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TITLE: Prognostic Value of Telomere DNA Content in Ductal Carcinoma *in Situ*

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14. ABSTRACT
Critically shortened telomeres cause genomic instability both in vivo and in vitro and thus drives changes in gene expression. Reduced telomere DNA content (TC) is associated with reduced survival in breast and prostate cancers. We hypothesized that TC could be a unique prognostic marker in DCIS. The goals of this project were: 1) determine if TC can be used to predict outcome in a retrospective study of DCIS, 2) determine if alterations in telomere homeostasis can induce changes in gene expression that could be the basis of novel prognostic markers. TC has been determined in microdissected tissues enriched for DCIS or histologically normal adjacent tissues from a cohort of non-recurrent DCIS. Efforts to obtain a recurrent DCIS samples are ongoing. A model system has been developed to examine gene expression changes in response to alterations in telomere homeostasis. Telomere binding proteins TRF2, a dominant negative allele of TRF2, and hTERT were expressed in primary human mammary epithelial cell populations from four donors. Microarrays were performed on these cell populations. Over-expression of TRF2wt and to a lesser extent, TRF2dn induces the expression of Cox 2 mRNA ($p=0.05$) and protein in an activin A-dependent manner. Activin A, a member of the TGF-β superfamily, induces the phosphorylation and activiation of both p38 MAPK and Smad2/3. Both these proteins can affect the level of Cox 2 mRNA. Exogenous activin A results in an increase in activated p38 and Cox 2. Treatment of cells with the p38 inhibitor SB203580 leads to a decrease in Cox 2 protein. In vivo, high Cox 2 expression is associated with reduced TC in DCIS ($p>0.0009$).

15. SUBJECT TERMS
Telomere, Breast Cancer, hTERT, TRF 2

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Statement of Work:

Task 1. **Perform a retrospective investigation of the relationship between TC and outcome in DCIS.**

a. *(Mos 1-3)* Identify 100 women for whom archival tissue is available utilizing the Bay Area Breast SPORE DCIS Study Registry.
b. *(Mos 2-30)* Recover 60 archival DCIS and DCIS-adjacent normal tissue, histologically confirm tissues and measure telomere DNA content with the slot blot assay.
c. *(Mos 24-30)* Repeat ambiguous experiments
d. *(Mos 30-34)* Perform statistical analysis of the data using two-sample non-parametric Wilcoxon test and Kaplan-Meier analysis
e. *(Mos 34-36)* Write manuscript describing the results of the study

Task 2. **Perform a prospective investigation of the relationship between TC and gene expression.**

a. *(Mos 2-24)* Isolate human mammary epithelial cells (HMEC) from reduction mamoplasty specimens and propagate in culture.
b. *(Mos 2-24)* Infect HMEC with hTERT, TRF2, and dominant negative allele of TRF2
c. *(Mos4-24)* Extract DNA and measure telomere DNA content with the slot blot assay.
d. *(Mos 2-24)* Extract RNA and perform microarray analysis
e. *(Mos 2-24)* Perform analysis of microarray data utilizing the Cluster and TreeView programs coupled to Graphical Gaussian Models.
f. *(Mos 24-34)* Evaluate the expression of candidate genes in DCIS and adjacent normal breast tissue using immunohistochemisty.
g. *(Mos 34-36)* Write manuscript describing the results of the study.

INTRODUCTION: The widespread use of mammography lead to a 500% increase in the diagnosis of ductal carcinoma *in situ* (DCIS) from 1983 to 1992 (*1*). However, only a fraction of these lesions progress to invasive cancer making it important to identify tumor markers that discriminate high-risk patients, whose disease is likely to progress, from low risk patients, who can be successfully treated with less invasive modalities. It is known that critically shortened telomeres give rise to genomic instability both *in vivo* and *in vitro,* (*2-5*) thereby driving alterations in gene expression. We have recently shown that reduced telomere DNA content (TC) was associated with decreased survival in prostate (*6*) and breast cancers (*7*). Remarkably, reduced TC was also associated with survival in tumor adjacent histologically normal breast tissue (*7*). Collectively these findings demonstrate that alterations in TC may occur early in the neoplastic process and are likely to impact clinical outcome in cancer. Thus we hypothesized that TC will be a unique and highly informative prognostic marker in DCIS. The purpose of the proposed project is twofold. First, determine if TC can be used to predict clinical outcome in a retrospective study of DCIS. Second, determine if loss of telomeric DNA can induce specific changes in gene expression. Genes whose expression is consistently altered have the potential to be novel prognostic markers.

PROGRESS RELATIVE TO THE STATEMENT OF WORK:

TASK ONE: Perform a retrospective investigation of the relationship between TC and outcome in DCIS.

A study population of 60 women diagnosed with ductal carcinoma *in situ* was identified in cooperation with the UCSF Cancer Center Tissue Core. The study population was selected such that half the women developed recurrent DCIS or progressed to invasive breast cancer within 10 years of their original diagnosis. Since tissues housed at the UCSF Cancer Center Tissue Core are collected from multiple sites, it was not possible to obtain a separate block of genetically matched histologically

normal breast tissue for each donor. Instead, a pathologist used an H&E reference slides from each block to define regions of DCIS and normal tissues within each block. Tissues were microdissected using Leica LCM microscope to produce populations enriched for either DCIS or histologically normal cell types. DNA was purified from the microdissected tissues and telomere content was determined using the slot blot assay as previously described (*8*). Since the DCIS lesions are typically small (less than 5 mm) it was not always possible to obtain sufficient quantities of DNA for the analysis.

Non-recurrent cases were analyzed first. Following analysis of the non-recurrent group of tissue, the UCSF Tissue Core determined that the amount of tissue required for this study (125um) was too great, and requests for recurrent tissues were denied. We tried to decrease the amount of tissue required by using *in situ* hybridization techniques to measure telomeres in a single 5um tissue section. Although preliminary data suggested that this method was reliable, subsequent experiments showed that the assay lacked reproducibility. For example, telomere content measured on serial sections of the same lobule was statistically different. It is likely that confounding factors related to specimen age and fixation caused these inconsistencies. We are attempting to obtain another cohort of specimens in order to address the relationship between TC and outcome in DCIS.

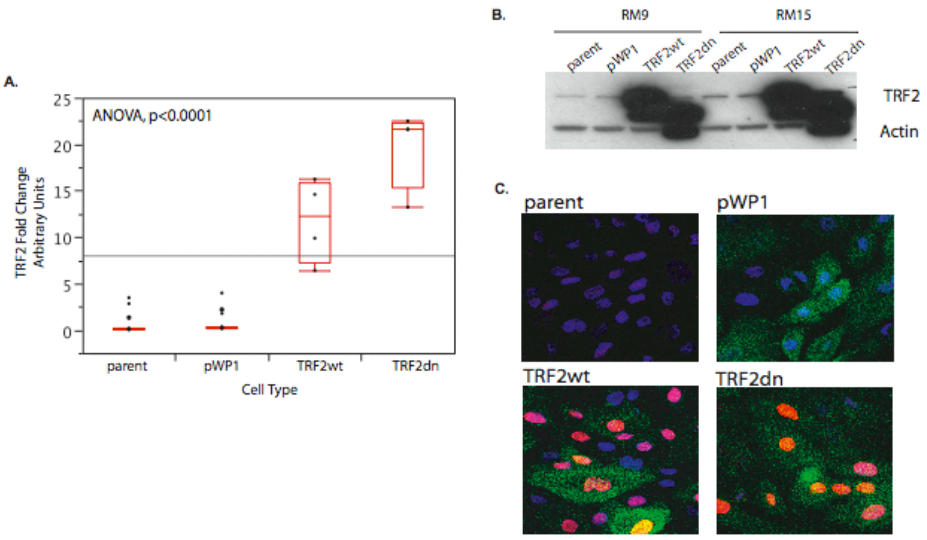
In total, TC was measured in DNA isolated from 23 individuals with non-recurrent DCIS. There was no association between TC and a variety of clinical and tumor variables including patients' age at surgery, ethnicity or menopausal status nor with tumor size or grade. However, as described subsequently, there was a statistically significant association between TC in the DCIS lesion and Cox 2 expression as measured by immunohistochemistry (figure 5).

TASK TWO: Perform a prospective investigation of the relationship between TC and gene expression.

In order to assess gene expression changes related to telomere status an *in vitro* model system has been developed. Human mammary epithelial cells (HMEC) were isolated from reduction mammoplasty specimens. As previously reported, HMEC grow for approximately 20 to 30 population doublings in culture before growth arrest. A subpopulation of HMEC, termed variants or vHMEC, is able to silence p16 through promoter hypermethylation and continue to proliferate an additional 20 to 50 population doublings until reaching a second growth arrest termed agonescence (*9*). In addition to loss of p16 and extended proliferation, vHMEC acquire the types of genomic instability that are consistent with malfunctioning telomeres; phenotypes that have been associated with early events in breast cancer. In fact, vHMEC have been found *in vivo* in histologically normal breast epithelia from disease-free women and within and surrounding DCIS (*10*).

Telomere integrity can be modulated by the telomere binding proteins hTERT and TRF2. hTERT, the catalytic subunit of telomerase, lengthens human telomeres and is up-regulated in cancer. TRF2 functions to stabilize the telomere superstructure and acts as a scaffold protein for a number of other critical telomere proteins. Loss of TRF2 function through the exogenous expression of a dominant negative allele of TRF2 (TRF2dn) induces a telomere-specific DNA damage response. vHMEC derived from four donors have been engineered to express each of these telomere binding proteins or vector controls. Each of the cell lines generated from the four donors have been characterized and shown to express the gene and protein of interest (figure 1). DNA and RNA have been purified from these cell lines and telomere content has been determined. Gene expression changes have been evaluated in a non-biased fashion using microarrays.

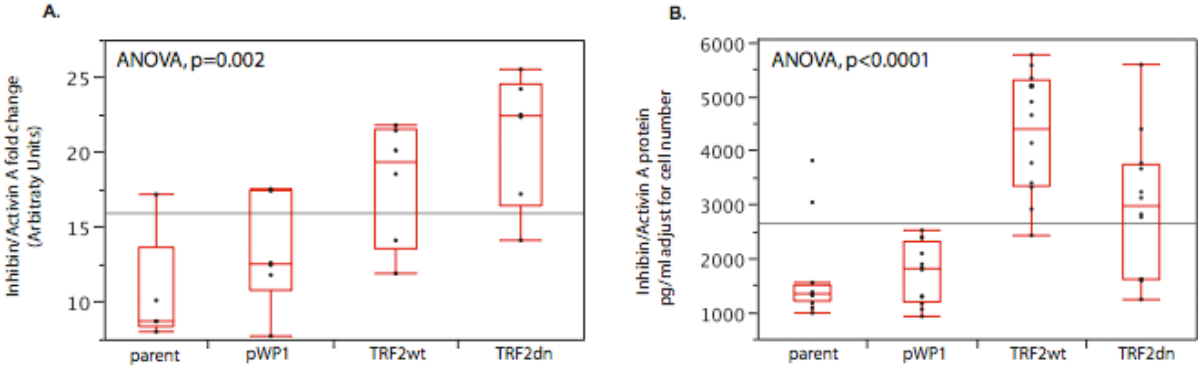
Figure 1: Ectopic Expression of TRF2 in Human Mammary Epithelial Cells



(A) Box plot of TRF2 gene expression in each of the treatment groups was determined with Q-PCR and is shown relative to Gus B levels. P value was calculated with ANOVA. (B) Representative immunoblot of TRF2 and actin (loading control) from two donors. At high levels, TRF2 appears as a doublet. (C) Immunocytochemistry for TRF2. Nuclei are stained with DAPI (blue) cells infected with lenti virus express GFP (green) and TRF2 (red).

There were few statistically significant changes in gene expression in the samples. Each of the treatment groups was more similar to its donor than each other; IE parent, vector, hTERT, TRF2 wt and TRF2dn from donor 15 were more similar to each other than parent vector, hTERT, TRF2wt and TRF2dn from donor 16. However, in total 34 genes' expression was statistically altered in cells expressing TRF2wt, TRF2dn or hTERT when compared to vector and parental controls. Preliminary data suggests that components of the TGF-β pathway (thrombospondin 1, inhibin/activin A, LTBP2) are differentially regulated in the hTERT, TRF2 wt and TRF2 dn expressing cell lines. At present studies have focused on two genes, inhibin/activin A and Cox 2 in vHMEC over-expressing either TRF2wt or TRF2dn. The inhibin/activin A gene encodes a protein product that can be part of a heterodimer to produce inhibin A or a homodimer to produce activn A. As previously stated activin A and its antagonist inhibin A are part of the TGF-β superfamily and play critical roles in cell proliferation, differentiation and apoptosis (*11-13*). Q-PCR was used to validate the microarray data and shows that the inhibin/activin A gene is statistically up-regulated in vHMEC expressing TRF2wt or TRF2dn (ANOVA, p=0.002). Since this gene can produce two different proteins with opposing functions, the levels of inhibin α (the second component of the inhibin A heterodimer) were also examined by Q-PCR. There is no change in the level of inhibin α in cells over-expressing the TRF2wt or TRF2dn proteins. Thus, it is likely that the protein product of this gene is the homodimer of the inhibin/activin A gene, activin A. Activin A levels in parent, vector, TRF2wt and TRF2dn cells from all four donors were measured using ELISA. There was a statistically significant increase in the level of secreted activin A in vHMEC over-expressing TRF2wt and to a lesser degree in vHMEC over-expressing TRF2dn (ANOVA, p<0.0001, figure 2).

Figure 2: TRF2 Expression is Associated with an Increase in Activin A mRNA and Protein.

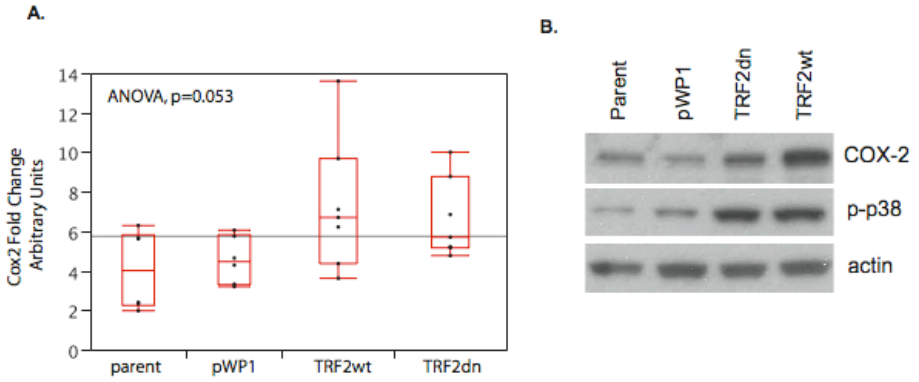


(A) Box plot showing Inhibin/activin A mRNA levels measured with Q-PCR. The fold change of the inhibin/activinA gene relative to the standard, GusB, in each of the treatment groups from all four donors is shown. (B) Box plot showing Activin A protein level from triplicate samples measured using ELISA and expressed as pg/ml adjusted for cell number. The change in activin A protein in conditioned media from each of the four treatment groups is shown in panel B. P values were calculated using ANOVA and are shown in each panel.

Like other members of the TGF-β superfamily, binding of activin A to its receptor leads to receptor activation through autophosphorylation. The receptor then phopshorylates the smad2/3 complex which is then translocated into the nucleus, once in the nucleus smad2/3 acts as transcriptional regulator of a number of genes, including endoperoxide H synthase or cyclooxygenase 2 (Cox 2). Activin signaling also results in the phosphorylation of p38 MAPK (*11, 12, 14*). Phosphorylated p38 stabilizes the Cox 2 transcript, thereby increasing the level of the Cox 2 protein in vHMEC (*15*).

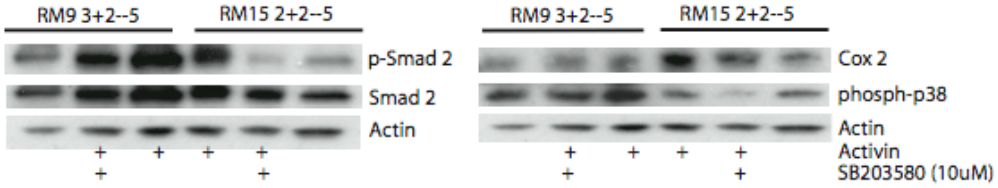
Microarray data demonstrated that Cox 2 mRNA expression is up-regulated in vHMEC expressing TRF2wt and to a lesser degree in TRF2dn. Cox 2 induction and the resulting PGE2 production have been shown to regulate many of the phenotypes that contribute to tumor initiation and malignant progression such as proliferation, apoptosis and invasion in epithelial cells and endothelial cell migration and angiogenesis and host immune evasion (*16, 17*). In breast epithelium, Cox 2 expression may be an early event in the carcinogenic process, with elevated expression common in invasive lesions and in an even greater fraction of DCIS (*10*). The levels of Cox 2 mRNA in parent, vector, TRF2wt and TRF2dn cells was validated using Q-PCR and is statistically up-regulated in vHMEC expressing TRF2 wt (ANOVA, p-0.053). Cox 2 protein level was accessed with immunoblotting in the same cell populations and is also up regulated (figure 3).

Figure 3: TRF2wt and TRF2dn Over-expression is Associated with an Increase in Cox 2 mRNA and Protein



(A) Box plot showing Cox 2 mRNA expression as measured with Q-PCR. The fold change of Cox 2 expression relative to standard, Gus B, is shown for all four donors. P value was calculated using ANOVA. (B) A representative Immunoblot for Cox 2, phospho p38 and actin (loading control) protein is shown.

Figure 4: Exogenous Activin Induces Cox 2 in Human Mammary Epithelial Cells in a Phosphop-38 Dependent Manner. vHMEC derived from two donors were treated with either activin A (0.01ug/ml) alone or in combination with the phospho-p38 inhibitor SB203580. Immunoblotting was preformed to access the levels of phospho-smad2, total smad 2, phospho-p38, Cox 2 or actin (loading control).



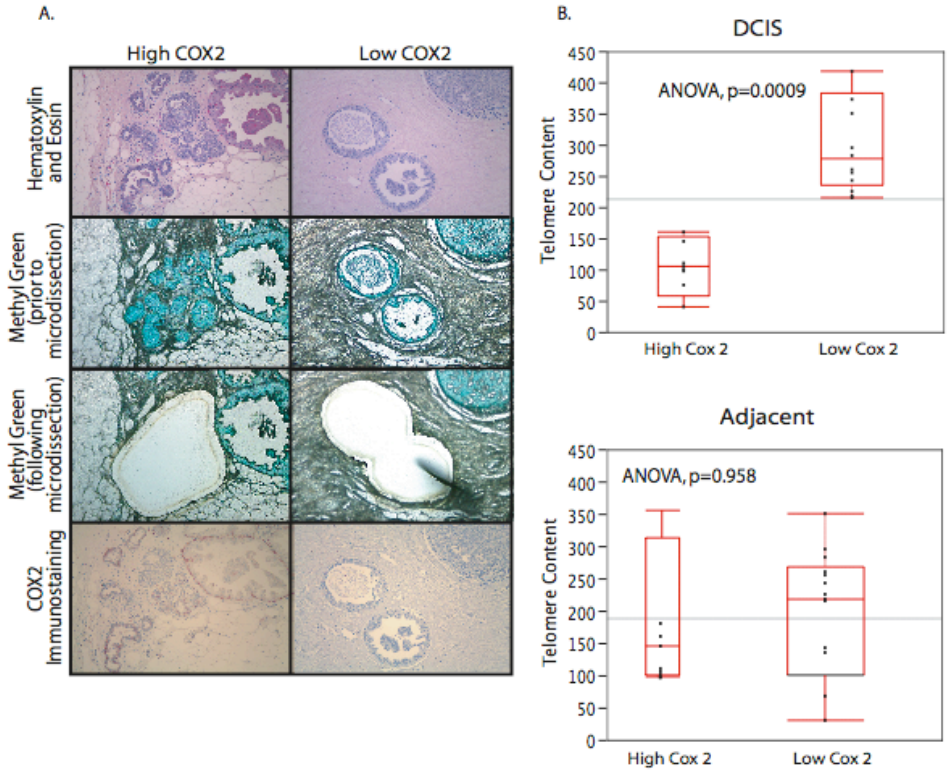
Treatment of unifected vHMEC cells with exogenous activin leads to the phosphorylation of p38 and a phospho-p38 dependent increase in Cox 2 protein, suggesting that the induction of Cox 2 expression observed in the cells over-expressing TRF2wt and TRF2dn is likely mediated by activin and phospho-p38 (figure 4). Further experiments are underway to determine if this supposition is indeed correct. Additionally, Cox 2 dependent phenotypes: levels of PGE2, apoptotic resistance and motility will be evaluated in these cell populations.

Since *in vitro* experiments suggests that TRF2 can modulate Cox 2 expression, we examined the relationship between Cox 2 expression and TC in the non-recurrent DCIS cohort described in specific aim one. TC was measured, as described (*8*) in DNA purified from cell populations enriched for

either DCIS or adjacent tissue using microdissection. Cox 2 expression was determined using immunohistochemistry. Briefly, a 5μm section, immediately adjacent to microdissected tissue sections, was utilized for Cox 2 IHC. Antigen retrieval was performed with EDTA-microwaving and the antibody/antigen complexes were visualized using the vector elite staining kit and AEC chromagen. There was a striking association between telomere content and Cox 2 expression in the DCIS lesions (p=0.0009). In contrast, Cox 2 expression and telomere content were not associated in adjacent histologically normal tissues from the same donors (p=0.985). There was no association with Cox 2 and any other clinical parameter including: patients' age at surgery, ethnicity or menopausal status nor with tumor size or grade.

Figure 5: Telomere Content is Associated with Cox 2 Expression in DCIS

(A) Tissue sections were stained with H&E and used as a reference for microdissection. Serial sections were used for microdissesction or IHC for Cox2 protein as described above. An example of tissues expressing high and low Cox 2 are shown. (B) Box plots showing the relationship between telomere content (y-axis) and Cox 2 staining intensity (x-axis) is DCIS or the adjacent normal tissues. Cox 2 expression was scored in a blinded fashion. High Cox 2 expression was defined as intense staining in at least 60% of the epithelial cells. Low Cox 2 expression was intense staining in zero to 59% of epithelial cells. P values were calculated using ANOVA and are shown in each box plot.



KEY RESEARCH ACCOMPLISHMENTS:

- Activin A mRNA and protein are up-regulated in vHMEC from all four donors that over-express TRF2 wt and to a lesser degree TRF2 dn.
- Cox 2 mRNA and protein are up-regulated in vHMEC from all four donors that over express TRF2 wt and TRF2 dn.
- Exogenous activin A can induce phospho-Smad 2, phospho-p38 and Cox 2 in vHMEC.
- Treatment of vHMEC over-expressing TRF2wt with the phospho-p38 inhibitor SB203580 results in a reduction of Cox 2 protein.
- Telomere content correlates with Cox 2 expression in DCIS, but not adjacent normal tissue.

REPORTABLE OUTCOMES:

- Manuscript: "TRF2 Overexpression Induces Cox 2 in Breast Epithelial Cells *In Vivo* and *In Vitro*" in preparation
- Abstratct: "Telomere Content in DCIS and Hitologically Normal Adjacent Cells" Era of Hope, Philadelphia, June 2005
- Primary vHMEC lines over-expressing TRF2wt, TRF2dn and hTERT from four different donors have been generated
- Dr. Colleen Fordyce has been supported by this training grant. She has gained expertise in confocal and laser-capture microscopy, culture and infection of primary human cells, breast pathology and histology, microarray hybridization and analysis and quantitative PCR.

CONCLUSIONS: At present there is no reliable method to determine which DCIS lesions will remain indolent and which will progress to invasive breast cancer (*1, 18, 19*). Effective identification of patients with aggressive DCIS lesions would make it possible to match treatment modalities to each patient's disease, thus eliminating unnecessary treatment-related side effects and minimizing treatment-related expense. Telomere content (TC) has potential as a novel prognostic marker in breast cancer and likewise, may predict outcome in DCIS. Here we have examined the relationship between TC in non-recurrent DCIS and adjacent histologically normal tissue. At this time we have not been able to obtain recurrent DCIS specimens that would allow us to address the hypothesis that TC can be used to differentiate recurrent and non-recurrent DCIS at the time of diagnosis. However, we hope to be able to complete this objective and are trying to obtain suitable tissues from sources outside of UCSF.

We have also developed a model system to assess the effects of changes in telomere stability on gene expression. Preliminary data suggests that members of the TGF-β superfamily are differentially regulated in human mammary epithelial cells exogenously expressing hTERT, TRF2 wt or TRF2 dn. To date, experiments have focused on examining the relationship between exogenous expression of TRF2 wt and TRF2 dn, activin A and Cox 2. Preliminary data suggests that TRF2 over-expression results in the induction of the Cox 2 protein is activin A and phosoph-p38 dependent fashion. Experiments are underway to confirm or refute these preliminary data. Likewise, investigation of the role of hTERT in this pathway is ongoing. It is anticipated that these data will be the basis of a manuscript that will be submitted in the coming months.

It has been shown that TC has prognostic potential in breast and prostate cancer. Understanding the cellular consequences of alterations in telomere homeostasis may provide novel therapeutic targets and prognostic markers.

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