS and more specifically peroxide-mediated toxicity. Therefore, in addition to the total intracellular glutathione levels we also investigated the effect on mitochondrial GSH. Neo and HT1 cells were treated with 300 μM H_2O_2 for 4 and 8 hrs following which the mitochondrial fractions were isolated and glutathione levels were assessed. Results show that the rate of reduction of GSH levels following H_2O_2 treatment was much slower in HT1 cells than in the Neo cells (Figure S3B).

Since the GSH/GSSG ratios were higher in HT1 cells as compared to the Neo cells, the level and activity of enzymes in the glutathione regulation pathway were assayed next. Glutamate cysteine ligase (GCL) catalyzes the rate limiting reaction in GSH synthesis. The catalytic subunit of this ligase, GCLC, confers the total enzymatic activity to GCL. Thus its expression was analyzed by Western blotting following treatment with 300 μM H_2O_2 from 0 to 8 hrs. In HT1 cells, the upregulation of GCLC was detected as early as 2 hrs and remained induced for 6 hrs after treatment as compared to the Neo cells in which a late and transient upregulation was observed 6 hrs following exposure to H_2O_2 (Figure 2B). This could contribute to the increased GSH synthesis and the increased GSH/GSSG ratio upon hTERT overexpression.

Next, the activities of Glutathione Peroxidase (GPx) and Glutathione Reductase (GR), important regulators of glutathione levels, were assessed. The net GPx (Figure 2C) and GR activity were significantly higher in HT1 cells as compared to Neo cells (Figure S3C).

hTERT expression regulates redox status of peroxiredoxins:

The thiol-dependant peroxiredoxins (Prx) and thioredoxins play a critical role in mopping out cellular peroxides. Though Prx I and Prx II are catalytically less efficient than GPx or catalase, they are more abundantly found in the cytosol and display a greater affinity for H_2O_2. This makes them prime candidates as regulators of H_2O_2 signaling (26). Prx III is localized mainly to the mitochondria and serves as one of the main antioxidant defenses against peroxides at the mitochondria. By studying the rate at which these peroxiredoxins might be returned to their active state in cells the cellular Prx activity could be indirectly assessed (27). We show here that, whereas the levels of Prx I, II and III remained unchanged following treatment with H_2O_2 in Neo and HT1 cells, the amount of hyperoxidised peroxiredoxins diminished more rapidly in the HT1 cells as compared to Neo cells (Figure 2D). The process of recovery from the hyperoxidised state without changes in total expression has been suggested as a process of regeneration of native peroxiredoxins from the hyperoxidized forms (27, 28). Substantiating these findings, hTERT silencing in SH-SY5Y cells demonstrated a slower regeneration of Prx from the hyperoxidised Prx SO_3, compared to the cells transfected with scrambled siRNA (Figure S3D).

hTERT overexpression results in improved mitochondrial function:

Given that hTERT expressing cells exhibited a significantly lower mitochondrial $O_2^{\cdot-}$ we next asked if hTERT expression improved mitochondrial function. As such, the mitochondrial localization pattern of hTERT, which is largely known to reside in the nucleus was first studied. Indeed, hTERT was localized to the mitochondrial fraction with significantly higher expression in HT1 cells as compared to the Neo cells (Figure 3A). Furthermore, we provide evidence that hTERT localizes to the inner (IMM) as well as outer mitochondrial membrane

(OMM) fractions (Figure 3B). These findings are in agreement with a recent report by Haendeler et al showing a similar localization pattern of hTERT in HEK cells (17).

Given the reduction in mitochondrial $O_2^{\cdot-}$ levels in hTERT overexpressing cells and the localization of hTERT to the mitochondria coupled with the functional significance of COX in mitochondrial respiration, the role of hTERT in mitochondrial respiration with regards to COX was investigated next. As expected, HT1 cells displayed an increase in COX activity with a more rapid oxidation of ferrocytochrome *c* substrate (Figure 3C).

***hTERT expression impairs ROS-induced cytosolic acidification and downstream mitochondrial outer membrane permeabilization*:**

Having established the inhibitory effect of hTERT on intracellular ROS production, we next set out to investigate the mechanism underlying this protective anti-oxidant effect. We have previously demonstrated that H_2O_2 added exogenously or triggered endogenously upon drug exposure is a stimulus for cytosolic acidification, which creates a permissive intracellular milieu for apoptotic execution (9, 23). Therefore, the effect of hTERT expression on ROS-induced cytosolic acidification was assessed using the fluorescent pH probe 2, 7-Bis (2-carboxyethyl)-5, 6- carboxyfluorescein (BCECF). Whereas exposure of Neo cells to H_2O_2 or the ROS-inducing agent, C1, triggered significant cytosolic acidification (ΔpH= -0.80 units), the drop in intracellular pH was much less pronounced in HT1 cells (ΔpH= -0.196 units) (Figure 4A, 4B).

Cytosolic acidification has been shown to signal the translocation of Bax to the mitochondria followed by the release of pro-apoptogenic factors (23) Following treatment of Neo and HT1 cells with H_2O_2 or C1, cytosolic and mitochondrial enriched fractions of Neo and HT1 cells were prepared and analyzed by Western blot for the release of mitochondrial proteins as well

as Bax activation. Results show that overexpression of hTERT significantly blocked mitochondrial translocation of Bax as well as the release of pro-apoptotic proteins from the mitochondria (Figure 3D). Since the release of pro-apoptogenic proteins from the mitochondria suggested mitochondrial membrane permeabilization, the mitochondrial transmembrane potential ($\triangle\psi_m$) was assessed next. The drop in $\triangle\psi_m$ following treatment with H_2O_2 or C1 was greater in Neo cells as opposed to HT1 cells (Figure 4C). This partial rescue from the drop in $\triangle\psi_m$ with hTERT overexpression provided further support that hTERT expression endows cells with a survival advantage against ROS mediated insults by inhibiting mitochondrial permeabilization. Corroborating that, hTERT overexpressing HT1 and HT2 cells were relatively refractory to the death inducing activity of H_2O_2 or C1, compared to the control cells (Figure 5A-D). To further characterize the loss of cell viability, cell cycle analysis was performed in Neo and HT1 cells following exposure to 150μM and 300 μM H_2O_2 for 18 hrs to determine the sub-G1 pool. Indeed, the percentage of sub G1 population was lower in HT1 (6.2% and 12.9%) cells as compared to Neo cells (16.27% and 21.84%) at 150 μM and 300 μM H_2O_2 (Figure 5C). This was further supported by significantly improved ability of HT1 cells to form colonies following H_2O_2 or C1 treatment in contrast to Neo cells (Figure 5D).

DISCUSSION

The recent discovery of the novel anti apoptotic role of hTERT, independent of its telomerase activity has added a new perspective to re-evaluate the anti-telomerase and specifically anti-hTERT strategies (6, 7, 29, 30). The present study provides strong evidence that increased hTERT expression not only reduces the total basal and mitochondrial ROS levels, but also significantly antagonizes the increase in cellular ROS in response to both exogenous (H_2O_2) and endogenous (C1) ROS triggers. These data are in agreement with recent reports linking hTERT expression in MRC 5 cells to reduced cellular ROS levels (4, 17, 31).

Mechanisms of redox regulation by hTERT:

Given hTERT's localization to the mitochondria and its ability to antagonize mitochondrial ROS coupled with the functional significance of COX in mitochondrial respiration, here the role of hTERT in mitochondrial respiration with regards to COX was investigated. Interestingly, cells overexpressing hTERT have greater basal COX activity as compared to the mock-transfected Neo cells, which could suggest an improved mitochondrial efficiency; more efficient mitochondria could lead to reduced ROS production and could further account for the reduced $O_2^{\cdot-}$ levels in hTERT overexpressing cells. Since hTERT has been shown to localize to the inner and outer mitochondria (Figure 3B) a direct interaction with COX which is an inner mitochondrial protein might be feasible too. Alternatively, it is possible that hTERT might modulate COX activity in an indirect manner by binding to other COX interacting proteins, such as Bcl-2.

The second mechanism by which hTERT seems to modulate intracellular redox status is by improving cellular antioxidant capacity. In this regard, GSH via its sulfhydryl group has been shown to play a critical role in cellular defense against toxic insults. Indeed, the GSH-GSSG oxidation-reduction cycle is regarded as pertinent in conserving the structural and functional

viability of the cells (32), and agents that modulate the cellular GSH concentrations have been shown to alter the transcription of other detoxification enzymes such as glutathione S transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1) as well as affect cell proliferation and death mechanisms (33, 34). As such, the GSH/GSSG redox state can be viewed upon as the mirror of cellular redox status and alterations in the cellular GSH/GSSG ratio can have immense significance on cell survival (35, 36). Here, the demonstration of an enhanced GSH/GSSG ratio in a variety of cell lines strongly implicates a role for hTERT in modulating glutathione levels in the cells and conferring survival advantages. The elevated GPx activity and a lack of difference in GR activity in HT1 cells raised the question on how GSH levels might have been regulated to maintain an elevated GSH/GSSG ratio in HT1 cells.

GSH loss and oxidation have been associated with an increased expression of the rate-limiting enzyme of GSH synthesis, glutamate cysteine ligase (GCL), and several other detoxification systems. This prompted further analysis on the catalytic subunit of glutamate cysteine ligase (GCLC) which catalyses the rate limiting step of GSH synthesis (35, 37). The results demonstrated an early and sustained induction of GCLC in response to H_2O_2 in HT1 cells. This was in clear contrast to the transient and late activation of GCLC observed in mock-transfected cells. Since GCLC is critical for GSH synthesis, an early and sustained induction of GCLC following oxidative stress in hTERT expressing cells would certainly help to antagonize a rapid decline in cellular GSH levels, thus contributing to their higher GSH/GSSG ratios. The increased basal GPx and GR activity could contribute to the lowered basal cellular ROS levels in hTERT overexpressing cells. Assessment of GPx activity or GR activity showed no further changes even when exposed to oxidative stress triggers (Data not shown).

The assessment of Prx I, II and III reflected no significant changes in their total levels after H_2O_2 treatment. Interestingly, the study of the hyperoxidised forms of Prx I, II and III revealed a faster decline in the overall hyperoxidised forms of the Prx in HT1 cells. The hyperoxidation of Prx could lead to the inactivation of Prx and their regeneration would require repair proteins such as sulfiredoxins and/or sestrins. In this regard, several studies have shown the regeneration process by monitoring the total and hyperoxidized forms of Prxs and used these varied forms of Prx to assess cellular Prx activity (27, 38). Since the total amount of Prx remains unchanged over the same time course, our data suggest that the hyperoxidized forms are not targeted for degradation and most probably regenerated to their active forms. Cumulatively, the more rapid decrease in the inactive form, as has been observed in hTERT overexpressing cells, might also mean a larger pool of active Prx available to target cellular peroxides in the cells.

Perturbation of cellular redox status alters intracellular milieu and cell fate:

Studies have shown that an increase in intracellular H_2O_2 can lead to cytosolic acidification, which can subsequently engage the mitochondria mediated pathway of death signaling via Bax conformational change and mitochondrial translocation culminating in effective death execution (9, 23). In the current study, H_2O_2-induced cytosolic acidification was significantly reduced in hTERT overexpressing cells, which impeded Bax translocation and mitochondrial outer membrane permeabilization. This could have led to the protection of hTERT overexpressing cells from the apoptotic stimuli as demonstrated by the reduction in cell death and the improvement in colony forming ability of hTERT overexpressing cells. This is consistent with a recent report demonstrating the protective effect of hTERT on receptor-mediated death signaling [tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL)] in NB4-LR1 cells, but not against cisplatin- or etoposide-induced cytoxicity

(39). In contrast, several studies have shown in other cell lines that hTERT expression is able to protect cells from the cytotoxic effects of cisplatin (40-42). As such, it is worth mentioning that ROS generation via p53-mediated p38alpha MAPK is a mechanism by which cisplatin induces apoptosis. (43). Thus in a p53 defective cell line such as NB4-LR1, although cisplatin could induce cytotoxic effects via other mechanisms such as DNA damage, its ability to elicit a death response via the induction of ROS could be diminished. As such, our data underscore the significance of hTERT expression in diminishing ROS-induced cytotoxicity, and the protective effect of hTERT could be a function of the intrinsic differences in the cell's ability to engage ROS-mediated death effector pathways triggered by drug treatment.

Taken together, the discovery of hTERT's ability to modulate intracellular ROS by altering antioxidant status and mitochondrial function highlights for the first time a ROS-mediated mechanism by which hTERT expression endows cancer cells with a survival advantage by blunting apoptotic signals. By implication, cancer cells with intrinsically higher levels of hTERT might be refractory to chemotherapeutic regiments that function via ROS-mediated mechanisms for efficient death execution (Figure 6). Hence, under such circumstances, alternative approaches such as targeting hTERT or the cellular antioxidant defenses might help to enhance chemosensitivity of cancer cells. Though it's early to hazard a conjecture on the mechanism that hTERT employs to regulate these antioxidant defenses, a possible candidate would be NF- κB, a key cellular transcription factors which engages in a unique cross talk with ROS. NF- κB is modulated by cellular redox status and has been shown to transcribe several antioxidant factors including MnSOD and γ-glutamyl-cysteine synthetase – the rate limiting enzyme for GSH synthesis (44-46) and also enhance the transcription of AP1's downstream target genes such as sulfiredoxin (47, 48). Several studies have also reported a physical interaction between hTERT and NF-κB. Akiyama and group have

demonstrated that the active NF-κB p65 complex could chaperone hTERT from the nucleus into the cytosol (49, 50). In addition, it has also been shown by GG Sharm *et al* that ectopic hTERT expression can upregulate expression of NF- κB by over 6 folds. They also showed that this occurred via a mechanism that did not involve direct binding with the promoter regions of NF-κB. Our unpublished data also shows higher levels of nuclear p65 in HT1 cells as compared to Neo cells. In addition, HT1 cells show a significantly higher level of MnSOD, which is a transcriptional target of NF-κB (data not shown). Given the keen association between the active NF-κB complex and hTERT at the nucleus, it is tempting to speculate that this interaction might be able to influence NF-κB's binding affinity to the DNA and the transcription of the NF-κB driven antioxidant genes. Data presented here together with recent evidence underscore the broad biological consequences of hTERT expression aside from its essential function in telomere maintenance and clearly illustrate the need for new therapeutic strategies to target the novel functional consequence of increased hTERT expression.

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FIGURE LEGENDS

Figure 1: Stable hTERT overexpression reduces basal intracellular ROS. (A) hTERT expression in untransfected HeLa cells (ATCC), p-Babe-Neo transfected cells (Neo) and 2 independent heterogeneous populations of hTERT-transfected cells (HT1, HT2) were assayed by Western blot analysis. Beta actin was used as a loading control. Basal levels of intracellular ROS were detected by flow cytometry in HeLa Neo, HT1 and HT2 cells using the probes (B) DCHF-DA and (C) MitoSox RED and the average G-Mean values were computed. Neo and HT1 cells were exposed to (D) 300 μM of H_2O_2 or 100 μg/ml of C1 for 3 hrs following which cellular ROS was assessed using the probe DCHF-DA. The density plot is data from 4 independent experiments. (* $p<0.01$, **$p<0.005$)

Figure 2: hTERT overexpressing cells express enhanced glutathione antioxidant defense system and a more rapid recovery of peroxiredoxins from the hyperoxidised state. Neo and HT1 cells were treated with 300 μM H_2O_2 from 0 to 8 hrs following which (A) intracellular GSH/GSSG ratio was computed or whole cell lysates were prepared and subjected (B) immunoblot analysis of GCLC or (D) Prx I, II, III, Trx I and Prx SO_3. The antibody recognizes Prx I/II SO3 and Prx III SO_3. (C) Neo and HT1 cells were used to prepare the sample for the GPx activity assay as described earlier and the fold difference was calculated. Data shown are mean ± SD of at least three independent experiments and (C) was performed in duplicates. (* $p < 0.05$, **$p<0.005$)

Figure 3: hTERT localizes to the mitochondria and hTERT expression improves mitochondrial COX activity. Neo and HT1 cells were fractionated and the mitochondrial hTERT levels were then assessed in Neo and HT1 cells (A). Mitochondrial fractions of HT1 cells were subjected to 50μg/ml Proteinase K to remove the OMM. The fractions were then

subjected to Western blot analysis of hTERT, Bcl-2, VDAC and prohibitin (B). 30 x 10^6 cells were fractionated and the COX activity was assessed in the mitochondrial fractions. The data shown are mean ± SD of at least 3 independent experiments. (* $p < 0.05$) (D) Neo and HT1 cells were treated with 300μM H_2O_2 or 100 μg/ml C1 for 12 hours and fractionated to obtain the cytosolic and mitochondrial fractions which were subjected to Western blot analysis of Bax and Cyt C with VDAC and Cu/Zn SOD as mitochondrial and cytosolic loading controls. (WCL: whole cell lysate)

Figure 4: hTERT expression reduces ROS induced cytosolic acidification, Bax translocation, and release of pro apoptogenic proteins. Neo and HT1 cells were treated with 300 μM H_2O_2 (A) or 100 μg/ml of C1 (B) for 4 hours following which, changes in intracellular pH (pHi) was assessed. (C) Neo and HT1 cells were treated with 300 μM of H_2O_2 or 100μg/ml of C1 for 6 hrs after which the cells the mitochondrial membrane potential was detected by flow cytometry using the probe $DIOC_6$. All data shown are mean ± SD of at least 4 independent experiments (*$p < 0.05$).

Figure 5: hTERT overexpression inhibits H_2O_2 mediated apoptosis. HeLa ATCC, Neo, HT1 and HT2 cells were treated with H_2O_2 (A) or C1 (B) in a dose dependant manner and cell viability was assessed by crystal violet assay at 18 hours. (C) Neo and HT1 cells were treated with 150μM and 300μM H_2O_2 and stained with propidium iodide for DNA fragmentation analysis by flow cytometry (* % of sub-G1 fraction) or exposed to (D) 300μM H_2O_2 or 100 μg/ml of C1 following which colony forming assay was performed as described earlier. All data shown are mean ± SD of at least three independent experiments (*$p < 0.05$).

Figure 6: Underlying mechanism of hTERT-mediated protection against oxidative stress induced cell death. At the nucleus, hTERT's interaction with transcription factors (TF) such as NF-κB may offer an explanation to the enhanced antioxidant defenses (e.g. GSH, Mn SOD) and lowered cellular ROS levels that is observed with hTERT expression. Exogenous ROS triggers have been shown to trigger nuclear export of hTERT. hTERT can be imported from the cytosol into the mitochondria by interacting with mitochondrial import proteins (IP) such as Tom 20 where it may enhance mitochondrial activity and reduce mitochondrial ROS production. Antagonism of an increase in cellular ROS levels can impede downstream pro-apoptotic events such as cytosolic acidification, dissipation of mitochondrial membrane potential (ψρm) and the release of pro-apoptogenic factors (PA) thus protecting from cell death.

Figure 1

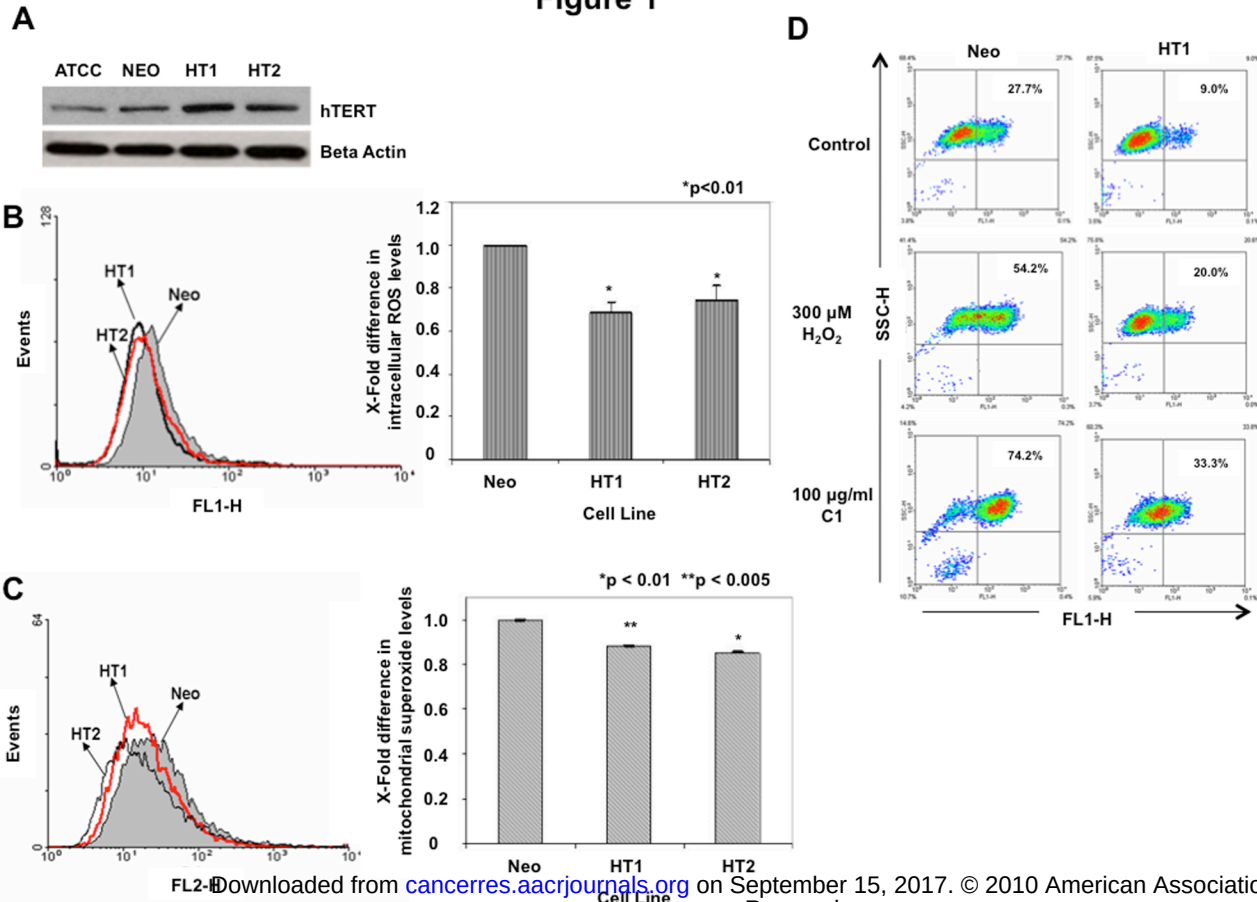
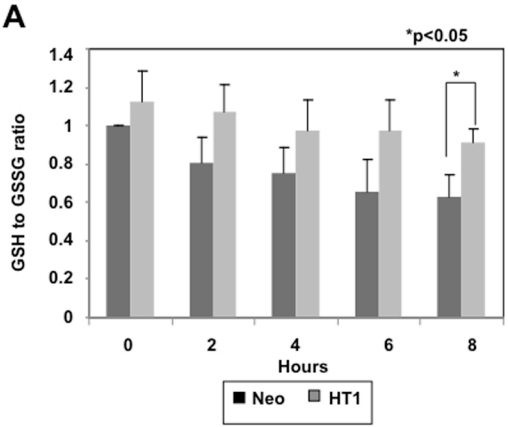
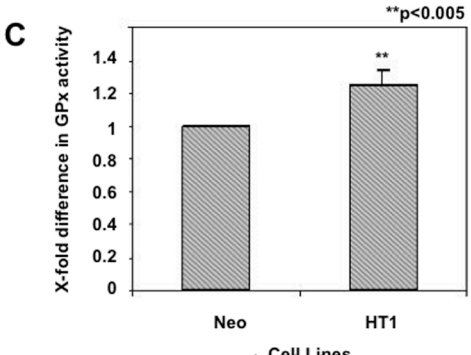
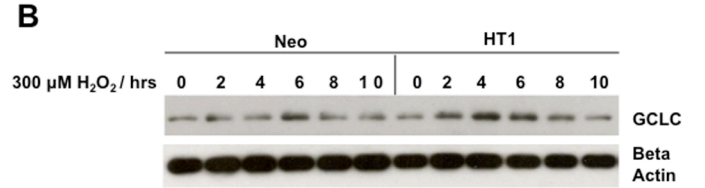
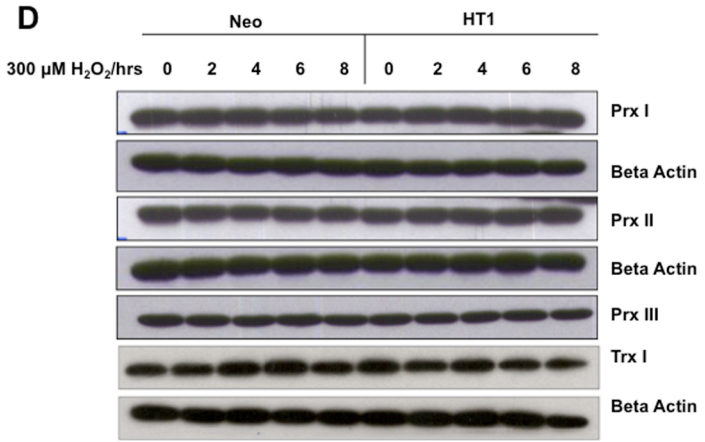


Figure 2









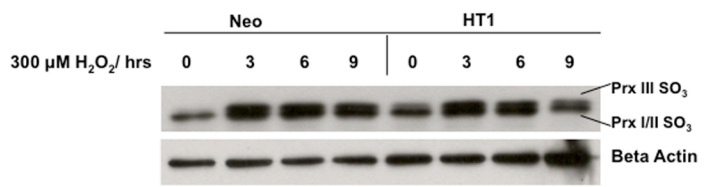
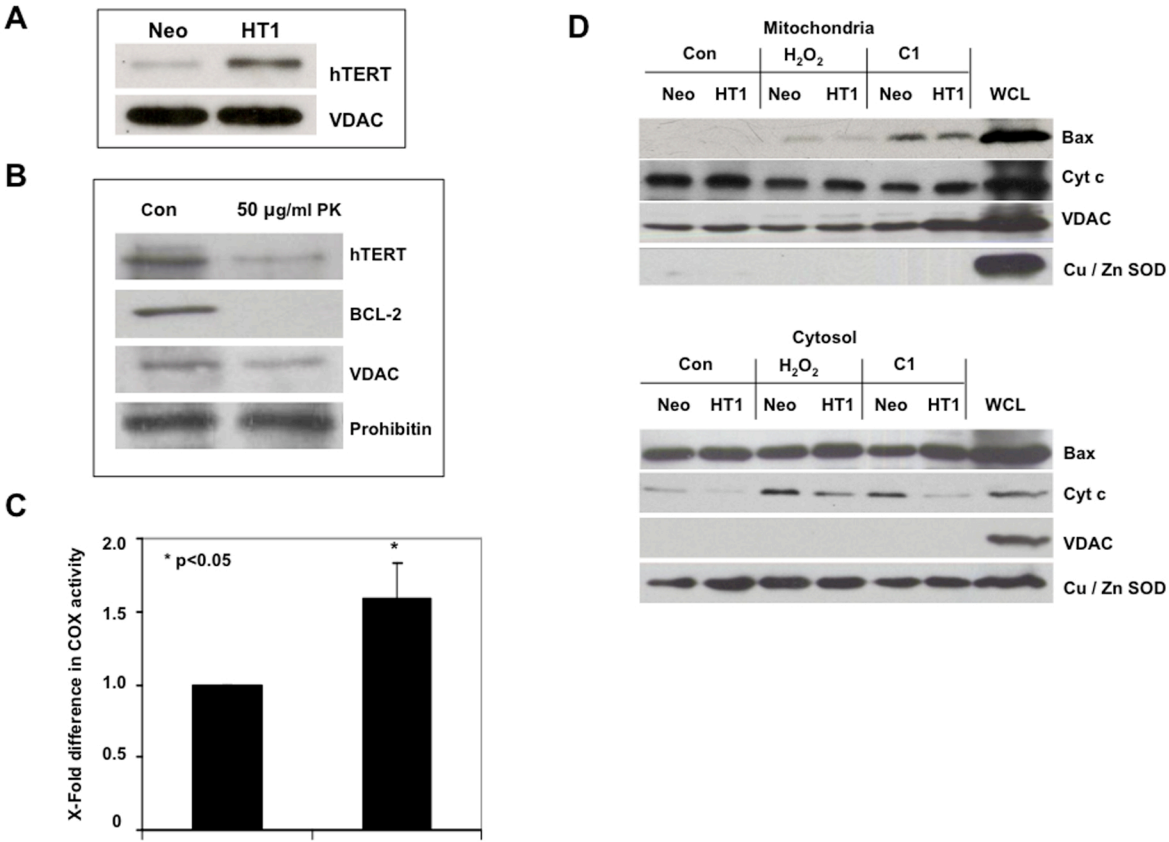


Figure 3



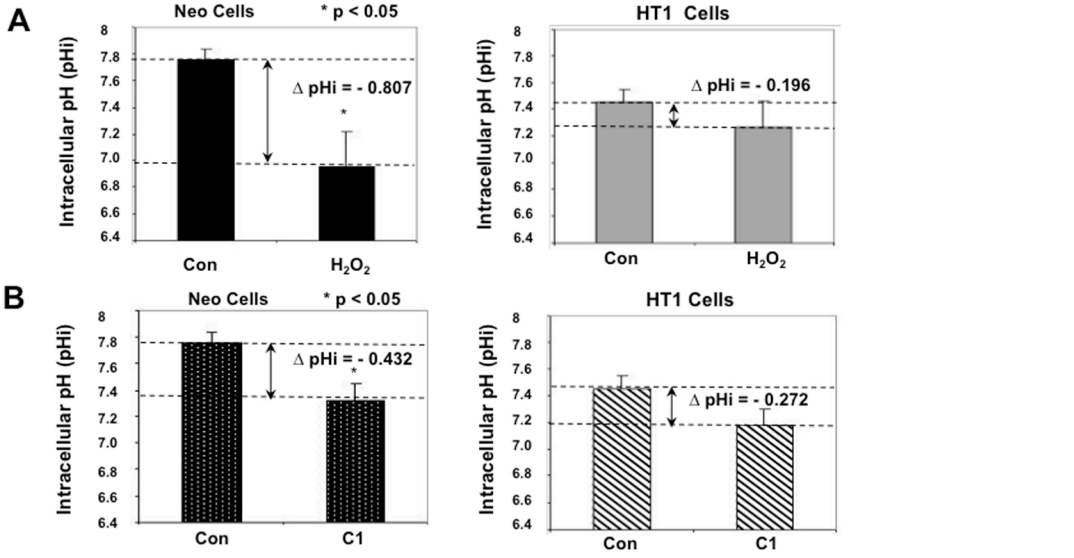


Figure 4

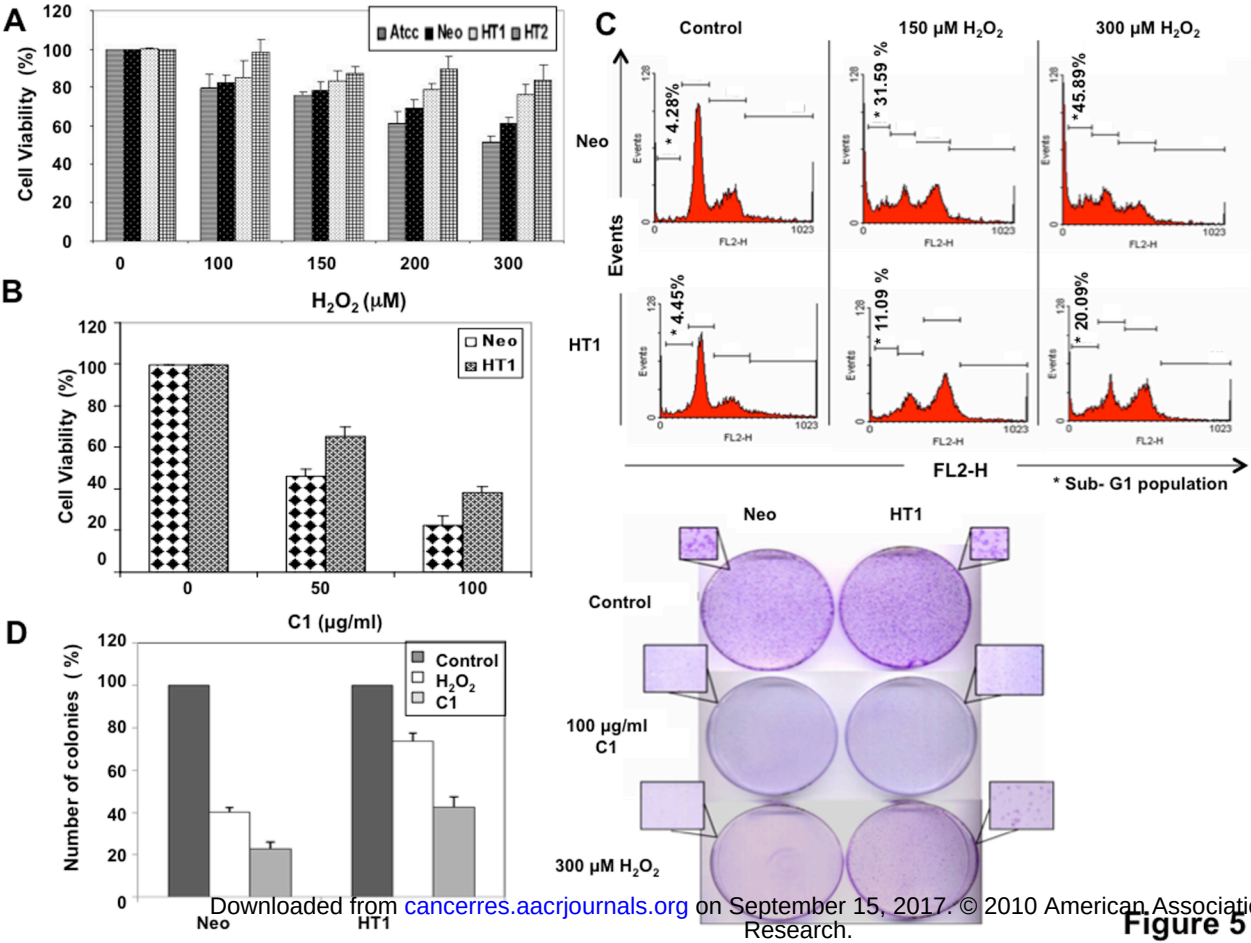
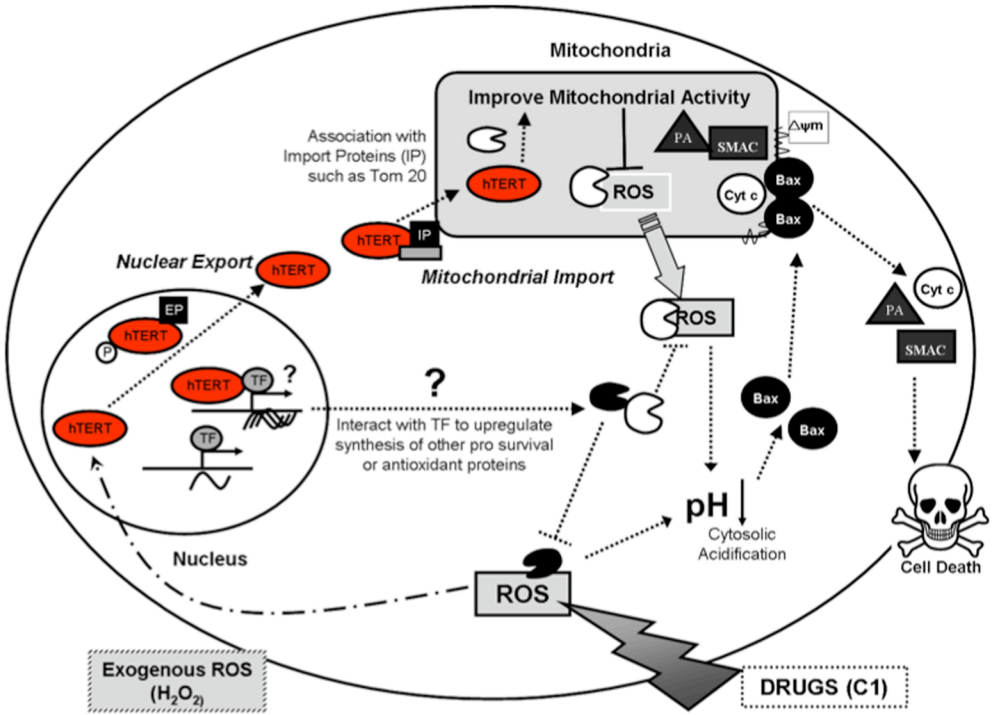


Figure 5

Figure 6



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hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells

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