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14. ABSTRACT
Telomerase activity is associated with over 90% of human breast cancers and is necessary for continued tumor cell growth, making it an ideal target for inhibition therapy. However, pharmacologic inhibitors of telomerase have not been as effective as expected. As such, our objective here is to identify novel telomerase interacting proteins and define their functional relationship to telomerase in order to provide additional targets for telomerase inhibition in breast cancer. In addition to the results that we reported in the previous annual report, we have found that molecular chaperones specifically associate with telomeres and that the interaction is likely through telomere binding proteins, as well as telomerase. We have confirmed that hsp90 associates with the TRF-2 telomere binding protein and are currently defining the functional significance of this interaction. In addition, we are screening for association of the hsp90/p23 complex with other telomere proteins, some of which may be specific for DNA repair. We are continuing to test additional telomerase-specific antibodies for discovery of novel telomerase interacting proteins, with the hope that biologically relevant proteins will be identified to provide additional targets for directed telomerase inhibition as a means to treat breast cancer.

15. SUBJECT TERMS
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Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human breast cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (**Weinrich et al 1997**). Telomerase is an obvious chemotherapeutic target (Shay and Bacchetti, 1997). Telomerase activity requires its two core components, hTERT and hTR, to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (Holt et. al., 1999). We have previously demonstrated that chaperones are essential for optimal telomerase assembly *in vitro* (**Holt et. al., 1999**) and that Hsp90 itself remains associated with the functional telomerase complex (**Forsythe et. al., 2001**) (see Figure 1).

In a human cancer progression model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with cancer progression, which is defined as increased aggressiveness *in vivo* (**Akalin et. al., 2001**). These findings indicate that increased expression of the Hsp90 chaperone complex with the associated activation of telomerase activity may be important steps in cancer formation (**Holt et. al., 1999; Akalin et. al., 2001**). While telomerase in cancer progression has been widely studied (reviewed by Shay and Bacchetti, 1997), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

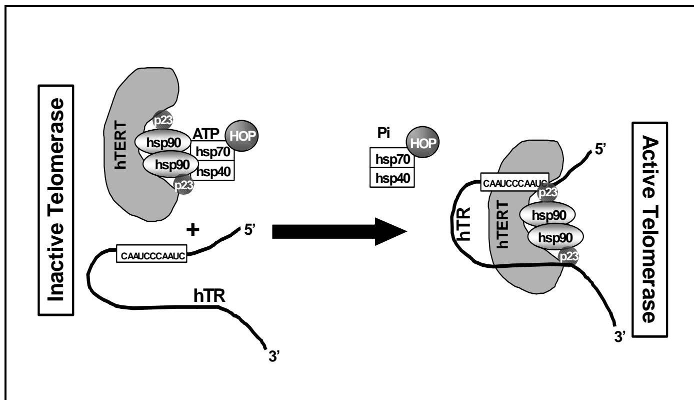

Figure 1. The hsp90 complex is required for assembly of active telomerase. Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]

Body

Rationale: The overall goal of this proposal is to identify mammary-specific telomerase interacting proteins that will serve as targets for direct and indirect inhibition of telomerase and determine the consequences of these interactions during breast cancer progression. Our proposed proteomic experimentation represents an innovative approach toward the discovery of proteins associated with telomerase, and identification of proteins using mass spectrometry will be validated by standard molecular and cellular techniques to assess the functional and biological significance of the interaction. The proposed experiments will allow further understanding and characterization of the mechanisms of telomerase/telomere structure and function as it relates specifically to breast cancer and will facilitate innovative techniques and protocols for early detection and treatment of breast cancer.

***Objective #1*: Define the regulation of telomerase by the identification of mammary-specific telomerase interacting proteins using a proteomic approach.**

The initial experiments in the first annual report were designed to familiarize ourselves with immunoprecipitating human telomerase using a variety of antibodies, most of which have proven to be useful for precipitating telomerase activity but not for detectable hTERT protein or chaperones (data not shown). We originally settled on the 2C4 monoclonal antibody (Novus Biologicals) (Masutomi et al. 2003), which has been shown to effectively detection hTERT by Western and immunohistochemistry, but have moved to a Rockland hTERT antibody, which has been shown to be more effective for immunoprecipitation and does not cross-react with nucleolin as is the case for the Novus IgM hTERT antibody (Wu et al., 2006). Clearly, this has set our work back a few months, if not longer, in that we were mostly detecting nucleolin rather than hTERT in our immunoprecipitation assays. During this time, we were also assessing proteins that were bound to hsp90 in breast tumor cells as an alternative to finding telomerase interactors specific for mammary-derived cells.

One of the clues that originally suggested that hsp90 may have other, telomere-specific binding partners came from the data in Figure 2, where we show that hsp90 and p23 are associated with the telomere using a ChIP assay, and that this link may be independent of their function in assembling telomerase. We have previously shown that hsp90 and p23 remain functionally associated with telomerase after assembly of the active enzyme (**Forsythe et al., 2001**). However, we have been unable to show telomerase is associated with telomeres using the ChIP assay, no matter the specific hTERT antibody used (Figure 2). Our data indicates that chaperones are found at the telomerase and may have binding partners unrelated to telomerase.

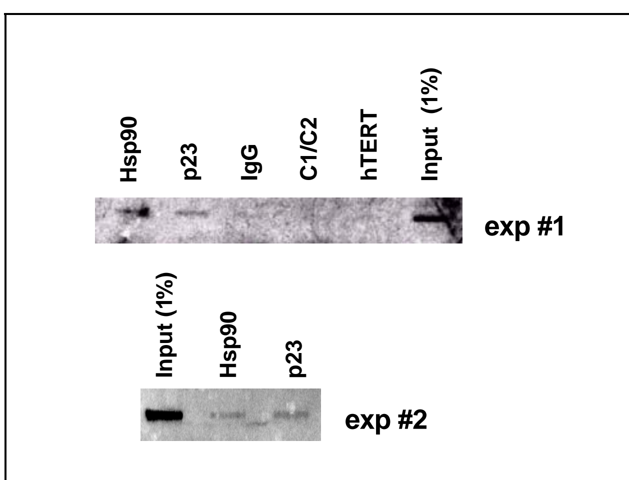

Figure 2: Molecular chaperones interact with telomeres. Chromatin Immunoprecipitation assays (ChIP) shows that in two independent experiments, hsp90 and p23 interact with telomeric DNA. Cells were crosslinked, DNA was sheared, followed by immunoprecipitation with indicated antibodies and reverse crosslinks. Sample is slot blotted and probed with telomere specific probe. Input is sheared DNA (1%). Note that hTERT antibodies show no telomere precipitation, suggesting either poor antibodies or more likely, very low number of hTERT molecules at the telomere in unsynchronized cells.

Given the ChIP assay results, we hypothesized that chaperones were able to interact with the integral telomere binding proteins, TRF-1 and TRF-2. MCF-7 cells were

extracted and subjected to co-immunoprecipitation with hsp90, hsp70, and p23 antibodies, followed by Western analysis. Figure 3 shows that hsp90 and hsp70 appear to interact with the TRF-2 protein, while no interaction was detected between either TRF-2 and p23 (Figure 3) or between TRF-1 and any of the molecular chaperones (data not shown).

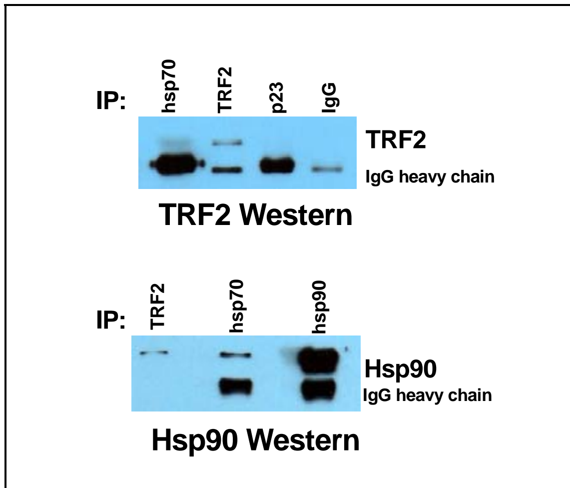

Figure 3. Chaperones interact with the integral telomere binding protein, TRF-2. Top panel is a western of chaperone proteins Hsp70 and p23 IP probed with TRF2 antibody, showing an interaction of TRF2 and Hsp70. Bottom panel shows an Hsp90 western after IP with Hsp90, Hsp70 and TRF2. Hsp90 and Hsp70 are known to interact, and an interaction between Hsp90 and TRF2 can be seen. IgG is used as a negative control.

To assess mammary specificity of this interaction, we tested for the TRF-2/hsp90 interaction in non-breast-derived cells, 293 human embryonic kidney cells and the BJ foreskin fibroblast normal cell line. No interaction between hsp90 and TRF-2 was observed in either cell line or the hTERT over-expressing BJ cell line (Figure 4). While this is clearly very preliminary data, it is possible that this interaction between hsp90 and TRF-2 is specific for breast tumor cells, although the rationale for this is unclear and significantly more cell types need to be tested by multiple methods for this claim to be even remotely accurate biologically. We are currently testing prostate cancer cells, normal mammary epithelial cells, other normal fibroblasts, colon and lung cancer cells, and other breast tumor-derived cell lines to define the specificity of this interaction.

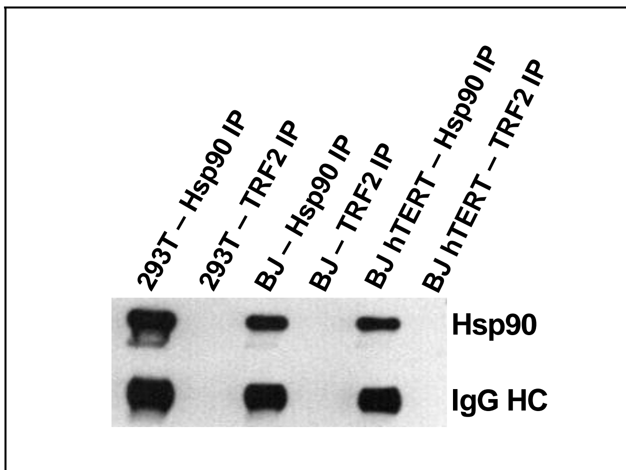

Figure 4. TRF-2 does not appear to associate with hsp90 in non-mammary-derived cells. The basic design of the experiment is to immunoprecipitate the hTERT telomerase protein subunit from either of the model systems (HME and/or MCF-7 with ectopic telomerase), run the protein on a gel (1D or 2D) or HPLC, and determine the differentially precipitated bands compared to cells with low or no telomerase activity. Chemical cross-linking the proteins may be done prior to IP to increase the detection of associated proteins.

Key Research Accomplishments

1-cell culture models of telomerase over-expression have been created, in both a normal mammary cell strain and a breast tumor cell line (as in the 1st annual report).
2-a new hTERT antibody has been assessed and will be used for future experiments.
3-identification of a novel chaperone target, the telomere binding protein TRF-2.
4-potential specificity of the chaperone/TRF-2 interaction as this is not found in HEK and fibroblast cells.

Recommended Changes to the Proposed Work Based on Additional Findings

While we will continue along the proposed experimentation lines, we have added to the goals by generating GFP-hTERT MCF-7 cell lines in order to have a visual selectable marker as well as another epitope for precipitation. The use of the GFP-hTERT is important as it has been previously shown that this fusion protein is fully functional, not only in *in vitro* telomerase assays but also in cells by maintaining telomere lengths (Wong et al., 2002). We will be utilizing positive controls for telomerase binding, proteins already known to associate with hTERT, namely hsp90, p23, and 14-3-3σ. Elimination of gels (i.e. use of HPLC and electrospray technology) may be necessary for ideal detection of telomerase interacting proteins. Because of the issues with antibody specificity for hTERT (versus nucleolin), we have had to alter our protocols and alter our protocols to adequately test a variety of antibodies. We are currently in the process of finalizing these experiments, which will be reported in the final DOD report in the summer of 2007. Lastly, because the reagents for hTERT precipitation are suboptimal, we will be continuing with the hsp90/p23 association with telomere binding proteins, whether those proteins are associated with repair or specifically bound to the telomere itself. This will provide an understanding of the mechanistic basis for chaperone inhibition and its relationship with the damage that occurs at the telomere, as we have previously shown (**Compton et al., 2006**).

Reportable Outcomes

Manuscripts

Compton,S.A., L.W.Elmore, K.Haydu, C.K.Jackson-Cook and S.E.Holt. 2006. Induction of NOS-Dependent Telomere Shortening after Functional Inhibition of Hsp90 in Human Tumor Cells. Molecular and Cellular Biology 26:1452-1462.

Poynter,KP., L.W.Elmore, and S.E.Holt. 2006. Telomeres and telomerase in aging and cancer: lessons learned from experimental model systems. Drug Discovery Today: Disease Models. 3:155-160.

Abstracts/Presentations

Holt,S.E. Era of Hope: Department of Defense Breast Cancer Research Meeting. Philadelphia, PA. June 2005.

Poynter,K.R., L.W.Elmore, and **S.E.Holt.** Era of Hope: Department of Defense Breast Cancer Research Meeting. Philadelphia, PA. June 2005.

Elmore,L.W., X.Di, C.Dumur, **S.E.Holt**, and D.A.Gewirtz. Era of Hope: Department of Defense Breast Cancer Research Meeting. Philadelphia, PA. June 2005.

Elmore,L.W., X.Di., E.A.Gaskins, D.A.Gewirtz, and **S.E.Holt.** AACR Special Conference. La Jolla, CA. September 2005.

Elmore,L.W., S.C.Henderson, X.Di., D.A.Gewirtz, and **S.E.Holt.** WISDM Research Day, VCU, Richmond, VA, April 2006.

Poynter,K.R., L.W.Elmore, and S.E.Holt. WISDM Research Day, VCU, Richmond, VA, April 2006.
Elmore,L.W., S.C.Henderson, X.Di., D.A.Gewirtz, and **S.E.Holt.** The Laboratory of Human Carcinogenesis International Symposium, April 2006
Elmore,L.W., S.C.Henderson, A.T.Bright, X.Di., D.A.Gewirtz, and **S.E.Holt.** Telomere and Genome Stability, Villars-su-Ollon, Switzerland, August 2006.
Poynter,K.R., L.W.Elmore, and S.E.Holt. Watt's Research Day, VCU, Richmond, VA, October 2006.

Invited Seminars

Holt,S.E. Mount Desert Island Stem Cell Symposium, MDIBL, Salisbury Cove, ME. August 2006.
Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2006.
Holt,S.E. Pathology Grand Rounds, MCV/VCU, Richmond, VA. February 2006.
Holt,S.E. Dermatology Grand Rounds, MCV/VCU, Richmond, VA. November 2005.
Holt,S.E. Aquatic Animal Models of Human Disease, University of Georgia, Athens, GA. October 2005.
Holt,S.E. University of Delaware, Newark, DE. October 2005.
Holt,S.E. Pathology Grand Rounds, MCV/VCU, Richmond, VA. September 2005.
Holt,S.E. Mount Desert Biological Laboratory, Salisbury Cove, ME. August 2005.
Holt,S.E. Massey Cancer Center, MCV/VCU, Richmond, VA. July 2005

Development of Cell Lines

We are have generated MCF-7 lines using the GFP-hTERT fusion protein (Wong et al., 2002) and will use these for GFP immunoprecipitations for telomerase interacting proteins.

Funding Applied For

P.I.: Shawn E. Holt, Ph.D.
Title: Development of an In Vitro Breast Cancer Progression Model System using Primary Mammary Cells and In Vivo Selection
Agency: Department of Defense Breast Cancer program (grant # **W81XWH-04-0551)**
Amount:
Duration: 3/1/07-5/31/09

Conclusions

Overall, we have had issues with hTERT precipitation and are finalizing results related to characterizing a number of antibodies for the telomerase protein in terms of immunoprecipication, immunocytochemistry, immunohistochemistry with primary breast cancer samples, and Western analysis, all of which should be available for the final DOD annual report. In the interim, we have adapted some of our studies to test the hypothesis that the members of the hsp90 chaperone complex are involved with telomere structure and function, independent of telomerase. We have found hsp90 and p23 at the telomere and that hsp90 and hsp70 associate with the integral telomere binding protein, TRF-2, but not TRF-1, which also binds specifically at the telomere. We have preliminarily shown that this TRF-2/hsp90 interaction appears specific for mammary cells, although many other cell and tissue types will need to be tested before this conclusion can be made. Therefore, we have expanded our proposal to defining the role of chaperones in telomere structure and function, in addition to the discovery of telomerase associated proteins specific for breast cancer. Understanding the regulatory mechanisms related to telomeres and telomerase in mammary-related cells will facilitate the development of improved therapeutic strategies specifically targeting breast tumor cells.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Shawn E. Holt, Ph.D.	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The Colorado College, Colorado Springs, CO	B.A.	1985-1989	Biology
Texas A&M University, College Station, TX	Ph.D.	1989-1994	Genetics
The University of Texas Southwestern, Dallas, TX	Postdoc	1994-1998	Aging and Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

A. Positions and Honors

Positions:

1998-2003 Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2003-present Associate Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University, Richmond, VA
1998-present Member, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA
2002-present Adjunct Faculty, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA
2002-present Member, Molecular Biology and Genetics Program, Virginia Commonwealth University, Richmond, VA
2003-present Director, Graduate Studies and Education, Department of Pathology, Virginia Commonwealth University, Richmond, VA

Honors:

2000-2003 The V Foundation Scholars Program, Cary, NC ($100,000 award)
1996-1998 NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX
1994 Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX
1994 Outstanding Student Government Member, Texas A&M University, College Station, TX
1988-1989 Dean's List, The Colorado College, Colorado Springs, CO
1988 Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987 Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987 Outstanding College Students of America

B. Selected Peer-Recieved Publications (over the past 4 years, from a total of 52)

Forsythe,H.L. J.L.Jarvis, J.W.Turner, L.W.Elmore, and **S.E.Holt.** 2001. Stable association of hsp90 and p23 with human telomerase. J Biol. Chem. **276**:15571-15574.

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Book Chapters (from a total of 4)

Elmore,L.W. and **S.E.Holt**. 2001. Cell Proliferation, Telomerase and Cancer. Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease. Elsevier Science, Inc. pp. 89-102.
Elmore,L.W. and **S.E.Holt**. 2003. Diagnosis and Treatment of Human Disease Using Telomerase as a Novel Target. Cancer Drug Discovery and Development, Humana Press, pp.361-370.

C. Research Support

Active
P.I.: Kennon R. Poynter
Mentor: Shawn E. Holt, Ph.D.
Title: Mechanisms of telomerase inhibition using small inhibitory RNAs and induction of breast tumor cell sensitization
Agency: Department of Defense Breast Cancer program
Duration: 4/1/04-3/31/07

P.I.: Lawrence F. Povirk, Ph.D. (Co-I, 5% effort, Shawn E. Holt, Ph.D.)
Title: Tyrosyl-DNA phosphodiesterase and oxidative DNA damage
Agency: NIH
Duration: 6/1/04-5/31/09

P.I.: Shawn E. Holt, Ph.D.
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program
Duration: 3/1/04-2/28/06

Completed
P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense
Duration: 12/18/01-12/17/04