Journal of Student Research (2015) Volume 4, Issue 1: pp. 99-103
Research Article

# The Effect of Increased Intracellular Calcium on the Localization of the Catabolic Subunit of Telomerase, hTERT, in HeLa Cells

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In cancer cells, telomeres are constantly repaired by telomerase allowing them to avoid reaching a critical length where the cell becomes senescent. Overtime, aerobic respiration in the mitochondria can create an increase in intracellular reactive oxygen species (ROS) that causes telomerase reverse transcriptase (hTERT), the catabolic subunit of telomerase, to exit the nucleus. Without hTERT in the nucleus, telomerase cannot function to repair telomere ends, inducing senescence. One effect of increased ROS production is the activation of calcium channels leading to an increase in intracellular calcium. We attempted to mimic this ROS induced increase in intracellular calcium levels by administering 0.5uM of Thapsigargin to HeLa cells in order to determine if an increase in calcium levels alone would be sufficient to cause hTERT to exit the nucleus. Immunofluorescene was used to visualize fluorescent intensities of hTERT in the nucleus after administration of the drug in conjunction with visualization of Ran as an indicator of functional nuclear transport. We predicted thapsigargin would disrupt nuclear transport and cause hTERT to exit the nucleus. Image J software was used to compare nuclear fluorescence intensities between treated and control cells. Our results indicated that administration of thapsigargin caused a significant decrease in nuclear levels of hTERT in conjunction with decreased nuclear levels of Ran. Our findings could provide a potential method to induce cellular senescence in cancer cells by inducing mislocalization of hTERT and Ran.

**Keywords:** telomerase, TERT, calcium; ROS, thapsigargin; HeLa cells; senescence

## Introduction

Telomeres are highly repetitive DNA sequences (TTAGGG) that function to protect the ends of chromosomes from being degraded during each replication (Indran, 2011). Each time the cell replicates, telomeres shorten in length because the lagging strand of DNA cannot be fully replicated (Feng, 1995). Once the telomeres shorten to a specific length they reach their critical point and the cell becomes senescent and no longer divides (Indran, 2011). The enzyme telomerase functions in the nucleus of reproductive cells and stem cells to maintain telomere length, preventing shortening by adding the specific repeated nucleotide sequences (Indran, 2011).

The function of telomerase is dependent on two components, a catalytic protein and an RNA template (Cong, 2002). hTR, the RNA component of telomerase provides template sequences of TTAGGG repeats (Indran, 2011). Human telomerase reverse transcriptase (hTERT) catalyzes the addition of telomeric sequences to the ends of chromosomes using the hTR template (Broccoli, 1995). While this process occurs in embryonic cells, it does not function in somatic, proliferating or terminally differentiated cells causing these cell to only divide approximately 40-60 times before becoming senescent (Indran, 2011). Senescent cells produce hTR RNA, but no hTERT mRNA, and thus no active hTERT protein (Cong, 2002). In most cancerous cells, hTERT expression is reactivated allowing telomerase to remain active using the hTR RNA present (Cong, 2002). However, this misexpressed telomerase can still be inactivated through mechanisms that alter hTERT expression or cause it to no longer be present in the nucleus (Cong, 2002). For example, oxidative stress in the form of reactive oxygen species, ROS, can lead to the inactivation of telomerase via mislocalization of hTERT (Henke, 2013).

ROS are oxygen containing chemically reactive charged radicals, such as oxygen ions and peroxides (Henke, 2013). The presence of ROS in cells is the by-product of aerobic respiration in the mitochondria that build up over time as a result of aging. Increased ROS due to cellular metabolism is known to cause hTERT to leave the nucleus, which prevents telomerase function, ultimately leading to cellular senescence (Henke, 2013). The cellular effects of ROS that may lead to telomerase export are numerous. Of particular interest to our work is the fact that this increased oxidation due to ROS alters intracellular calcium levels by activating L-type calcium storage channels (Rosenberger, 2007). In addition to increased intracellular calcium levels, another downstream effect of ROS is the disruption of nuclear transport (Kelly, 2007). Specifically, the GTPase, Ran, is mislocalized from the nucleus to the cytoplasm due to increased oxidative stress in the cell (Kelly, 2007). The Ran GTPase gradient in a cell is crucial for the nuclear transport of many molecules including hTERT (Haendeler, 2003). Specifically, Ran GTP is typically at high concentrations within the nucleus where it induces formation of export complexes and disassembly of import complexes (Kelly, 2007).

In our experiment, we intended to determine if increased calcium levels alone would be sufficient to induce nuclear export of hTERT. If altered calcium levels can disrupt hTERT function in this manner, it implies that one possible way to induce cellular senescence in cancer cells would be through drugs that alter cellular calcium concentrations. To determine if altered calcium alone is sufficient to induce cytoplasmic mislocalization of these proteins, the drug thapsigargin, which blocks specific pumps that allow an influx of calcium to the sarcoplasmic and endoplasmic reticulum, was used to mimic the increased calcium levels caused by increased levels of ROS (Thastrup, 1990). We

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treated HeLa cervical cancer cells with thapsigargin and visualized the location of hTERT and Ran via immunofluorescence. Specifically, the location of Ran was used as a control to ensure that thapsigargin treatment was sufficient to disrupt nuclear transport. Nuclear intensities of Ran or hTERT were then compared between cells treated with Thapsigargin and vehicle control cells using the program image J. Higher nuclear fluorescent intensity values correlate with high levels of either the proteins hTERT or Ran in the nucleus.

We predicted that thapsigargin treatment of HeLa cells would cause hTERT to be absent from the nucleus. Our results supported this hypothesis, in that lower nuclear intensities were observed for both Ran and hTERT in cells treated with thapsigargin. The findings from our experiments show a potential mechanism to induce the onset of cellular senescence in cancer cells by potentially decreasing nuclear telomerase activity due to hTERT mislocalization allowing telomeres to reach their critical point and enabling the cells to enter senescence.

**Methods:**

*Cell Culture*

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). 75% confluent cells were split onto 12 well coverslips and grown for 24 hours prior to drug treatment and immunofluorescence.

*Immunofluorescence*

0.5uM of Thapsigargin was administered to treatment group HeLa cells in 12 well plates for 15 or 30 minute exposures. The drug was removed by washing with 1XPBS and the cells were fixed in 70ul droplets of 3% Formaldehyde (Malinckroft, Paris, KY), permeabilized with 0.5% Trition X-100 (G Bioscience, St. Louis, MO), and blocked with 0.5 mL of 5% FBS (G Bioscience, St. Louis, MO). Primary rabbit IgG antibodies for hTERT (Santa Cruz, Dallas, TX) at a 1:50 concentration were added and primary mouse IgG antibody for Ran (BD Transduction Laboratories San Jose, CA) at a 1:100 concentration for 60 minutes. Secondary antibodies at a 1:200 concentration for hTERT (Santa Cruz, Dallas, TX) and a 1:100 concentration for Ran (BD Transduction Laboratories San Jose, CA) at were added for 60 minutes. The coverslips were then mounted in vectashield (Vector Labs, Burlingame, CA) and visualized under a Leica DM 750 fluorescence microscope.

*Image Quantification*

Image J software(Rasband, 1997-2014)was used to compare nuclear intensity between treatment group cells and control cells by determining corrected total cell fluorescence (CTCF) of the nucleus with the NII plug-in the nucleus. First, the plug-in was used to outline each nucleus in the DAPI images. The nucleus was then outlined in both the green and red fluorescence images. A small outline of the background was needed to be used in the equation to calculate CTCF in each cell to determine Ran and hTERT nuclear intensities. CTCF was found by using the equation: Integrated Density – (Area of selected cell X Mean fluorescence of background readings) from values given from the program. 100 cells for each group were analyzed from 3 independent immunofluorescence trials for a total of 300 cells per group.

**Results**

Ran and hTERT are localized to the cytoplasm by thapsigargin. In order to determine if Ran and hTERT exit the nucleus in response to thapsigargin, visualization of the proteins' location was determined in cells with and without the drug added. Immunofluorescence for Ran and hTERT was performed on HeLa cells treated with 0.5µM of the drug thapsigargin for 15 and 30 minute exposures as well as on control HeLa cells with no drug added (Figure 1). DAPI staining was used to visualize the location of the nucleus as a reference for staining of Ran and hTERT (Figure 1A). hTERT appears predominantly nuclear in control cells and localized to the cytoplasm in treatment group cells (Figure 1B). Ran also appears to be predominantly nuclear in control cells but becomes localized to the cytoplasm in the both the 15 and 30min treatment groups (Figure 1C).

Image J software was used to determine and quantify the fluorescence nuclear intensities of Ran and hTERT by using a plug-in that determines the corrected total nuclear cell fluorescence which we used solely on the nucleus by using the DAPI reference images to determine nuclear area (Figure 2A). Average nuclear intensity values for Ran and hTERT were calculated using 500 cells per treatment (Figure 2B and 2C). Significantly higher levels of both nuclear hTERT and Ran fluorescence were observed in the control group HeLa cells as compared to the 15 and 30 minute Thapsigargin treatment group cells (Figure 2B and 2C). Lower amounts of nuclear fluorescence in the treatment groups, compared to the control, suggest that the proteins Ran and hTERT have exited the nucleus in response to an increase in intracellular calcium induced by the drug Thapsigargin. Both groups were shown by an Anova test to be significantly lower than the control.

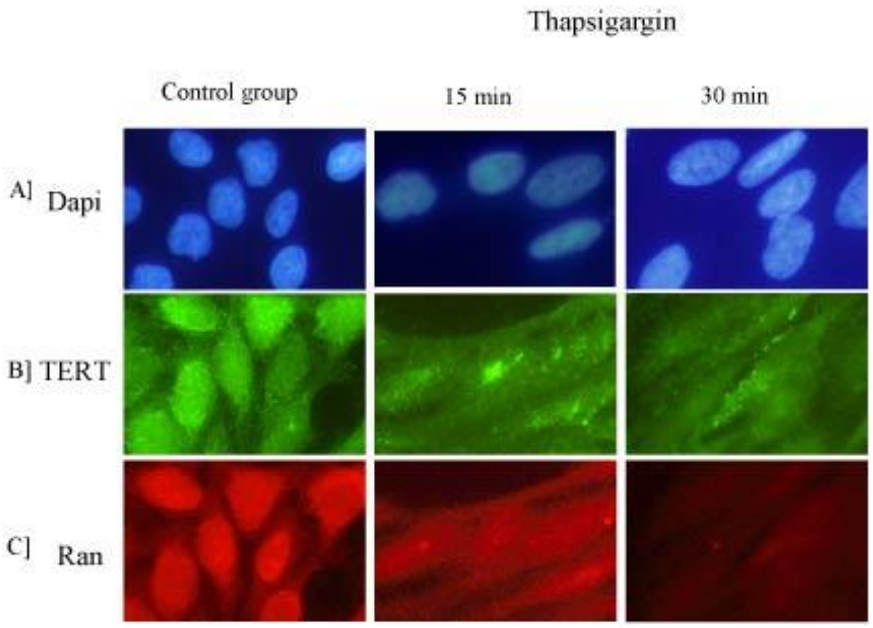


**Figure 1:** Ran and hTERT accumulate in the cytoplasm after treatment with thapsigargin. Representative images of immunofluorescence for control HeLa cells and HeLa cells treated with 0.05µM of thapsigargin or a control of DMEM media alone. A) DAPI staining indicates nuclear position B) hTERT begins nuclear and becomes cytoplasmic upon thapsigargin treatment and C) Ran becomes cytoplasmic after treatment with thapsigarin.

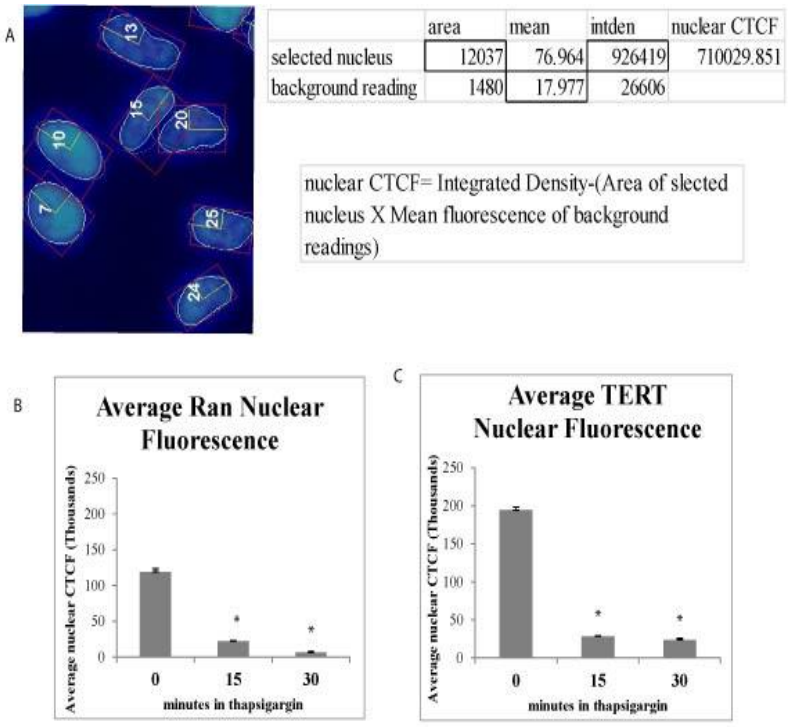


**Figure 2:** Average levels of nuclear Ran and hTERT fluorescence intensity in each thapsigargin treatment group and control cells. A) Representative nuclear outlines of cells and data using Image J to determine nuclear intensities from the equation: Integrated Density – (Area of selected nucleus X Mean fluorescence of background readings). Integrated density is the total amount of fluorescence determined from pixel values. The amount of fluorescence in the background is multiplied by the area of the selected nucleus and then subtracted from the total nuclear fluorescence. B) Significantly higher levels of nuclear Ran fluorescence were found in control cells compared to cells exposed to thapsigargin for 15 and 30 min. C) Significantly higher levels of nuclear hTERT fluorescence was found in control cells compared to cells treated with thapsigargin for 15 and 30 min exposures. Error bars represent standard error. An asterisk indicates a significant difference between the control cells and the treatment groups.

## Discussion

HeLa cells treated with hydrogen peroxide display increased hTERT nuclear export due to the increase in ROS levels (Singhapol, 2013). Export of hTERT from the nucleus allows it to shuttle to the mitochondria where it functions to decrease ROS levels therefore preventing potential oxidative damage to cellular DNA (Singhapol, 2013). Because one of the known cellular outcomes of altered ROS levels is an increase in intracellular calcium levels, we sought to determine the location of hTERT in cells where only calcium levels were increased rather than the entire spectrum of ROS induced changes. Thus, we were able to determine if change in calcium concentration alone was enough to cause export of hTERT from the nucleus.

We observed the cellular localization of the nuclear transport factor Ran and hTERT via immunofluorescence in HeLa cells following treatment with the calcium amplifier, thapsigargin. Quantitatively determined nuclear fluorescence intensities showed that significant levels of Ran and hTERT exited the nucleus in response to thapsigargin (Figure 2). Specifically, lower levels of nuclear fluorescence (<25,000 was considered low levels while >100,000 was considered high levels) for Ran and hTERT were detected in treated HeLa cells (Figure 2B and 2C). We propose that, without the catalytic subunit hTERT in the nucleus, the holoenzyme telomerase would no longer able to function properly leading to shortened telomeres and cellular senescence in response to altered cellular calcium.

The translocation of hTERT from the nucleus to the cytoplasm has been shown to cause a decrease in telomerase activity in other studies that altered cellular calcium through various mechanisms (Liao 2007). The reFIP protein, which that releases intracellular calcium by causing the ER to release stored calcium, causes hTERT to exit the nucleus in A549 human lung adenocarcinoma cells (Liao 2007). Similar results were observed using thapsigargin in A549 cells (Liao 2007). Interestingly, thapsigargin treatment, while disrupting localization, did not alter hTERT protein expression, which was very stable (Rosenberger 2007). This stability of hTERT expression highlights the large variation in mechanism of action by which increased calcium concentration inactivates hTERT as described in the current literature. For example, translocation of hTERT from the nucleus to the cytoplasm was not induced by the reFIP protein in H1299 human lung carcinoma cells even though telomerase activity was affected (Liao 2007). The loss of telomerase activity in those cells was likely due to downregulation of the hTERT protein (Liao 2007).

Further evidence that increased calcium levels inhibit telomerase activity in a variety of ways comes from studies of A549 cells treated with a range of natural and synthetic compounds that release $Ca^{2+}$ from the ER (Rao, 2010). While TRAP assay results showed that telomerase activity decreased due to the release of intracellular calcium, in these cells hTERT location did not change, remaining primarily nuclear (Rao, 2010). Once again in this study it is thought that protein expression was the cause for alteration of telomerase and results of multiple studies clearly indicate that the location of hTERT is not always altered with an increase in calcium rather, in some cases transcription of hTERT is downregulated instead. However, both these mechanisms alter telomerase activity in response to increased calcium (Rao, 2010). It is possible that different mechanisms of telomerase inactivation induced by altered calcium alterations may be dependent on the particular combination of cell type and drug treatment. While some cells may cause reduced expression of hTERT, others could induce posttranscriptional regulation by shuttling hTERT out of the nucleus.

Here we provide evidence that the exit of hTERT from the nucleus induced by thapsigargin in HeLa cells may result from a disregulation of Ran GTPase (Figure 1C and 2C). Oxidative stress has previously been shown to cause nuclear export of hTERT by activating Ran GTPase (Haendeler, 2003). Therefore it is possible that calcium alone may be able to activate Ran GTPase in a similar manner. Nuclear export of hTERT is dependent on CRM1 which functions with Ran GTPase to shuttle the protein out of the nucleus (Seimiya, 2000). Our results show that increased calcium levels cause Ran to exit the nucleus by disrupting the Ran GTPase gradient (Figure 1C). This alteration of Ran occurs in parallel with the accumulation of hTERT in the cytoplasm (Figure 1B). This evidence, together with the fact that hTERT is known to exit the nucleus in a Ran GTPase dependent manner indicate that increased calcium levels disrupt the Ran gradient which precludes the return of hTERT to the nucleus (Kelly, 2007). Therefore disruption of nuclear transport by thapsigargin would then lead to a disruption in telomerase activity in HeLa cells.

In conclusion we have shown that thapsigargin treatment in HeLa cells disrupts nuclear transport by mislocalizing Ran and causes hTERT to accumulate in the cytoplasm. Therefore it is possible that mislocalization of hTERT by strictly altering calcium levels in cancer cells has the potential as an anti-cancer therapy by disrupting telomerase function and limiting proliferation. However, it is also possible that hTERT may have a beneficial function for cancer cells outside the nucleus because it helps reduce ROS levels to prevent oxidative DNA damage. Despite this conflict of advantageous function in both the nucleus and cytoplasm, we propose that in HeLa cells if nuclear hTERT is not replaced by upregulation of the gene or returned inside the nucleus, the onset of cellular senescence is likely to occur.

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