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TITLE: The Role of Telomeric Repeat Binding Factor 1 (TRF1) in Telomere Maintenance and as a Potential Prognostic Indicator in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Kimberly S. Butler
Jeffrey K. Griffith, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico
Albuquerque, NM 87131-6003

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Kimberly S. Butler and Jeffrey K. Griffith, Ph.D.

E-Mail: kbutler@salud.unm.edu

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14. ABSTRACT

The aims of this study are to (i) determine the relationships between the telomere binding protein Telomere Repeat Binding Factor 1 (TRF1) and other telomere binding proteins, (ii) establish the potential of TRF1 as a surrogate marker for telomere content (TC) and as a potential clinical marker and (iii) characterize the relationship between of the telomere binding protein TRF1 and TC. Through examining the role of TRF1 in telomere length control and in breast cancer progression, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all specific aims; as outlined in the Statement of Work, are expected to be completed on schedule. The research is in progress.

15. SUBJECT TERMS
HUMANBREASTCANCERTISSUE,TELOMERES,TELOMERE-BINDINGPROTEINS, IMMUNOHISTOCHEMISTRY, RT-PCR, SLOT BLOT, SIRNA

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I. INTRODUCTION

The aims of this study are to determine the relationship of the telomere binding protein TRF1 to other telomere binding proteins, establish the potential of Telomere Repeat Binding Factor 1 (TRF1) as a surrogate marker for telomere content and as a potential clinical marker and further, to characterize the relationship of the telomere binding protein TRF1 to telomere content. Through examining the role of TRF1 in telomere length and in breast cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, Specific Aim 2 has been completed and a manuscript based on the data is being edited for submission. The student has received a no cost extension, which should allow the completion of Aim 1 and 3. Three of the tasks in Specific Aim 1 were delayed due to a technical issue, however this issue has been overcome and the data collection is in progress and expected to be completed prior to the end of the extension. Several of the tasks Specific Aim 3 were delayed due to necessary changes in techniques, however the new methods have been trouble shot and data collection is in progress. All Aims are expected to be completed by the end of the no cost extension.

Hypothesis and Rationale

Telomere Content (TC) has prognostic value in breast cancer; however the factors that control TC are poorly understood. In vitro studies have shown that high levels of TRF1 can stabilize short telomeres and the preliminary results suggest the levels of TRF1 mRNA are related to TC. Together this data suggests that TRF1 level may be directly related to telomere content and therefore be a potential biomarker for telomere length. However, TRF1 also has multiple interacting partners, TRF1 Interacting Nuclear Factor 2 (TIN2), Tankyrase, Telomere Repeat Binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which may modify the interaction between TRF1 and TC. If increases in TRF1 are partially responsible for decreased TC, a prognostic marker of poor outcome, then targeting TRF1 may be a good preventive treatment of breast cancer progression. However, it is also possible that the observed increase in TRF1 is a cellular reaction in response to low TC and therefore a good surrogate for TC. Theses two scenarios must be tested to evaluate the prognostic significance of TRF1 in human breast cancer. Therefore *I hypothesize that defining TRF1 levels using immunohistochemistry could provide a surrogate measure for TC that would be easily adaptable to the clinical setting*. To test this hypothesis I will assess the potential prognostic value of the TRF1 in human breast tumor samples. Additionally, I propose to characterize the relationship of TRF1 to TC, and to TIN2 and Tankyrase to further examine the relationship of TRF1 to TC. I will evaluate this hypothesis through three specific aims.

- **Specific Aim #1**
  Assess the potential of TRF1 protein levels as a surrogate for Telomere DNA Content (TC) in frozen and paraffin embedded breast tumor tissues.

- **Specific Aim #2**
  Assess the potential modification of the relationship between TC and TRF1 mRNA levels by TRF1 interacting protein 2 (TIN2) and Tankyrase in frozen human breast tumor samples.

- **Specific Aim #3**
  Examine the effects of increased TRF1 expression on TC and decreased TC on TRF1 expression in breast cancer cell lines.

II. KEY RESEARCH ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

- There exists an association between the levels of TRF1, TIN2 and POT1 mRNA within breast tumors, as measured by real-time RT-PCR.
- The levels of TRF1mRNA are not associated with the mRNA levels of the human telomerase reverse transcriptase (hTERT) mRNA or the levels of TRF2 mRNA within breast tumors.
- The levels of TIN2, TRF1, TRF2 and POT1 mRNA are all associated with telomere content.
- Visualized TRF1 and TRF2 distribution by Immunohistochemistry.
- Developed siRNA for hTERT.
- Developed TRF1 overexpression vector.
- Developed 2 drug treatments to increase TRF1, TIN2 and POT1 mRNA levels
- Developed a drug treatment which decreases telomere content without changing hTERT levels
- Assessed the relationship of TRF1 and TRF2 to cell cycle by flow cytometry
- Developed a western blotting procedure for TRF1 and TRF2 proteins

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the previous annual review, the PhD candidate has had continuing opportunities to work and interact with oncologists, pathologists and other PhD scientists who specialize in breast cancer. These interactions have occurred though tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. To the training in microscopy, cryosectioning and paraffin sectioning she received in the first year and second years of the award, she has received training in flow cytometry from the UNM Flow Cytometry resource.

On an educational level, the candidate has continued her involvement in the Biochemical Laboratory Methods Course and has written a section and co-instructed the upper-level undergraduate course. The candidate has aspirations of continuing her career in research and remaining in academia and felt teaching provided an opportunity to develop the essential teaching skills need for her chosen career.

IIc. PERFORMANCE ACCOMPLISHMENTS:

Experimental Milestones

Specific Aim 1: (7 tasks)

Task 1 Month 1-2 Completed in year 1

- Purify DNA from paraffin embedded breast tumor samples previously collected by our laboratory.

Task 2 Month 2-6 Completed in year 1

- Measure TC in paraffin embedded breast tumors samples.

Task 3 Month 6-12 Completed in year 1

- Optimize TRF1 antibody for use in frozen tissue and paraffin embedded breast tumor tissue.

  TRF1 antibody specificity has been demonstrated in breast cancer cell-line MCF-7 and conditions for antigen retrieval and staining have been determined.

Task 4 Month 12-14 **In Progress and Modified**

- Section frozen human breast tumor samples and stain with antibody to TRF1.

  In year 2, the student showed that the immunohistochemical staining using an antibody to TRF1 in breast cancer cell lines, has shown a relationship between cell cycle and intensity of TRF1 staining (Appendix A). During year 3, the candidate developed staining procedures for TRF2 and attempted to develop staining procedures for TIN2, both of which also demonstrated a relationship to Telomere Content and also demonstrate potential as a clinical tool. Staining conditions for TIN2 could not be found however, the staining protocol for TRF2 has been determined and both TRF1 and TRF2 stains are were done in frozen breast tissue. However, immunohistochemical staining of TRF2 also showed a relationship to cell cycle (Appendix A). The relationship between the cell cycle and telomere associated proteins, TRF1 and TRF2, immunohistochemical staining was investigated by the student using flow cytometry (Appendix B). Flow cytometry revealed that the levels of TRF1 and TRF2 are constant throughout the cell cycle however, the staining pattern changes from defuse nuclear staining during most of the cell cycle to bright dots during mitosis. This change is staining pattern precludes the use of TRF1 and TRF2 as a clinical marker. The student as decided to attempt to assess the relationship of TRF1 and TRF2 to telomere content by western blot in frozen breast tissue.

Task 5 Month 13-14 Completed in year 2

- Assess relationship between normalized TRF1 mRNA levels and TRF1 staining intensity.

Student demonstrated that the levels of TRF1 mRNA as measured by quantitative real-time PCR relate to the staining intensity of TRF1 visualized in various breast cancer cell lines by immunohistochemical staining.

Task 6 Months 14-24 **Modified**

- Section paraffin embedded breast tumor tissues and stain with antibody to TRF1.

TRF1 and TRF2 staining has been shown to be dependant on the cell cycle, precluding its use as a clinical immunohistochemical stain. The student has decided to answer the question of the relationship of TRF1 and TRF2 to clinical markers and TC by western blot. Western blot is highly unreliable in paraffin embedded tissue so the student will focus on frozen tissue.

Task 7 Months 12-30 **Initiated and modified**

- Score sections stained with TRF1 antibody and compare to TC data, histological markers and survival data.

Due to the relationship of TRF1 to cell cycle, which precludes the use of TRF1 as a clinical immunohistochemical stain, the student has decided to determine the protein level of TRF1 and TRF2 by western blot. The procedure for the western blots has been troubleshot and blots are currently being performed (Appendix C). The levels of TRF1 and TRF2 will then be compared to TC data, histological markers and survival data as previously intended.

Specific Aim 2: (4 tasks) Completed

Task 1 Month 1-2 Completed in year 1

- Extract RNA from frozen breast tumor samples already collected by our laboratory. Design and order Tankyrase and TIN2 primers and probe.

RNA was extracted from 36 breast tumors. Primers for Tankyrase and TIN2 were designed.

Task 2 Month 2-4 Completed in year 1

- Optimize Tankyrase and TIN2 RT-PCR

TIN2 RT-PCR was optimized, however Tankyrase primers picked up both Tankyrase 1 and the analog Tankyrase 2. The expression levels of these two proteins are quite different and Tankyrase 2 is highly expressed and functionally not associated with telomere management. Assessment of Tankyrase by RT-PCR yielded an inconclusive result in all experiments. RT-PCR experiments to determine Tankyrase mRNA levels have been placed on hold pending new methods to delineate these two analogs at the molecular level. Recent studies have determined the regulation of Telomere length by proteins to involve a number of complexes, which include TRF1 and TIN2, and also include Tankyrase, POT1 and TRF2. As levels of Tankyrase mRNA could not be

determined and POT1 and TRF2 levels may be associated with TRF1 mRNA levels and involved in telomere content determination, RT-PCR reactions were optimized for TRF2 and POT1 as well.

Task 3 Month 4-7 Completed in year 1

- Measure Tankyrase and TIN2 mRNA levels by RT-PCR in RNA extracted from frozen breast cancer samples.

  Tankyrase mRNA levels could not be assessed; however TIN2, POT1 and TRF2 mRNA levels were assessed in 36 frozen breast tumor samples.

Task 4 Month 7-12 Completed in year 1

- Analyze association between Tankyrase and TIN2 mRNA levels with TC and TRF1 mRNA expression.

  Tankyrase mRNA levels could not be assessed so no comparison was possible. TIN2 mRNA levels showed a strong association with TC and TRF1 levels as well as two other telomere binding proteins; POT1 and TRF2.

Specific Aim 3: (6 tasks)

Task 1 Month 12-15 Completed in year 2

- Design and test small interfering RNAs (siRNA) of human Telomerase Reverse Transcriptase (hTERT)

  The candidate designed several siRNAs against hTERT and tested these siRNAs. The siRNAs showed a reduction in hTERT expression by quantitative real time PCR.

Task 2 Month 15-27 **In Progress and Modifed**

- Express siRNA of hTERT in breast cancer cell lines and examine TRF1 mRNA levels and TC by RT-PCR and slot blot over time.

  Student is currently following the slow telomere attrition using a slot-blot method of analyzing telomere content. In an effort to speed the loss of telomere, the student examined the use of drugs that stabilize the G-quadruplex structures that can be created in the telomere region. The use of the drug N,N′-*bis*[2-(1-Piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic Diimide (referred to as PIPER) causes rapid telomere attrition without changing hTERT levels. The student has completed short-term exposures to PIPER and is currently doing long term exposures to PIPER and is examining TRF1 mRNA by RT-PCR and TC by slot blot (Appendix D).

Task 3 Month 15-18 Completed year 2

- Design, generate and test TRF1 expression vector.

Student has designed and generated a TRF1 expression vector, which demonstrates an increase in TRF1 mRNA levels when examined by quantitative real time PCR.

Task 4 Month 18-30 **In Progress and modified**

- Overexpress TRF1 in breast cancer cell lines and examine TC levels by slot blot over time.

Previous experimentation completed in Specific Aim 2 has demonstrated that the level of TRF1 is coordinately regulated with the telomere-associated proteins TIN2 and POT1. Therefore, to examine the effects of changes of TRF1 on TC levels, it is necessary to change all three protein levels at the same time. To do this, the student examined the use of drug treatments to increase these three mRNA levels and has identified a drug, Dexamethasone, which selectively increases TRF1, POT1 and TIN2 without increasing TRF2 or hTERT (Appendix E). Treatment with TNF-alpha is also predicted to specifically increase TRF1, TIN2 and POT1. This student is currently treating cells with these drugs and collecting populations to examine the relationship of TRF1, POT1 and TIN2 to TC levels.

Task 5 Month 30-34 **In Progress**

- Analyze relationship between TRF1 and TC.

Preliminary data is completed and long term data is currently being collected.

Task 6 Months 30-36 **In Progress**

- Prepare and submit manuscripts.

Candidate is currently in the final editing stage of a manuscript based on Specific Aim 2 and has begun a paper based on the early data from Specific Aims 1 and 3.

Education and Training Milestones (6 tasks)

Task 1 Month 1-6 Completed in Year 1

- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste.

Student has examined various types of breast cancer and can recognize features of different tumors and tumor stages.

Task 2 Month 1-36 **Continuing**

- Attend tumor board meetings and monthly Cancer Research and Treatment Center Meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.

Student continues to attend tumor board and the Cancer Research and Treatment Center Meetings on a regular basis and has developed a working relationship with several of the doctors to allow further understanding of current treatments and clinical trials in breast cancer.

Task 3 Month 1-6 **Completed**

- Attend the University of New Mexico School of Medicine medical student training Neoplasia block.

Student attended all the lectures the Genetics and Neoplasia block and also acted as a tutor for the problem based learning sections of the Genetics and Neoplasia block.

Task 4 Month 6-12 **Completed**

- Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste.

Student has learned basic staining procedures and has gained understanding of the commonly used markers to determine treatment in breast cancer.

Task 5 Month 12-24 **Completed**

- Work with oncologists in the University of New Mexico Hospital to gain perspective on breast cancer.

Student has developed a working relationship with several doctors in the Cancer Center and has used these relationships to develop an understanding of patient care and current issues in breast cancer treatment.

Task 6 Months 12-36 **In Progress**

- Present ongoing work at local and national meetings

III. REPORTABLE OUTCOMES

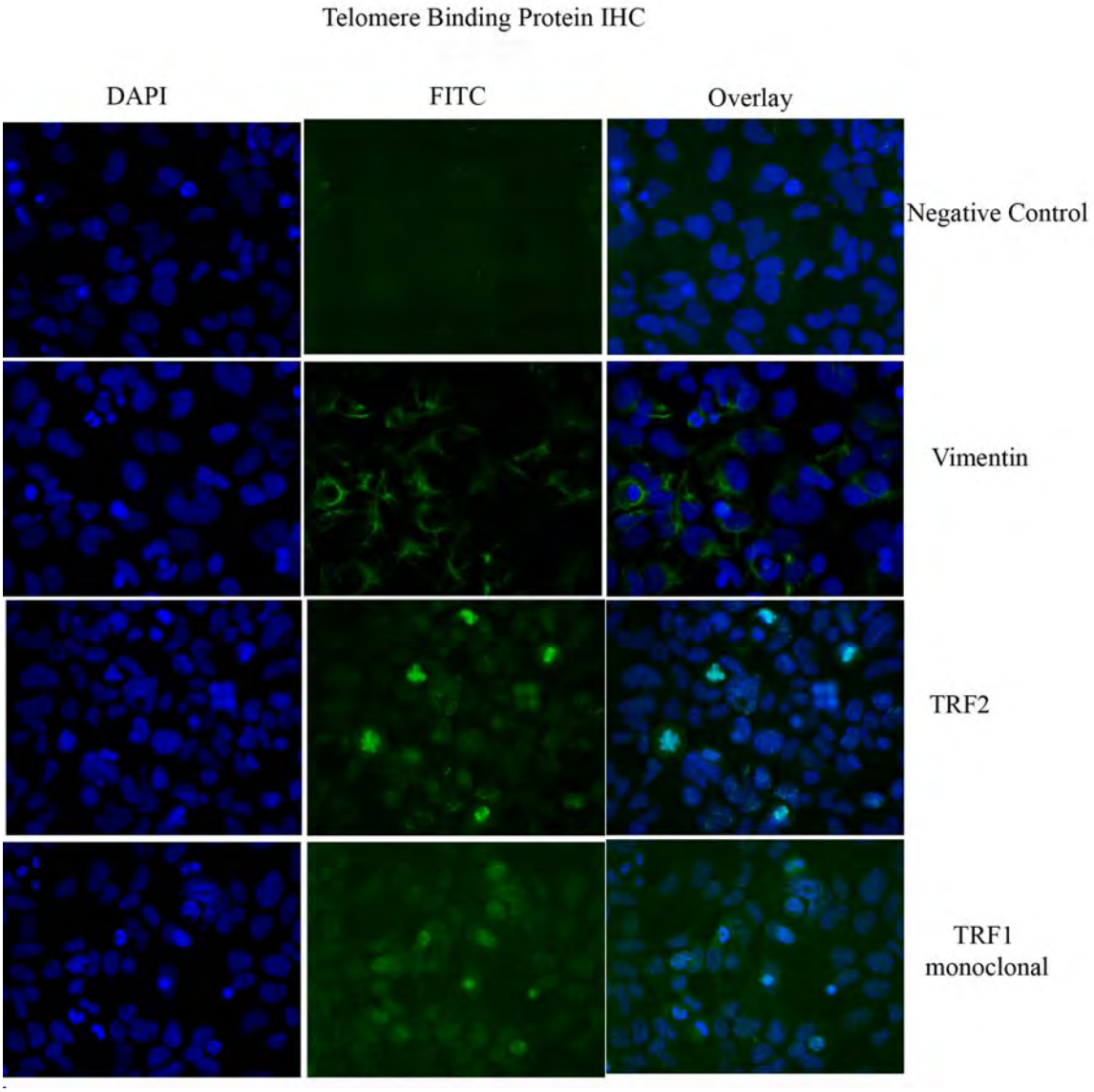
Presentations:

Sevilleta 2006 Department of Biochemistry and Molecular Biology Retreat, Invited Graduate Speaker. Sevilleta Wildlife Refuge, April, 23 2006. "Levels of Telomere-Associated Protein mRNAs Suggest Coordinate Regulation of Their Transcription." (Appendix F)

IV. CONCLUSIONS

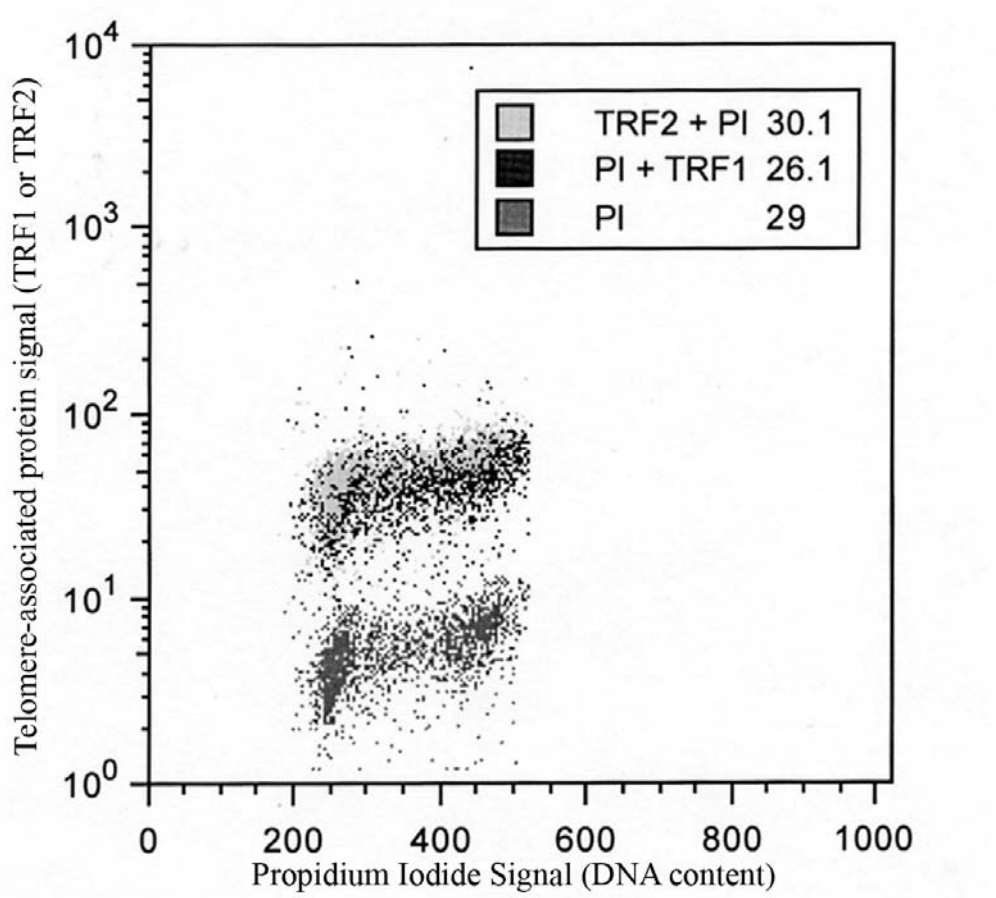
To date, all tasks; as outlined in the Statement of Work are on schedule. Two of the tasks in Specific Aim #1 were delayed due to a technical issue, however this issue has been overcome and data collection is in progress and expected to be completed by the end of the no cost extension. Specific Aim #2 has been completed and a manuscript based on the results from this body of work is nearing completion. The tasks for Specific Aim #1 and 3 have all been initiated and expected to be completed by the end of the no cost extension. The PhD candidate has completed her educational goals.

Appendix A



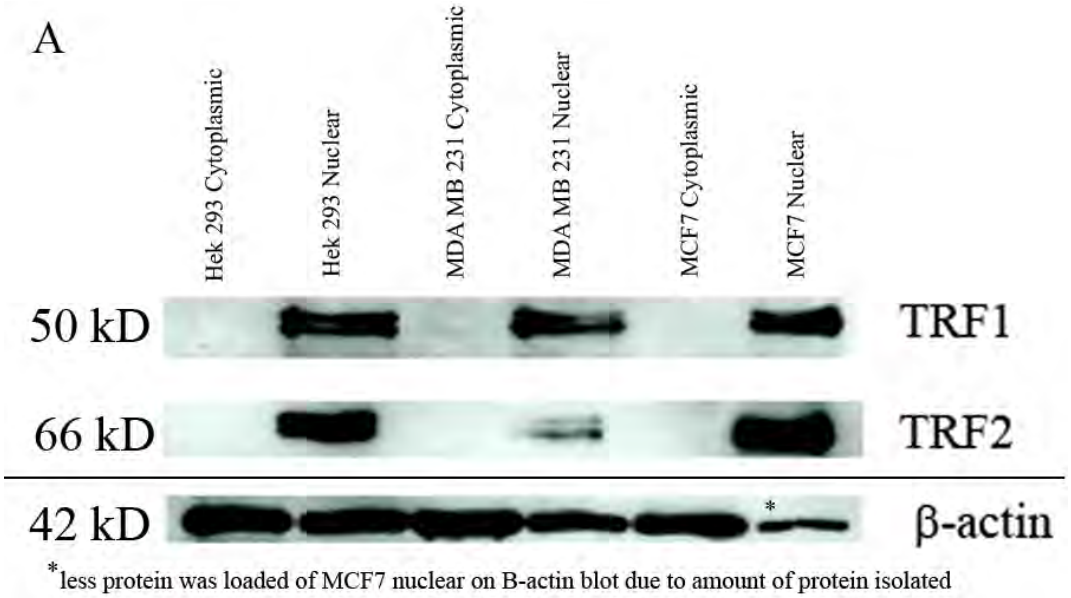
Immunohistochemical staining of TRF1 and TRF2 labeled with FITC and counter stained with DAPI. Vimentin is a cell surface marker. The brighter cells found in both the TRF1 and TRF2 fields are undergoing mitosis. This demonstrates the issue of using TRF1 or TRF2 as a clinical stain.

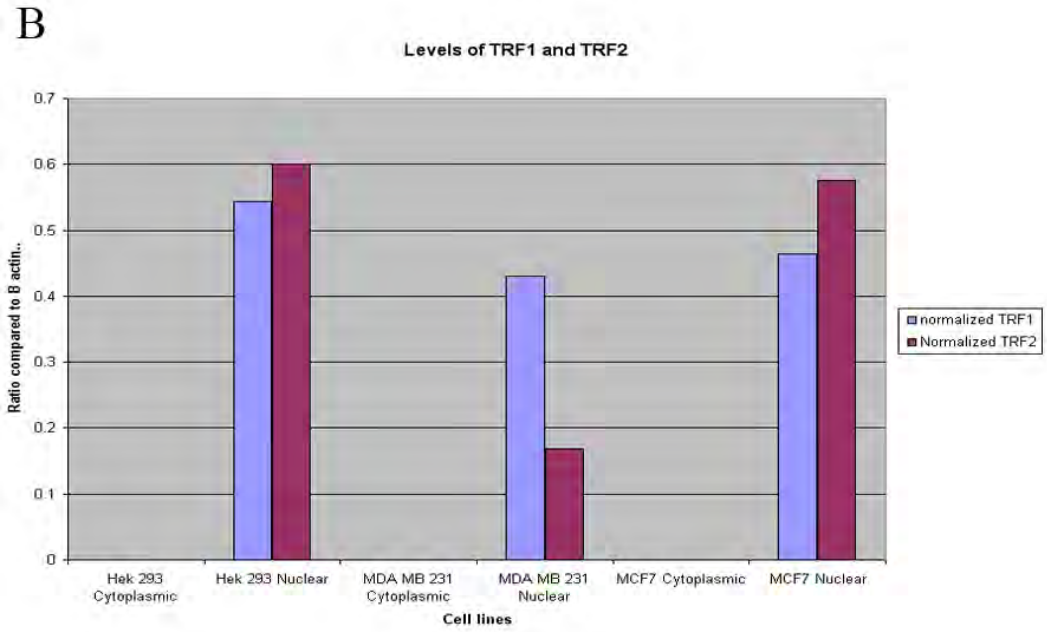
Appendix B



Comparison of Levels of TRF1 and TRF2 proteins to DNA content and Cell cycle. DNA is stained with propidium iodide. TRF1 and TRF2 proteins are stained with antibodies to TRF1 and TRF2 and secondarily stained with FITC. As the cells progress through the cell cycle, the DNA content increases and consequently the propidium iodide signal increases. The levels of TRF1 and TRF2 remain constant regardless of the point in the cell cycle or DNA content.

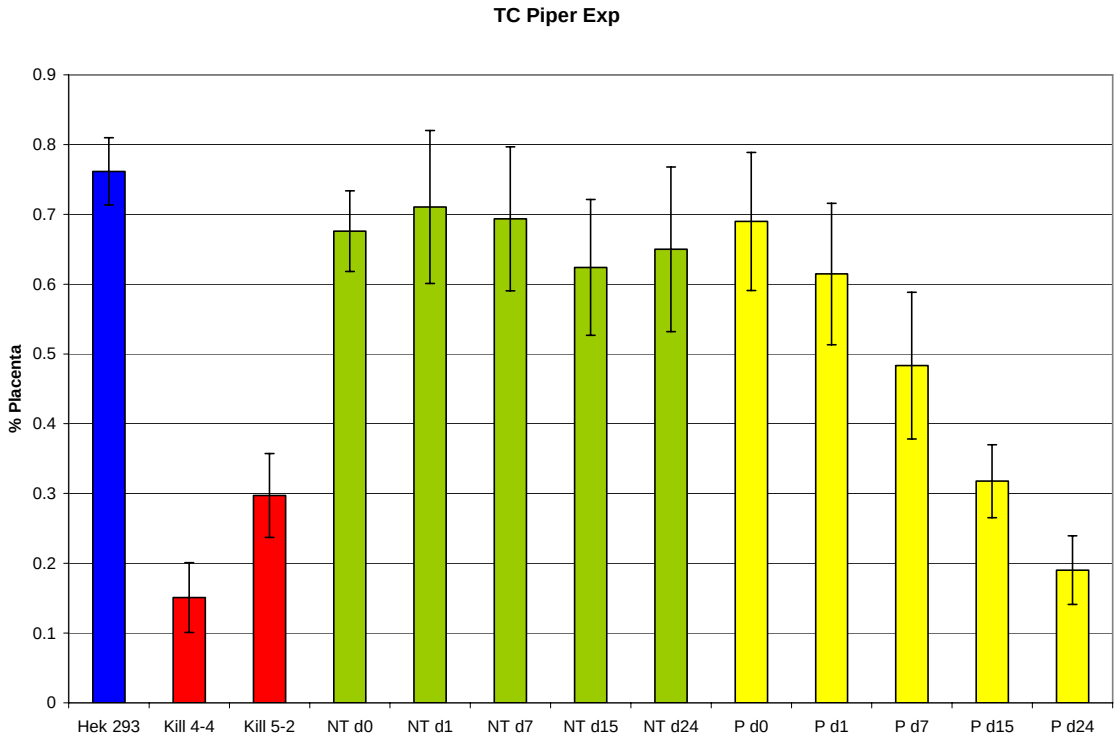
Appendix C





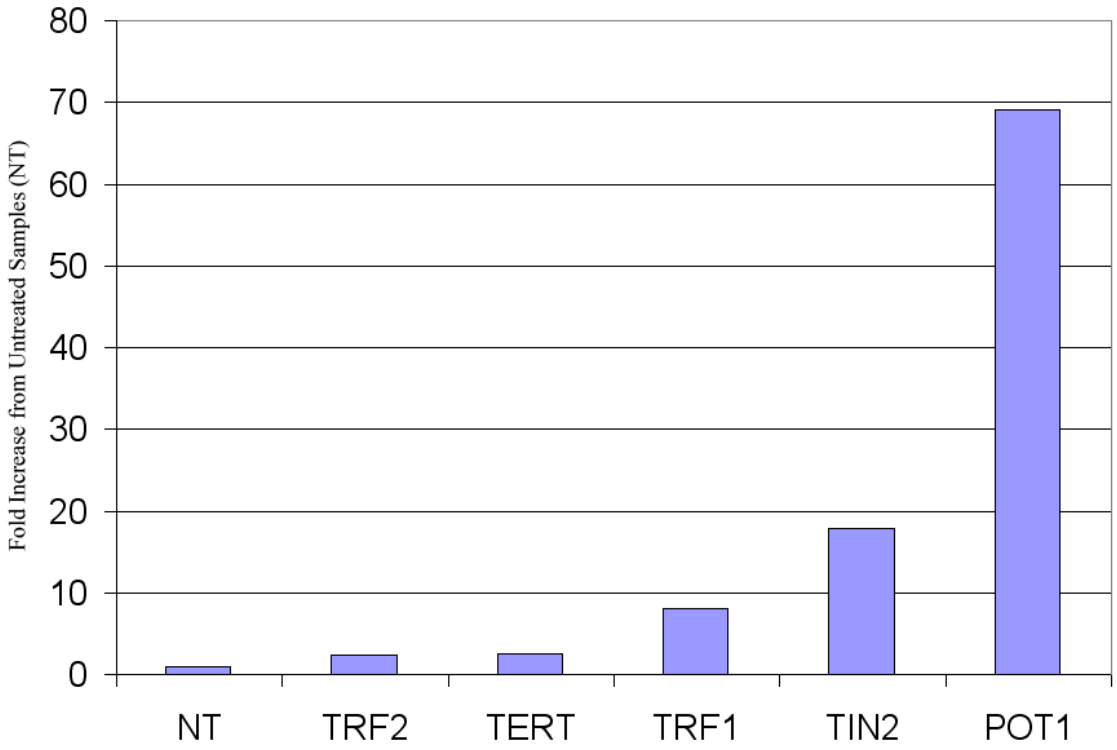
TRF1 and TRF2 levels in cell lines by Western Blot. A. Western Blot of TRF1 and TRF2 in MDA MB 231, MCF 7 and Hek 293 nuclear and cytoplasmic extracts. Beta-actin is used as a loading control. **B.** Analysis of densitometry of western blot from A. TRF1 and TRF2 were normalized to beta-actin levels. TRF1 and TRF2 antibodies are specific and only show proteins localized to the nucleus.

Appendix D



Treatment of Hek 293 cells with PIPER. Hek 293 cells were treated for 3 weeks with Piper. The blue column represents untreated Hek 293 cells prior to plating. The green columns are the non-treated controls cultured at the same time as the PIPER treated cells. The green columns represent day 0 (d0) to day 24 (d24), showing very little change in Telomere Content (shown as a percent of placenta control) over 24 days. The yellow columns show the same collection days but in PIPER treated samples. The PIPER treated samples show a steady decrease in telomere content over the 24 days of treatment. The red columns show two cell populations which died during PIPER treatment and show significant decrease in telomere content.

Appendix E



Expression of Telomere Associated Protein mRNAs when cells are treated with Dexamethasone. Cells were treated for 14 hours with Dexamethasone and then collect. The mRNA levels of TRF1, TRF2, TIN2, POT1 and TERT were compared to untreated controls. The untreated controls were set to 1 and the relative fold increase in each mRNA was calculated. Dexamethasone treatment selectively increased TRF1, TIN2 and POT1 mRNA levels.

Appendix F

Levels of Telomere-Associated Protein mRNAs Suggest Coordinate Regulation of Their Transcription

Telomeres are specialized protein-nucleic acid structures that stabilize the ends of chromosomes and prevent end-to-end fusions. Multiple proteins are necessary for proper telomere function and maintenance of telomeres in human cells, including telomere repeat binding factor 1 and 2 (TRF1, TRF2). The interaction between TRF1 and the telomere is modulated by its protein-binding partners, one of which is TRF1 interacting nuclear factor 2 (TIN2). The TRF1 complex promotes the binding of another telomere binding protein, protection of telomeres 1 (POT1), which prevents binding of telomerase, the telomere-elongating enzyme, to the telomere. A model of telomere length control has emerged, from previous studies in human cell lines, in which telomere-associated proteins act through two separate mechanisms, which are moderated by TRF1 and TRF2, to control telomere length. Based on this complex model, we hypothesize the existence of a coordinate regulation of transcription of the telomere-associated proteins.

Preliminary examination of the promoter regions of TRF1, TRF2, TIN2, POT1 and the protein component of telomerase (TERT) for similar transcription factor binding sites was performed using TF Site Scan, a computational program for the identification of transcription factor (TF) binding sites. Three transcription factors, NFAT5, GR and C/EBP, have potential binding sites in the promoters of TRF1, TIN2 and POT1 that are not present in the promoters of TERT or TRF2. Interestingly, each of these transcription factors has been previously linked to breast cancer progression or apoptosis. To further examine the relationships between the mRNA levels of the telomere-associated proteins, the relative mRNA levels of TRF1, TRF2, TIN2, POT1 and TERT was determined in 36 human breast tumors using quantitative real-time RT-PCR. No association was found between the mRNA levels of TRF2 and TERT with any of the other telomere-associated proteins. Linear regression revealed significant, positive, linear associations between the mRNA levels of TRF1 and TIN2 and POT1 ($p=0.014$ and 0.003 respectively). A significant, positive, linear association was also found between TIN2 and POT1 ($p=0.035$). The associations found between TRF1, TIN2 and POT1 were predicted by TF Site Scan and suggest the presence of coordinate regulation of some of the telomere-associated proteins.