Growth Factor-Mediated Telomerase Activity in Ovarian Cancer Cells

by

Yira Bermudez

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Pathology and Cell Biology College of Medicine University of South Florida

Major Professor: Patricia A. Kruk, Ph.D. Jin Q. Cheng, M.D., Ph.D. Santo V. Nicosia, M.D. Rebecca Sutphen, M.D.

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Table of Contents

List of Tables......................................................................................................................................... iv

List of Figures ......................................................................................................................................... v

List of Abbreviations ............................................................................................................................ viii

Chapter I  Introduction.........................................................................................................................1

Ovarian Cancer..................................................................................................................................... 1
  Origin of Ovarian Cancer from Ovarian Surface Epithelium ............................................................2
  Epidemiological Studies of Ovarian Cancer ....................................................................................... 3
  Genetic Predisposition for Ovarian Cancer ......................................................................................... 3
  Diagnostic and Prognostic Screening Modalities ............................................................................ 4
  Treatments ......................................................................................................................................... 6

Telomeres and Telomerase .................................................................................................................... 10
  Telomeres ......................................................................................................................................... 10
  The End Replication Problem and Telomere Hypothesis of Aging .............................................. 11
  Clinical Relevance of Abnormal Telomeric Shortening................................................................. 13
  Telomerase ....................................................................................................................................... 14
  Telomerase Confers Immortality and Chemotherapeutic Resistance ............................................ 16
  Telomerase Therapeutics for Cancer ................................................................................................. 18
  Telomerase Regulation in Cancer Cells .............................................................................................. 20
  Telomerase Regulation by Growth Factors ......................................................................................... 22

Central Hypothesis ................................................................................................................................. 26

Specific Aims ......................................................................................................................................... 26

Chapter II  VEGF and LPA Induce Telomerase Activity in Ovarian Cancer Cells......................... 27
  Abstract ............................................................................................................................................... 27
  Introduction ......................................................................................................................................... 28
  Materials and Methods ....................................................................................................................... 30
Cell Lines and Tissue Culture .................................................................30
Treatment with VEGF, LPA, FGF-2, TNF-α, TGF-β, anti-hVEGF Antibody,
CBO-P11 and U0126 .................................................................30
Telomerase Assay ............................................................................31
RT-PCR .................................................................31
Western Blot Analysis .................................................................32
Luciferase Reporter Assay .............................................................33
VEGF ELISA .............................................................................34
Statistical Analysis ......................................................................34

Results ..........................................................................................35
VEGF stimulates telomerase activity ...........................................35
VEGF induction of telomerase activity is transcription-dependent 36
VEGF-mediated telomerase activity is ERK 1/2-dependent ..........39
Proximal 976- to 378-bp regions are required for induction of hTERT promoter activity by VEGF ........................................41
LPA stimulates telomerase activity in ovarian cancer cells in a VEGF-dependent manner ........................................42
LPA-mediated telomerase activity is ERK 1/2-dependent ............45
Proximal 976- to 378-bp regions are required for induction of hTERT promoter activity by LPA ........................................47
VEGF- and LPA-induced hTERT promoter activation is Sp1-dependent ..................................................51

Discussion .....................................................................................52

Chapter III Vitamin E Suppresses LPA-Mediated Induction of Telomerase Activity in Ovarian Cancer Cells .................................................................57
Abstract ......................................................................................57
Introduction ................................................................................58
Materials and Methods .............................................................61
Cell Culture .................................................................61
PCR-ELISA Telomerase Assay .......................................................61
RT-PCR .................................................................62
VEGF ELISA .............................................................................63
SDS-PAGE and Western Blot Analysis ........................................64
Luciferase Reporter Assay .............................................................65
MTS Assay for Cell Viability ........................................................65
Statistical Analysis ......................................................................66

Results ......................................................................................66
Vitamin E suppresses telomerase activity ..................................66
Vitamin E decreases hTERT RNA expression .........................69
Vitamin E targets the -976 to -578 responsive regions of the hTERT promoter ..................................................71
List of Tables

Table 1       Growth Factor-Mediated Telomerase Activity................................. 23
List of Figures

Figure 1   End Replication Problem...........................................................................12

Figure 2   Telomere Length in Relation to Senescence, Crisis, andImmortalization..................................................................................................................13

Figure 3   Telomerase-Associated Proteins .................................................................16

Figure 4   Telomerase Confers Resistance to Caspase-Mediated Apoptosis ..........17

Figure 5   VEGF Induces Telomerase Activity in a Time- and Dose-DependentManner...................................................................................................................37

Figure 6   VEGF Induces Telomerase Activity .............................................................38

Figure 7   VEGF Increases hTERT Transcription Levels ........................................39

Figure 8   VEGF Induction of Telomerase Activity is ERK 1/2-Dependent ..............40

Figure 9   976- to 378-bp hTERT Promoter Regions are required for VEGFInduction of Telomerase Activity .............................................................................43

Figure 10  LPA Induction of Telomerase Activity is VEGF-Dependent...............44

Figure 11  LPA Induces Telomerase Activity via the ERK 1/2 Pathway ...............46

Figure 12  Proximal hTERT 976- to 378-bp Promoter Regions are Responsive toLPA-Induced Telomerase Activity .........................................................................48

Figure 13  VEGF- and LPA-Induced Telomerase Activity is Sp1-Dependent ..........49

Figure 14  VEGF and LPA Target Sp1 Binding Sites in hTERT Promoter ..........50

Figure 15  Proposed VEGF/LPA Regulation of Telomerase Activity in OvarianCancer Cells .............................................................................................................56
Figure 16 Vitamin E Suppression of Telomerase Activity is Dose- and Time-Dependent ................................................................. 68
Figure 17 Vitamin E Suppresses Telomerase Activity in Human Ovarian Cancer Cells ........................................................................................................................................ 69
Figure 18 Vitamin E-Mediated Suppression of Telomerase is Transcription-Dependent ................................................................................................................................. 70
Figure 19 The 976- to 578-bp hTERT Promoter Regions are Required for Vitamin E Suppression of Telomerase Activity................................................................. 71
Figure 20 Vitamin E Suppresses LPA-Mediated Telomerase Activity ................................................................................................. 74
Figure 21 Vitamin E Negatively Regulates LPA-Mediated hTERT Promoter Activity ................................................................................................................................. 75
Figure 22 Vitamin E Increases Chemotherapeutic Sensitivity in Ovarian Cancer Cells ................................................................................................................................. 76
Figure 22 (Continued) Vitamin E Increases Chemotherapeutic Sensitivity in Ovarian Cancer Cells ................................................................................................................................. 77
Figure 23 Vitamin E May Protect Telomerase-Negative Cells from Cisplatin-Mediated Cytotoxicity ................................................................................................................................. 82
Figure 23 (Continued) Vitamin E May Protect Telomerase-Negative Cells from Cisplatin-Mediated Cytotoxicity ................................................................................................................................. 83
Figure 24 Schematic Representation of VEGF-Induced Telomerase Activity and Vitamin E Suppression of LPA-Mediated Telomerase Activity .......... 88
Figure 25  EGF Regulation of Telomerase Activity is Time- and Dose-Dependent ............................................................................................... 101

Figure 26  EGF Stimulates Telomerase Activity in Human Ovarian Cancer Cells .... 102

Figure 27  EGF is Specific to Stimulate Telomerase Activity .................................. 103

Figure 28  EGF Stimulation of Telomerase Activity is Transcription-Dependent....... 104

Figure 29  EGF Stimulation of Telomerase Activity is Pyk2-Dependent.................... 106

Figure 30  EGF Stimulation of Telomerase Activity is Via the ERK 1/2 Signaling Pathway .......................................................................................... 107

Figure 31  EGF Drives hTERT Core Promoter Activity ............................................ 110

Figure 32  Pyk2 Drives hTERT Core Promoter Activity ............................................ 111

Figure 33  EGF-Mediated Telomerase Activity is c-Myc- and Sp1-Dependent......... 113

Figure 34  EGF Targets c-Myc and Sp1 Binding Sites in the hTERT Core Promoter ................................................................................................. 114

Figure 34  (Continued) EGF Targets c-Myc and Sp1 Binding Sites in the hTERT Core Promoter .......................................................................................... 115

Figure 35  Schematic Representation of the Proposed Signaling Pathway for EGF-Induced of Telomerase Activity in Ovarian Cancer Cells ................. 119
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP2</td>
<td>Activated Protein 2</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer Gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer Gene 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer Antigen 125</td>
</tr>
<tr>
<td>cDNA</td>
<td>Chromosomal Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant Negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Edg</td>
<td>Endothelial differentiation gene</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Oestrogen Response Element</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
</tr>
<tr>
<td>EST1A</td>
<td>Ever Shorter Telomeres 1A</td>
</tr>
<tr>
<td>EST1B</td>
<td>Ever Shorter Telomeres 1B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast Growth Factor 2</td>
</tr>
<tr>
<td>FHIOSE</td>
<td>IOSE cells derived from women with a family history of breast and/or ovarian cancer</td>
</tr>
<tr>
<td>GC or CpG</td>
<td>Guanine Cytosine Content</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein-Coupled Receptor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>Hsp 90</td>
<td>Heat Shock Protein 90</td>
</tr>
<tr>
<td>hTERT</td>
<td>Telomerase Reverse Transcriptase Component</td>
</tr>
<tr>
<td>hTR</td>
<td>Telomerase RNA Component</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>IGF-binding protein 2</td>
</tr>
<tr>
<td>IOSE</td>
<td>SV-40 Large T Antigen-Transfected Ovarian Surface Epithelial Cells</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MTL</td>
<td>Mean Telomeric Length</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factors</td>
</tr>
<tr>
<td>OR</td>
<td>Oestrogen Receptor</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian Surface Epithelium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI-3-K</td>
<td>Phosphotidyl Inositol 3-Kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Proline-Rich Tyrosine Kinase 2</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RTK</td>
<td>Tyrosine Kinase Inhibitors</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective ER Modulator</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specific Protein 1</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TEP1</td>
<td>Telomerase-Associated Protein 1</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TIN2</td>
<td>TRF1-Interacting Nuclear Protein 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TRF1</td>
<td>Telomeric Repeat Binding Factor 1</td>
</tr>
<tr>
<td>TRF2</td>
<td>Telomeric Repeat Binding Factor 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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Growth Factor-Mediated Telomerase Activity in Ovarian Cancer Cells

Yira Bermudez

ABSTRACT

Ovarian cancer is the leading cause of gynecological cancer death in the United States. Even though no single genetic alteration can be attributed to all ovarian cancers, 90% of ovarian tumors express telomerase, a ribonucleoprotein that elongates telomeric (TTAGGG)_n repeats de novo. In normal somatic cells, telomerase is absent. In cancer cells, the re-expression of telomerase allows senescence to be bypassed contributing to cellular immortalization, a key step for cellular transformation, making telomerase a potentially important target for therapeutic intervention.

Ovarian cancer cells secrete vascular endothelial growth factor (VEGF) and lysophosphatidic acid (LPA) that feedback through their receptors present on ovarian cancer cells to promote cell growth. Since telomerase can be regulated by growth factors, I examined VEGF regulation of telomerase activity and the possible contribution of LPA as an upstream regulator of VEGF-mediated telomerase activity in ovarian cancer. My data reveal that both VEGF and LPA upregulate telomerase activity by ERK 1/2-dependent transcriptional activation within the -976 to the -378 bp hTERT promoter regions where Sp1 is one of the major mediators of VEGF- and LPA-induced transactivation of hTERT. It also identifies telomerase as a novel molecular target of
LPA as well as a target of VEGF in non-endothelial cells. In addition I found that, vitamin E, a dietary supplement able to degrade and suppress LPA activity, consistently abrogates LPA-mediated telomerase activity through transcriptional inhibition of the hTERT -976 to -578 bp promoter regions.

Lastly, since epidermal growth factor (EGF) promotes ovarian surface epithelial (OSE) cell growth and EGF receptors are frequently constitutively activated in ovarian cancers, the potential contribution of EGF in the regulation of telomerase activity was also examined. While none of the ovarian cancer cell lines examined produced large amounts of EGF, EGF stimulation of telomerase activity was mediated by Sp1 and c-Myc transcription factors within the hTERT core promoter in an ERK 1/2 /Pyk2-dependent manner.

In conclusion, my research shows differential regulation of telomerase activity by growth factor and/or anti-oxidant nutraceuticals. In the future, these factors may be exploited as adjuvant therapy for improved chemotherapeutic benefit to decrease the mortality associated with ovarian cancer.
Chapter I

Introduction

Ovarian Cancer

Ovarian cancer, the “silent killer”, presents a serious clinical challenge because symptoms do not develop or are not recognized until the disease is in its advanced stages, contributing to poor prognosis. It is the fourth leading cause of cancer death among women after lung, breast, and colorectal cancer [1] and is associated with a 1.7% lifetime risk [2]. It is estimated that 22,220 new cases are diagnosed in the United States (US) annually and that 16,210 women die annually from this disease [1]. Five-year survival rates for ovarian cancer vary based on stage and grade of the tumor [1]. In the United States, the overall survival rate for stage I is 93%, stage II is 70%, stage III is 37%, and stage IV is 25% [1]. However, only about 20% of patients are diagnosed at stage I, whereas approximately two-thirds of patients are diagnosed at stage III or IV [1]. Currently for ovarian cancer, there is no known preinvasive disease condition and, unfortunately, ovarian cancer is relatively asymptomatic until later stages. Even when symptoms do present, they are often mistaken as gastrointestinal problems or menopausal symptoms. Therefore, there is often a delay in diagnosis, accounting for predominance of cases that are identified in late stages of the disease.
**Origin of Ovarian Cancer from Ovarian Surface Epithelium**

Over 85% of ovarian tumors arise from the ovarian surface epithelium (OSE) [3-5]. Epithelial ovarian cancers are thought to arise from the single layer of cells that covers the ovary or that lines cysts immediately beneath the ovarian surface [3-5]. These cells are generally quiescent, but proliferate following ovulation to repair the defect created by rupture of a follicle [6-8]. In addition, cyst formation is more frequently observed in the later reproductive years, which coincides with the average age of ovarian carcinoma [3]. When these cysts form, they are usually sealed off from the surface similar to a crypt-like environment exposing them to doses of hormones and growth factors different from those cells still on the ovarian surface [9].

In support of these observations, examination of the OSE of ovaries removed prophylactically from women with a familial history of breast and ovarian tumors have revealed numerous histologic changes. Such changes include epithelial papillomatosis, numerous inclusion cysts, deep invaginations, epithelial pseudostratification, and hyperactive stroma [8]. Since the OSE originates in the coelomic epithelium overlying the gonadal ridge [9], the OSE is embryologically related to other gynecological tissues [10], including the epithelial lining the oviduct, uterus and vagina. Consequently, ovarian tumors often mimic these tissues derived from the coelomic epithelium and are classified as serous, endometrioid and mucinous ovarian cancers [11].

*In vitro* models also support the origin of ovarian cancer from the OSE. For instance, Godwin *et al* demonstrated that repeated passaging, which mimicked post-ovulatory repair-driven OSE proliferation, could transform rat OSE cells in culture [12],
while separate studies by Nicosia et al and Auersperg et al also demonstrated how the highly proliferative capacity of rabbit and human OSE cells can lead to formation of preneoplastic lesions [13-15]. In addition, epithelial irritation by external contaminants, such as fibers, can promote tumorigenesis [16]. Since the OSE maintains a direct connection with the external environment, the hypothesis of external irritants causing ovarian cancer initially gained support because women commonly used asbestos-contaminated talcs for genital hygiene [16].

**Epidemiological Studies of Ovarian Cancer**

Epidemiological studies also support the hypothesis that ovarian neoplasms arise primarily from the OSE. A well accepted theory is the “incessant ovulation” hypothesis described by M.F. Fathalla in 1971 [17]. This study observed that nulliparous women had a higher incidence of ovarian cancer than multiparous women by 30 to 60% [18]. In addition, studies by Lowry et al and Francheschi et al showed that oral contraceptive use or breast feeding are protective against ovarian cancer as they suppress ovulation [19, 20]. In summary, this hypothesis proposes that repeated traumatic rupture and expeditious repair of the OSE during each ovulatory cycle could result in the accumulation of genetic mutations that allow for transformation of these cells [17].

**Genetic Predisposition for Ovarian Cancer**

Most epithelial ovarian tumors are sporadic, with familial or hereditary patterns accounting for 5 to 10% of all malignancies [21]. Two genes often associated with genetic predisposition to ovarian cancer are BRCA1 and BRCA2 located on
chromosomes 17 and 13, respectively [22]. These mutations are passed via autosomal dominant inheritance. Women who have a mutation in the BRCA1 gene are associated with a lifetime risk of ovarian cancer as high as 28 to 44% while the risk for women with a BRCA2 mutation is as high as 27% compared to the general population [23].

Ovarian carcinoma may also arise in families with hereditary non-polyposis colorectal cancer (HNPCC) or Lynch II syndrome [24, 25]. The two most frequently mutated genes in HNPCC are hMSH2 and hMLH1, and the risk of ovarian carcinoma is raised in carriers of mutations in either of these genes [24, 26]. The cumulative risk of ovarian cancer in HNPCC families is more than 12% and they are frequently diagnosed at a younger age than the general population [26, 27]. In addition, most tumors are low stage, well differentiated or moderately differentiated carcinomas [26, 27].

**Diagnostic and Prognostic Screening Modalities**

Currently, there is no standardized strategy for early detection of ovarian cancer. However, potential screening modalities including the use of cancer antigen 125 (CA125) and lysophosphatidic acid (LPA) as diagnostic tools, and vascular endothelial growth factor (VEGF) as a prognostic tool has been explored.

CA125 is a glycoprotein that is expressed by tissues derived from coelomic epithelium, which includes cells of the pleura, pericardium, peritoneum, and mullerian tissue [28]. It is not found in normal ovarian epithelium, but it is overexpressed by serous and mucinous papillary tumors [29]. However, since CA125 is expressed by tissues derived from coelomic epithelium, the value can be elevated with any condition that causes peritoneal or mesothelial inflammation. Furthermore, in ovarian cancer patients,
CA125 levels are not elevated above normal ranges in over half of the patients with early-stage disease [30, 31]. Due to the nonspecific nature of CA125 expression, it has not been shown to be effective as a sole screening modality in the detection of early ovarian cancer.

In contrast, LPA, a natural occurring phospholipid, is found elevated in plasma and ascites of ovarian cancer patients. Two studies by Xu et al and Sutphen et al showed that patients with ovarian cancer had significantly higher plasma LPA levels as compared with the control group. Nine of ten patients with stage I disease had elevated levels as well as in all 24 patients with stage II-IV disease [32]. In contrast, corresponding CA125 values only showed elevation above 35 U/mL in two of the nine patients with stage I disease, and only 13 of the 24 patients with stage II-IV disease had elevated CA125 levels [32]. In regards to healthy controls, five of the 48 patients had elevated serum levels of LPA and 4 of the 17 patients with benign gynecologic conditions had elevated serum levels of LPA [32]. The Sutphen et al study in 2004 corroborated LPA levels to be elevated preoperatively in ovarian cancer patients as compared with control samples [33]. In addition, they studied 22 cases with both preoperative and postoperative samples to determine whether LPA could also be used as a prognostic tool [33]. They found that postoperative levels of total LPA were significantly lower than preoperative levels ($p \leq 0.05$) [33]. Furthermore, Sutphen et al proposed to detect LPA by surface-enhanced Raman scattering (SERS) using silver nanoparticles to eliminate the need for partial purification of samples by thin layer chromatography (TLC) prior to analysis using chromatography and mass spectrometry assays in cancer screening, which will allow for
a more specific and sensitive detection of LPA [34]. In conclusion, these studies show LPA as a potential biomarker for ovarian cancer.

VEGF, an important proangiogenic regulator [35, 36], has been shown to be elevated in ovarian cancer cyst fluid as compared with benign, borderline, or functional cysts [37]. Several studies have shown that patients whose disease recurred, progressed, or who died from the disease displayed significantly higher tumor staining of VEGF suggesting a correlation between induced neoangiogenesis and tumor progression and/or metastases [38-42]. Therefore, high VEGF levels in ovarian cancer cyst fluid may represent an indicator of malignancy and tumor progression in the ovary.

Treatments

Despite initial tumor response rates of 80% to first-line taxane- and platinum-based chemotherapy and surgical debulking [43, 44], most women with advanced ovarian carcinoma will ultimately develop drug-resistant disease [45, 46]. The use of second-line chemotherapeutic agents, such as doxorubicin, topotecan, gemcitabine, tamoxifen, and vinorelbine, can lead to a response rate of approximately 15% to 25% [47]. Therefore, it is imperative that novel and/or improved therapeutic modalities for ovarian cancer are developed.

In response, molecular targeting strategies for cancer therapy are being explored because of their potential to specifically target the tumor when combined with current conventional chemotherapy modalities. Since 35 to 70% of ovarian carcinomas overexpress EGF receptor (EGFR) [48], and VEGF is elevated in ascites of ovarian
cancer patients [37], these are two promising molecules in the treatment of ovarian cancer.

EGFR is dysregulated in many malignancies and its signaling pathway plays a pivotal role in controlling tumor growth and progression, apoptosis, and angiogenesis [49, 50]. In addition, dysregulation of EGFR has been associated with chemoresistance and poor prognosis [51]. Monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors (TKIs) are the most promising and well studied EGFR inhibitor strategies in the treatment of malignancies refractory to conventional chemotherapy. They both target the same receptor, but their mechanisms of receptor inhibition are different. Monoclonal antibodies block the extracellular ligand-binding portion of the EGFR and interfere with its activation. In contrast, TKIs block induction of the intracellular tyrosine kinase-mediated signaling pathways.

Phase II clinical trials of Tarceva® (OSI-774, erlotinib), a small-molecule EGFR tyrosine kinase inhibitor, demonstrated an acceptable safety profile during moderately long-term administration in 34 women with EGFR-overexpressing ovarian tumors treated with OSI-774 by daily oral dosing [52]. Randomized trials are needed to further evaluate OSI-774’s potential in the treatment of ovarian cancer in conjunction with conventional chemotherapy.

Similar techniques are being implemented to target the tumor’s vasculature, which nourishes the tumor by supplying oxygen and nutrients, allowing it to grow and metastasis [35, 36]. Vascular disrupting agents (VDAs) work by causing the endothelials cells, cells that line the inside of blood vessels, to change shape and collapse in order to
shut off blood flow to tumors, leading to cell death [53, 54]. To date, two types of VDAs, small molecule and ligand directed are being pursuit. Small molecule VDAs damage the structure of endothelial cells by interfering with the cells’ cellular scaffolding, or tubulin, important in helping cells maintain their shape, or by releasing tumor necrosis factor, which leads to the collapse of blood vessels [53, 55, 56]. Ligand-directed VDAs use antibodies, peptides, or growth factors to target toxins or procoagulants to the tumor endothelium.

One small molecule VDA in clinical trials with advanced ovarian cancer patients is combretastatin A-4 phosphate (CA4P), which triggers a change in the shape of the endothelial cells lining tumor blood vessels, blocking the flow of blood to a tumor and depriving it of oxygen and nutrients essential to its survival. In a Phase Ib study that combined CA4P with the chemotherapy drugs paclitaxel or carboplatin, six of nine patients with advanced ovarian cancer showed 50% tumor shrinkage [57]. In summary, small molecule VDAs are attractive because of their specificity in targeting the tumor vascular system. Future progress including advanced clinical trials will enable these agents to reach cancer patients either as single agents or in combination therapy.

The antiangiogenic agents are the second group of vascular targeted therapies. The antiangiogenic group differs from the VDAs in that the antiangiogenic group interferes with new vessel formation and therefore has a preventive action, requires chronic administration, and is likely to be of particular benefit in early stage or asymptomatic metastatic disease [58].
One prospective antiangiogenic agent against ovarian cancer is bevacizumab, a humanized recombinant antibody that prevents vascular endothelial growth factor binding and inhibits angiogenesis and tumor growth. Randomized trials have shown statistically significant improvements in progression-free and overall survival when bevacizumab is combined with chemotherapy in several solid tumors. In the Gynecologic Oncology Group 170-D study, 63 patients with recurrent ovarian cancer were treated with 15 mg/kg of bevacizumab intravenously every three weeks [59]. The overall response rate was 17.7% and 38.7% of patients were progression free at 6 or more months [59]. Since antiangiogenic therapies require high dosages, current data suggest that antiangiogenic agents such as bevacizumab may be best used upfront or in combination with chemotherapy in patients going through their initial chemotherapy.

Future directions lie in understanding the molecular mechanism(s) of action of these promising drugs currently undergoing clinical evaluation. Increased understanding of their molecular targets and cell signaling processes involved might aid in improving the current drugs and/or assist in the development of second generation of these agents.
Telomeres and Telomerase

Telomeres

Early studies by Muller and McClintock showed the ends of chromosomes are capped by telomeres to prevent chromosome fusions [60, 61]. In humans, telomeres are usually 10 kb long and consist of guanine-rich repetitive DNA sequences of (TTAGGG)<sub>n</sub> [62, 63]. Even though the majority of the telomere is double stranded, there is a single stranded 3’ overhang that forms a loop as it folds back on the double stranded telomeric DNA and base pairs with a duplex region of telomeric repeats to form a “t (telomere)-loop” [64]. Concurrently, a portion of the 5’ to 3’ strand along the length of the telomere is displaced to form the “D (displacement)-loop” [64]. The duplex region of the telomeric DNA framework allows the direct binding of two main telomeric proteins, telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), which are important in telomere length regulation, integrity, and t-loop formation [65-67]. TRF1 functions in telomere length regulation, controlling the access of telomerase to the telomere termini [65], whereas TRF2 functions independently to protect telomeres from non-homologous end joining and activation of DNA repair or DNA damage response pathways [66, 68].

Many protective functions are attributed to telomeres such as protecting the chromosomes ends from nuclease degradation, end-to-end fusion, ligation, and recombination [69]. In addition, a well-studied function attributed to telomeres is their ability to influence cellular replicative capacity of a cell, solving what is known as the “end replication problem”.
The End Replication Problem and Telomere Hypothesis of Aging

In the 1970's, as the mechanisms responsible for DNA replication were becoming better understood, it became clear that DNA polymerase could not fully synthesize the 3' end of linear DNA. In 1972, Watson explained this as the end-replication problem [70].

As depicted in Figure 1, DNA replication is a semi-conservative process in that the parental strands of DNA are separated and both are used as templates for the new daughter strands. As the parental strands are unwinded by the helicases, a new daughter strand of DNA, leading strand, is formed in a 5’ to 3’ direction with an RNA primer attached to the parental strand of DNA to provide the DNA polymerase a starting point. Since DNA polymerase can only synthesize from the 5’ to 3’ direction, the complementary 3’ to 5’ daughter strand, lagging strand, is synthesized in small pieces called Okazaki fragments, each requiring an RNA primer, as the parental strand is unzipped. As a consequence, DNA polymerase is unable to completely replicate the 3’ DNA end resulting in the loss of 50 to 200 base pairs of telomeric DNA with each successive round of replication [71, 72] (Figure 1). Therefore, erosion of this non-coding DNA, the telomeres, acts as a mitotic clock, determining the number of replications allowable during a cellular lifespan [71, 72]. Upon reaching a critically shortened telomeric length, the cellular lifespan reaches its limit, known as the Hayflick Limit [73], and the shortened telomeres would then signal the cell to senesce in support of the telomere hypothesis of aging [74, 75].

As normal cells reach the limit of their lifespan through telomere shortening, most cells exit the cell cycle and enter a state called senescence [74]. Occassionally, a few
cells will bypass senescence and continue to proliferate. At the end of this extended lifespan, cells experience genetic instability highlighted by chromosomal fusions and aneuploidy, then enter “crisis” [73, 76]. Escape from crisis is a spontaneous, rare event that gives rise to immortal cells, such as cancerous cells, capable of maintaining a stable telomeric length. In order to escape from crisis, telomerase activation or reactivation is important to maintain a stable telomeric length (Figure 2).

---

**Figure 1  End Replication Problem [77]**

During DNA replication, leading and lagging DNA strands separate. Using the parental strand as a template, DNA polymerase copies the leading strand in the 5’ to 3’ direction. Since DNA polymerase cannot synthesize in the 3’ to 5’ direction, the lagging strand is copied one segment at a time as the DNA strands unwind. An RNA primer is laid down and the DNA polymerase extends this primer making an Okazaki fragment. The primer is then removed and the DNA segments are ligated. The lack of DNA at the very end of the 5’ does not allow an Okazaki fragment to be ligated. Therefore, the lagging strand is an incomplete replication of the parental 3’ end.
Figure 2  Telomere Length in Relation to Senescence, Crisis, and Immortalization

The Hayflick limit is the finite replicative capacity of normal somatic cells. In order to bypass the Hayflick limit, telomerase has to be activated or reactivated. Germ cells express telomerase until they undergo differentiation. In regenerative tissues, cells such as lymphocytes are capable of reactivating telomerase to enter a proliferative state. In contrast, most somatic cells are telomerase negative and continuously experience telomere erosion with each round of replication. Once cells reach a critically shortened telomere length, most cells lose their proliferative potential and senesce. Nonetheless, a few cells overcome senescence and continue to proliferate with the risk of experiencing genetic instability and crisis. When stabilization of telomere length is achieved by telomerase reactivation, cells spontaneously escape crisis allowing this cell population to become immortal.

Clinical Relevance of Abnormal Telomeric Shortening

Telomere shortening during aging occurs in a variety of human tissues and organs including dermal fibroblasts [78], peripheral blood cells [79], liver [80], and spleen [81]. Most of these cells, tissues, and organs are mitotically active [81], except for the liver that shows very little mitotic activity indicating that there must be factors other than cell
division modulating the attrition of telomeres during aging [80]. In addition, the kinetics of telomere shortening during aging are not linear [82]. For instance, telomere shortening is accelerated in peripheral blood cells in young infants, plateaus in older children, and slowly decreases in adults [83].

Accelerated telomeric loss is related to several clinical diseases including colitis ulcerosa [84], dyskeratosis congenital [85], and Fanconi anemia [86]. However, different mechanisms have been suggested as the cause of accelerated telomere shortening in these diseases.

In colitis ulcerosa, it is suggested that elevated cell turnover leads to accelerated telomere shortening possibly limiting the regenerative capacity of affected organs [84]. In the autosomal dominant form of dyskeratosis congenital, telomerase activity is inhibited because of an 821 bp deletion at the 3’-end of the hTR gene resulting in accelerated telomeric loss [87]. Lastly, cells from patients with Fanconi’s anemia have high levels of intracellular radical oxygen species thought to induce telomere breaks, thereby shortening telomeres [86]. In conclusion, although the telomere represents a mechanism to regulate proliferation in normal cells, a mechanism that could counter or accelerate telomere shortening could modify replicative capacity.

**Telomerase**

Telomerase activity is associated with an increased proliferative capacity. It can be detected in germline cells [88, 89] and the proliferative cells of renewal tissues such as hematopoietic cells, activated lymphocytes [90-93], and basal cells of the epidermis [94, 95]. In addition, telomerase is detected in embryonic tissues, but its activity is repressed
as cells exit the cell cycle and differentiate [88, 96, 97]. Consequently, normal somatic cells do not typically express high levels of telomerase activity.

Telomerase is the multi-subunit ribonucleoprotein (RNP) enzyme that elongates telomeric DNA at its 3’ overhang [98, 99]. Telomerase consists of the human reverse transcriptase catalytic subunit (hTERT), the human RNA template (hTR) for telomeric sequence synthesis, and a number of telomerase associated proteins, which are important in the assembly of telomerase RNA into stable RNP. hTR is a member of small nucleolar RNA molecules termed box H/ACA RNAs, which is an RNA motif with a consensus structure that includes a hairpin stem, a hinge sequence, a second hairpin stem and an ACA sequence, essential for precursor RNA 3’ end processing and mature RNA accumulation [100]. The subunits hTR and hTERT associate to form a complex tetramer composed of two RNA subunits (hTR) and two catalytic subunits (hTERT) [101, 102]. In addition to these core components, other proteins including telomerase-associated protein 1 (TEP1), heat shock proteins 90 (hsp90), molecular chaperones p23, and dyskerin are also included in the telomerase complex (Figure 3). TEP1 is important in the stabilization and recruitment of hTR and hTERT in vitro [103]. The molecular chaperones p23 and hsp90 are essential for active telomerase assembly [104]. Dyskerin binds to the hTR H/ACA motif to stabilize the telomerase RNA [105]. Recently, EST1A and EST1B proteins have been shown to associate with telomeres and bind telomerase in vitro [106, 107]. Furthermore, overproduction of EST1A affects telomere length and capping by inducing anaphase bridges due to chromosome ends (Figure 3) [106, 107].
Telomerase is composed of the catalytic subunit hTERT and the RNA component hTR. The subunits associate to form a complex tetramer composed of two RNA subunits (hTR) and two catalytic subunits (hTERT). In addition to these core components other proteins including TEP1, p23, hsp90, and dyskerin have been associated with telomerase even though the exact functions of these proteins in telomerase formation and/or regulation are not completely understood. Recently, homologs of the yeast Est1p protein, EST1A and EST1B have been shown to associate with telomeres and bind telomerase in vitro.

Telomerase Confers Immortality and Chemotherapeutic Resistance

Although telomerase re-activation is sufficient to extend cellular lifespan, hTERT expression is insufficient to trigger malignant transformation [109, 110]. However, immortalization contributes to increased susceptibility for cellular mutations eventually resulting in oncogenic transformation during prolonged cell proliferation. In addition, telomerase activation has been shown to increase chemotherapeutic resistance consistent with poor prognosis in many tumors types including colon, breast, gastric, cervical, and uterine, and ovarian [111-114]. For instance, Faraoni et al showed that telomerase activity in primary cultures of ovarian cancer cells was inversely related to chemosensitivity [114]. Also, Takahashi et al showed that all ovarian cancer patients responding to platinum therapy had low levels of telomerase whereas 50% of non-responders demonstrated elevated telomerase activity [115]. When Shoup et al studied
the effect of cisplatin sensitivity with concurrent treatment with telomerase inhibitors *in vitro*, they found that treatment of drug-resistant ovarian cancer cells, C13, with cisplatin and the telomerase inhibitor, 2’-O-(2-methoxyethyl) RNA caused a 61% reduction in C13 viability compared to controls and increased cisplatin-mediated cytotoxicity by 40% [116].

Furthermore, studies have suggested that telomerase mediates chemoresistance by conferring resistance to apoptosis [117-123]. For instance, a recent study in our laboratory showed that introduction of hTERT expression into telomerase-negative immortalized ovarian surface epithelial (IOSE) cells was sufficient and specific to confer resistance to drug-induced cytotoxicity (Figure 4) [118]. Consequently, since telomerase re-activation is a crucial step for cellular immortalization and malignant transformation, inhibition of telomerase may act as a tumor-suppressive mechanism and have clinical utility as adjuvant therapy for enhanced chemosensitization.

![Telomerase Confers Resistance to Caspase-Mediated Apoptosis](image)

**Figure 4**  Telomerase Confers Resistance to Caspase-Mediated Apoptosis [118]  
Control IOSE cells, IOSE cells transfected with hTERT cDNA, and IOSE cells transfected with both hTERT cDNA and dominant negative (DN) hTERT cDNA were treated ± 1 µM staurosporine (STS) and examined for telomerase activity by PCR-ELISA (right panel) and assayed for activated caspase 3 by Western blot analysis using actin as a loading control (left panel). Telomerase activity was expressed as the absorbance at 490 nm ± S.E.
Telomerase Therapeutics for Cancer

Therapeutic opportunities exist in which cancer cells can be efficiently targeted by telomerase inhibitors, while normal telomerase-expressing cells, such as stem and germline cells, remain unaffected as a result of their longer telomere lengths and slower rates of cell division. The most advanced telomerase therapeutic strategies include vaccines targeting telomerase, oligonucleotide-based therapeutics, and the use of a telomerase oncolytic virus.

Since telomerase is over-expressed in nearly all cancers, it is considered an endogenous target for strategies using vaccines that boost the immune system to attack cancer cells. Phase I and II clinical trial of patients with metastatic prostate cancer, demonstrated that cancer patients’ immune cells could be activated with a telomerase vaccine to kill their own cancer cells by using dendritic cells from patient’s blood, pulsed with hTERT RNA, then returned to the patient’s body where cytotoxic T-cells were instructed to kill tumor cells that expressed telomerase [124]. The results of this Phase I and II clinical trial showed that the immune responses of patients were strong as well as specific based on tests assessing the generation of telomerase-specific cytotoxic CD8+ T-lymphocytes as well as CD4+ T-lymphocytes [124]. This study and similar studies [125-129] have helped identify a clinical setting for testing immunotherapy against hTERT.

Since the template region of hTR must be accessible to bind to the telomeric repeats, it has been suggested that the 11-base template region of telomerase RNA should be an excellent target for direct enzymatic inhibition of telomerase activity [130]. One agent in Phase I and II clinical trials in patients with chronic lymphocytic leukaemia is
GRN163L, a telomerase RNA template antagonist agent [131-133]. It contains a
lapidated 13-mer thio-phosphoramidate that targets the hTR component of telomerase,
preventing it from forming an active complex with hTERT [131-133]. GRN163L is one
of the first generation of small-molecule telomerase inhibitors for the treatment of cancer.
Current data suggest it has the potential to be a universal anticancer agent with minimal
side effects.

The third telomerase therapeutic with great potential is the vector-mediated
approach of replicating oncolytic viruses to activate cell death (60-68). Oncolytic
viruses, which are tumor-selective viruses that mediate oncolytic effects on tumors, are
genetically modified viruses engineered to replicate in and kill targeted cancer cells. One
oncolytic virus that has demonstrated a high degree of specificity and effectiveness in
xenograft models is CG5757 [134]. This virus was generated by replacing the E1a and
E1b endogenous promoters with promoters derived from the human E2F1 and the hTERT
gene, respectively [134]. The E2F1 promoter is activated in retinoblastoma (Rb)-
defective tumor types, a pathway mutated in 85% of all cancers. Likewise, telomerase is
aberrantly expressed in 90% of tumors. In vitro studies showed that expression of E1a
and E1b genes was restricted to Rb-defective and hTERT-positive cancer cells and did
not replicate in normal cells [134]. In addition, in vivo studies using the bladder cancer
253J B-V mouse model showed the average tumor volume in animals treated with 4
consecutive daily intratumoral injections of CG5757 decreased to 72% of baseline,
compared to the control group that had an increase to 944% of baseline [134].
Furthermore, 50% of treated animals had complete regression of the 253J B-V tumor
Therefore, these data demonstrate that CG5757 has strong tumor selectivity and antitumor efficacy in cancers that are Rb-defective and hTERT (telomerase)-positive.

**Telomerase Regulation in Cancer Cells**

Although emerging telomerase-targeted therapy is a promising and novel approach to cancer therapeutics, many gaps still remain in our understanding of the complexities of the regulation of telomerase. Therefore, it is important to understand its regulation in order to achieve optimal translational results.

The hTERT gene is located on the distal arm of chromosome 5p (5p15.33) [135, 136]. It consists of 16 exons and 15 introns across ~35 kb that is rich in GC content with no TATA and CAAT boxes, but contains binding sites for several transcription factors that may be involved in its regulation [137, 138]. Even though mutations are rare in this region, amplifications have been detected in some types of cancers, suggesting that increased copy number may be one mechanism that increases telomerase expression in human tumors [135, 139].

The most common post-translational modification associated with telomerase regulation is phosphorylation. Several kinases, including Akt and protein kinase Cα (PKCα) have been implicated in the regulation of telomerase [140-143]. Akt, a target of phosphotidyl inositol 3-kinase (PI3K), has been shown to activate telomerase *in vitro* by direct phosphorylation of hTERT [141]. Likewise, PKCα is capable of phosphorylating hTERT and consequently activating telomerase in breast cancer cells, while PKC inhibitors have an inhibitory effect on telomerase expression [140, 144]. On the other
hand, phosphorylation of hTERT by c-Abl tyrosine kinase has been shown to downregulate telomerase activity [145].

The hTERT promoter contains a number of regulatory sites including E-boxes, MT-box, Sp1, Myc, AP2 binding sites among others [137, 138, 146, 147]. The most studied of these transcription factors is c-Myc as a positive regulator of telomerase, while the c-Myc agonist, Mad, down regulates the hTERT expression [148-150].

In addition, hTERT has been reported to have increased DNA methylation in its promoter region in hTERT-positive cancer cells and lack methylation in normal hTERT-negative cells [151-155]. This pattern is inconsistent with observations that DNA methylation of promoter CpG islands is typically associated with gene silencing. On the other hand, other reports of hTERT promoter DNA methylation suggest that methylation of the hTERT promoter is associated with gene silencing [152, 153, 156-158]. However, a recent study by Zinn et al showed that there is increased DNA methylation in the hTERT promoter of cancer cells, but hTERT maintains an unmethylated region around the transcription start site allowing the continued expression of hTERT in cancer cells [159].

Characterization of the hTERT gene promoter has also revealed a number of potential binding sites for steroid hormone elements suggesting that steroid hormones may also be involved in regulating hTERT transcription [160-165]. One sex hormone that has been implicated in the development of a number of cancers, especially in breast and uterus, where it is a positive regulator of cell proliferation, is oestrogen [166, 167]. In normal cells, oestrogen exerts profound effects on tissue growth, differentiation and
regeneration in a variety of tissues, including reproductive organs, bone, the cardiovascular system and the central nervous system [168-172]. It has been recently shown that under physiological conditions, there is a significant association between the levels of oestrogen and telomerase activity [173]. It has also been shown that oestrogen rapidly upregulates hTERT gene expression and telomerase activity in several oestrogen receptor (ER) α-positive cancer cell lines [160, 161]. Interestingly, mutation of the two c-Myc binding sites located within the proximal 181 bp promoter region completely abrogates oestrogen activation of telomerase activity even though the oestrogen response element (ERE) is located at the -2677 of the hTERT gene promoter [160]. These findings suggest that oestrogen may regulate hTERT gene transcription by cis and trans mechanisms involving c-Myc [174, 175].

**Telomerase Regulation by Growth Factors**

Growth factors play important roles in both positively and negatively regulating proliferation of a variety of cell types in tissue growth, differentiation, and tumorigenesis. Research efforts have shown that growth factors also regulate telomerase activity.

Molecular regulation of telomerase activity by five growth factors have been extensively studied (Table 1). Epidermal growth factor (EGF) plays an important role in cell proliferation of a variety of cells types by binding to the EGF receptor (EGFR), which stimulates several signal transduction pathways leading to the transcriptional activation of a number of growth-related genes and transcription factors including c-fos, c-jun, and Ets [176, 177]. This signaling pathway activation also plays an important role in the development of many solid tumors [178]. Treatment of EGFR-positive cancer
cells with EGF increases telomerase activity within 24 hours of exposure, which is preceded by a rapid increase in hTERT production, suggesting a transcriptional effect on the hTERT promoter via the Ras/MEK/ERK signaling pathway [176]. In addition, it has been shown that overexpression of the MAP kinase downstream transcription factor, Ets, enhances EGF stimulation of telomerase activity [176, 178].

Fibroblast growth factor 2 (FGF2), also known as basic fibroblast growth factor (bFGF) is expressed in the embryonic central nervous system (CNS) [179] and it may be one of the main regulators of CNS development. It has mitogenic action on cortical progenitor cells and it also regulates the generation of neurons and glia [180-182]. Haïk et al showed that FGF2 dramatically upregulated telomerase activity in neural precursor cells through a possible post-transcriptional mechanism since hTERT mRNA levels were unaffected [183]. The study also proposed that FGF2-mediated upregulation of telomerase activity was mediated by the PI-3-kinase/Akt signaling pathway [183].

A third growth factor that has been associated with cell development, lifespan, and aggressiveness of various tumor types is insulin-like growth factor (IGF) [184, 185]. Studies have shown that IGF-1 stimulates telomerase activity, hTERT mRNA transcription, and protein expression in androgen-dependent and –independent prostate cancer cell lines [184]. IGF-binding protein 2 (IGFBP-2) also stimulates telomerase activity in prostate cancer cells even though it acts as a growth inhibitor in normal prostate epithelial cells [186]. Interestingly, MAP kinase signaling is also involved in IGFBP-2-mediated telomerase activity, but not in IGF-1’s [184, 186]. In addition, Akiyama et al showed that IGF-1 upregulated telomerase activity in multiple myeloma.
cells without altering the hTERT protein expression via the phosphatidylinositol 3’-kinase (PI3k)/Akt/nuclear factor κB (NFκB) signaling pathway [187].

Nerve growth factor (NGF) appears to be involved in the control of epithelial cell growth and differentiation [188]. Studies have shown that during NGF induction of cell proliferation in pheochromocytoma cells and prostate cancer cells, telomerase was significantly downregulated through the MAP kinase pathway [189-191].

Transforming growth factor-β (TGF-β) uniquely regulates telomerase activity in a tumor-specific manner. Treatment of human lung adenocarcinoma cells with TGF-β induces inhibition of telomerase and stimulation of the cancer cell senescence [192]. In addition, overexpression of TGF-β type II receptor (RII) in human colon carcinoma also leads to telomerase inhibition [193]. When RII is disrupted in human breast cancer cells, telomerase activity is increased [193]. The inhibition of telomerase activity by TGF-β correlates with decreased hTERT mRNA levels suggesting transcriptional repression of the hTERT promoter [138, 193]. However, studies of signaling pathways involved in TGF-β-mediated inhibition of telomerase activity have not yet been reported.

The role of telomerase in cancer cell growth and survival is evident. Although inhibition of telomerase may not result in immediate tumor death, the most rapidly proliferating tumors would undergo senescence eventually reducing patient tumor burden. In addition, telomerase inhibitor treatments could be most beneficial when used in conjunction with current, traditional chemotherapeutic agents, tyrosine kinase inhibitors (RTKs) and monoclonal antibodies targeting angiogenesis. Therefore, research studying telomerase regulation by growth factors important for cellular proliferation and
survival may produce novel tools specifically targeting the proliferative lifespan of tumorigenic cells.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Effect on Telomerase Activity</th>
<th>Possible Molecular Mediators</th>
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<tbody>
<tr>
<td>EGF [176,178]</td>
<td>Increases activity in telomerase-positive cells</td>
<td>EGFR, ERK1/2, Ets</td>
</tr>
<tr>
<td>FGF-2 [183, 194, 195]</td>
<td>Increases telomerase in HUVECs and primary cortical cultures</td>
<td>Akt/PI-3-kinase</td>
</tr>
<tr>
<td>IGF-I [184, 187]</td>
<td>Increases activity in prostate cancer cells</td>
<td>Androgen receptor, Akt kinase</td>
</tr>
<tr>
<td>IGFBP-2 [186]</td>
<td>Inhibits telomerase in normal prostate epithelial cells, Increases activity in prostate cancer cells</td>
<td>PI-3-kinase</td>
</tr>
<tr>
<td>NGF [190, 191]</td>
<td>Decreases telomerase in HUVECs, PC12 pheochromocytoma cells, and prostate cancer cells</td>
<td>MAPK</td>
</tr>
<tr>
<td>TGF-β [192, 193]</td>
<td>Decreases activity in colon and lung cancer cells, RII disruption in breast cancer cells increases activity</td>
<td>c-Myc, Smad proteins</td>
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Central Hypothesis

Telomerase activity is regulated by growth factors in ovarian cancer cells.

Specific Aims

Since both VEGF and EGF regulate ovarian cancer cell growth and survival, this study examined whether these two growth factors could regulate telomerase activity in ovarian cancer cells. Three specific aims were proposed to test this hypothesis:

- Evaluate telomerase regulation by VEGF and its positive upstream regulator, LPA.
- Since the nutraceutical, vitamin E, has been shown to degrade and suppress LPA activity, examine the potential of vitamin E to suppress telomerase activity by abrogating LPA-mediated telomerase activity.
- Define EGF-mediated telomerase activity in ovarian cancer cells.
Chapter II

VEGF and LPA Induce Telomerase Activity in Ovarian Cancer Cells

Abstract

Both vascular endothelial growth factor (VEGF) and lysophosphatidic acid (LPA) are secreted by ovarian cancer cells and are known to promote cancer cell growth though the exact molecular mechanism(s) are not completely understood. Since telomerase, a ribonucleoprotein expressed in 95% of ovarian cancer, plays an important role in cellular immortalization, growth, and tumor progression, it was examined whether telomerase is a molecular target of VEGF and LPA in ovarian cancer. My experiments show that: (1) both VEGF and LPA upregulate telomerase activity; (2) LPA induction of telomerase activity is VEGF-dependent; (3) VEGF and LPA induction of telomerase activity is ERK 1/2-dependent; and (4) Sp1 binding sites within the proximal 976- to 378-bp regions of the hTERT promoter are essential for VEGF- and LPA-induced hTERT promoter activity. Consequently, these data show the novel finding that VEGF can regulate telomerase in non-endothelial cells and that telomerase appears to be a novel molecular target of LPA.
Introduction

Ovarian cancer is the leading cause of gynecological cancer death in the United States [196]. Although a number of genetic changes have been identified in ovarian cancer, no single genetic alteration is common to all ovarian cancers [197, 198]. However, telomerase activity is found in 90% of malignant tumors, including ovarian cancer [199]. Telomerase is a ribonucleoprotein that consists of an RNA (hTR) component, a reverse transcriptase (hTERT) component and associated proteins that elongates telomeric (TTAGGG)_n DNA sequences de novo [89]. Both the protein catalytic hTERT and hTR subunits have been identified and cloned, and it is thought that the protein catalytic subunit, hTERT, is the limiting determinant of telomerase activity [102, 200]. In the ovary, telomerase is absent in normal ovarian surface epithelium (OSE) and pre-malignant lesions, but tumor cells from both ascites fluid and ovarian carcinomas express telomerase activity [89, 201].

Vascular endothelial growth factor (VEGF) is a homodimeric 34-42 kDa, heparin-binding glycoprotein growth factor that plays a predominant pro-angiogenic role [202]. Five common isoforms exist (VEGF_{121}, VEGF_{144}, VEGF_{165}, VEGF_{189}, VEGF_{206}) as a result of alternate gene splicing [203, 204]. VEGF_{165} is the major isoform produced by several normal and tumor cells. Once ligand bound, VEGFR-2, the major VEGF receptor involved in VEGF-mediated cell growth and angiogenesis, activates downstream signaling pathways, including mitogen activated protein kinase (MAPK) also known as extracellular signal-regulated kinase 1/2 (ERK 1/2) [205]. Recent reports indicated that a number of cancers including breast [206], prostate [207], leukemia [208], pancreatic...
and ovarian express VEGF receptors, VEGFR-1, VEGFR-2 and neuropilin, that function in an autocrine loop to promote tumor cell growth, migration, and survival.

Likewise, lysophosphatidic acid (LPA) is found in very high levels in plasma and ascites of ovarian cancer patients and as a consequence it is considered a potential diagnostic marker for ovarian cancer. LPA is the simplest glycerophospholipid that mediates several cellular responses, including smooth muscle cell contraction, regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis and reproductive functions. It interacts with at least three specific cell surface G-protein-coupled receptors of the endothelial differentiation gene (Edg) family, LPA1 (Edg-2), LPA2 (Edg-4), and LPA3 (Edg-7). Ovarian cancer cells, particularly, express LPA receptors, LPA1 and LPA2 while the normal ovarian cells only express LPA1. In addition, LPA stimulates VEGF production in ovarian cancer cells suggesting that LPA can act in both a paracrine and autocrine manner for ovarian cancer tumor growth.

Since VEGF and LPA are secreted by ovarian cancer cells and play important roles in cell growth and proliferation, VEGF regulation of telomerase activity and the possible contribution of LPA as an upstream regulator of VEGF-mediated telomerase activity in ovarian cancer were examined. My data show that both VEGF and LPA upregulate telomerase activity by ERK 1/2-dependent transcriptional activation of Sp1 binding sites within the 976- to 378-bp hTERT promoter regions. Furthermore, results show that LPA-mediated induction of telomerase activity is VEGF-dependent.
Materials and Methods

Cell Lines and Tissue Culture

Telomerase-positive ovarian carcinoma cell lines PA-1, SW 626, and one non-tumorigenic SV40 large-T antigen-transfected human ovarian surface epithelial (IOSE) cell line, FHIOSE 118, derived from normal ovarian surface epithelium were used [217]. Cells were cultured in Medium 199/MDCB 105 (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 µg/mL Gentamicin (GIBCO, NY). The cells were incubated at 37ºC with 5% CO₂ / 95% air.

Treatment with VEGF, LPA, FGF-2, TNF-α, TGF-β, anti-hVEGF Antibody, CBO-P11 and U0126

Each cell line was grown in serum-free media for at least 18 hours prior to treatment with 25 to 150 ng/mL VEGF dissolved in 0.1% BSA (BioSource, Camarillo, CA), 20 µg/mL Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA) dissolved in 1% BSA (Sigma Aldrich, St. Louis, MO), 20 ng/mL FGF-2 dissolved in 10 mM Tris pH 7.6 (Sigma Aldrich, St. Louis, MO), 1 ng/mL TNF-α dissolved in 5 mM Tris pH 8.0 (Sigma Aldrich, St. Louis, MO), 5 ng/mL TGF-β dissolved in 4mM HCl containing 0.1% BSA (Sigma Aldrich, St. Louis, MO), 0.1 mg/mL anti-hVEGF Ab dissolved in PBS (R&D Systems, Minneapolis, MN), 1.3 µM VEGF receptor inhibitor, CBO-P11, dissolved in Milli-Q water (Calbiochem, La Jolla, CA), or 10 µg/mL U0126 MAPK inhibitor dissolved in methanol (Cell Signaling, Beverly, MA). Cultures were harvested at various time intervals up to 24 hours.
Telomerase Assay

To quantitatively detect changes in telomerase activity levels, cells were assayed for telomerase activity using the telomerase polymerase chain reaction-sandwich enzyme-linked immunosorbent assay (PCR-ELISA) following the manufacturer’s protocol (Roche, Indianapolis, IN) and as previously described [218]. Briefly, cells were washed with DPBS, trypsinized, and centrifuged at 500 g for 5 minutes. Pellets were washed twice in DPBS, then resuspended in 150 µL of lysis buffer. The cell pellets were kept on ice for 30 minutes and then centrifuged at 100,000 g for 60 minutes at 4°C. Supernates were then assayed using the Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA) to determine protein concentration following detergent solubilization according to manufacturer’s instructions. To perform the telomerase PCR-ELISA within a linear range, all of the cells extracts equivalent to 3 µg of protein were used. Following PCR-ELISA, telomerase activity was detected using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and recorded as optical density ± S.E.

RT-PCR

To examine the contribution of transcriptional control of telomerase regulation by VEGF or LPA, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) studies were performed as previously described [218]. Total RNA was collected using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample. The cDNA samples were amplified using the Perkin-Elmer (Palo Alto, CA) GeneAmp kit. The hTERT primers used were hTERT-S (CGGAAGAGTGCTCTGGAGCCAA) and hTERT-
AS (GGATGAAGCGGAGTCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) and with β-actin primers Actin-S (CAGGTCATCACCATTGGCAATGAGC) and Actin-AS (GATGTCCACGT CACACTTCATGA) for an internal control. The amplified products were then separated by electrophoresis on a 1% agarose, stained with 1X SyberGreen (FMC Bioproducts, Rocklan, ME) and analyzed with the Kodak EDAS 120 Digital Analysis System. Net hTERT mRNA intensities from treated samples were normalized to their corresponding β-actin mRNA levels.

**Western Blot Analysis**

For Western blot analyses, cell lysates were prepared in CHAPS lysis buffer as previously described [219]. Protein concentrations of cell lysates were determined using the Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA) in accordance to the manufacturer’s instructions. Protein extracts (20 µg) were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) and separated via 12.5% SDS-PAGE and electroblotted onto Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ) by wet transfer. Immunoblotting was performed using antibodies against ERK 1/2 (1:1000) and phospho-ERK 1/2 (1:1000; Cell Signaling, Beverly, MA), and Sp1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin (1:5000, Sigma Aldrich, St. Louis, MO) was used as loading control. Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Blots were scanned and analyzed with Life Sciences GE Healthcare.
ImageQuant image analysis software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Values reported for target proteins were normalized to the blots' respective β-actin levels.

**Luciferase Reporter Assay**

To measure hTERT promoter activity, the full-length (pGL3-1375), and deleted hTERT promoter-luciferase constructs (pGL3-1175, -976, -776, -578, -378, -181) were used as previously described [220]. To determine whether the transcription factor, Sp1, was involved in VEGF- and LPA-induced telomerase activation, cells were transfected with 1 µM Sp1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA). SW 626 cells were serum starved for 24 h and then transiently co-transfected with 3 µg of DNA and 1 U of β-galactosidase cDNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) in serum- and antibiotic-free media. Cultures were replenished with serum 1h after transfection. Prior to collection, SW 626 cells were treated with ± VEGF (50 ng/mL for 4 hours), ± LPA (20 µg/mL for 24 hours), ± CBO-P11 (1.3 µM for 4 hours), and ± U0126 (10 µg/mL for 4 hours). Expression of luciferase was measured 48 h after transfection using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturers’ instructions. At the time of collection, cells were microscopically observed to ensure cell viability with no signs of apoptosis using parallel cultures transfected with green fluorescent protein (GFP). Transcriptional activity was expressed as relative luciferase activity ± S.E., after normalization with β-galactosidase activity.
VEGF ELISA

To measure VEGF production by cultured cells, equal amounts of conditioned media from cell lines treated with ± 20 µg/mL LPA for 24 hours were collected, centrifuged at 500 g for 5 minutes at room temperature to remove any cellular debris and the supernatants were frozen at -80°C until assayed using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions for VEGF165 levels. The enzymatic reaction was detected at 450 nm using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and the results expressed as pg/mL VEGF of triplicate experiments ± S.E.

Statistical Analysis

Samples for VEGF ELISA, telomerase PCR-ELISA, RT-PCR, and luciferase assays were run in triplicate, and the data were subjected to the Student’s t test for determination of statistical significance.
Results

VEGF stimulates telomerase activity

To determine whether VEGF mediated telomerase activity in ovarian cancer cells, time-dependent and dose-dependent assays were performed (Figures 5A and B). Telomerase activity was measured by PCR-ELISA for 0 to 24 hours following treatment with 0 to 150 ng of VEGF, respectively. Cells were incubated in media containing 0.1% FBS for 24 hours prior to culturing them in the presence or absence of VEGF. Figure 5A shows a gradual VEGF-mediated increase in telomerase activity peaking at four hours. Ovarian cancer cells PA-1 and SW 626 showed a 56% and a 63% increase in telomerase activity when treated for four hours, respectively. Fifty ng/mL VEGF elicited greatest increase in telomerase activity in PA-1 (54%) and SW 626 (55%) cells (Figure 5B). Different dosages of VEGF or treatment intervals did not have any effect on telomerase activity in telomerase negative FHIOSE 118 cells serving as control (Figures 5A and B).

To demonstrate specificity of VEGF for telomerase activity in ovarian cancer cells, cultures were either immuno-depleted of VEGF with anti-hVEGF antibody (Figure 6A), treated with FGF-2, TNF-α or TGF-β (Figure 6A, inset) or treated with VEGF receptor inhibitor CBO-P11, which blocks the binding of VEGF165 to its receptors (IC50 = 700 nM for VEGFR-1, 1.3 µM for VEGFR-2) (Figure 6B). Immuno-depletion with anti-hVEGF antibody in PA-1 and SW 626 cancer cells resulted in a moderate suppression of endogenous telomerase activity, 20% and 49%, respectively (Figure 6A). However, immuno-depletion significantly suppressed VEGF-mediated activation of telomerase activity in PA-1 (47%) and in SW 626 (56%) cancer cell lines (Figure 6A). In addition,
FGF-2, TNF-α and TGF-β failed to induce telomerase activity in either ovarian cell lines (Figure 6A, inset) indicating that not all growth factors generically increase telomerase activity in ovarian cancer cells. Furthermore, CBO-P11 suppressed endogenous telomerase activity in PA-1 (20%) and SW 626 (36%) cells (Figure 6B). CBO-P11 also inhibited VEGF-mediated activation of telomerase activity in both PA-1 (61%) and SW 626 (71%) cells (Figure 6B). Treatments with anti-hVEGF and CBO-P11 diluents (PBS and water) did not alter VEGF induction of telomerase activity. In addition, telomerase-negative FHIOSE 118 cells were unaffected by VEGF inhibition (Figures 6A and B) serving as control.

**VEGF induction of telomerase activity is transcription-dependent**

RT-PCR was employed to determine the relative levels of the telomerase catalytic hTERT mRNA in FHIOSE 118, SW 626, and PA-1 cells for VEGF-induced telomerase activity (Figure 7). Cancer cell lines PA-1 and SW 626 demonstrated an increase of 67% and 55%, respectively, in relative levels of hTERT-mRNA transcript after 4-hour VEGF treatment when normalized to actin mRNA levels (Figure 7). In contrast, VEGF failed to stimulate any hTERT transcription in the telomerase-negative FHIOSE 118 cells serving as control (Figure 7).
Figure 5  VEGF Induces Telomerase Activity in a Time- and Dose-Dependent Manner

Telomerase activity was measured in cultured human ovarian FHIOSE 118, PA-1, and SW 626 cells treated with 50 ng/mL VEGF and examined for VEGF stimulation of telomerase at various time intervals up to 24 hours (A) and treated with 0 to 150 ng/mL VEGF and collected at 4 hours (B). * indicates the statistical significance of telomerase activity in PA-1 and SW 626 cells treated with 50 ng/mL VEGF for 4 hours compared to untreated controls. Telomerase activity was measured by PCR-ELISA and expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Figure 6  VEGF Induces Telomerase Activity

To show specificity of VEGF to induce telomerase activity, cultures were immunodepleted of VEGF with anti-hVEGF antibody (A) or cells were treated with VEGF receptor inhibitor, CBO-P11 (B). FHIOSE 118, PA-1, and SW 626 cells were treated with ± 50 ng/mL VEGF for 4 hours along with ± 0.1 mg/mL anti-hVEGF (A) ± 1.3 µM CBO-P11 (B). Samples were also treated with the diluents, PBS and water, serving as controls. p values indicate statistical significance between VEGF treated cells vs. untreated cells (*), VEGF + anti-hVEGF or CBO-P11 vs. VEGF treated cells (**). FHIOSE 118 and SW 626 cell lines were also treated with either 20 ng/mL FGF-2, 1 ng/mL TNF-α or 5 ng/mL TGF-β and examined for telomerase activity (A, inset). Telomerase activity was measured by PCR-ELISA and expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Figure 7  VEGF Increases hTERT Transcription Levels

FHIOSE 118, PA-1, and SW 626 cells were incubated ± 50 ng/mL VEGF for 0, 30 minutes, 2 and 4 hours in triplicate and examined by RT-PCR for hTERT mRNA. Results are expressed as relative hTERT mRNA expression ± S.E. normalized to corresponding β-actin mRNA levels. p values indicate the statistical significance of hTERT mRNA levels in PA-1 and SW 626 cells treated with 50 ng/mL VEGF at 4 hours compared to their untreated controls (*).

VEGF-mediated telomerase activity is ERK 1/2-dependent

In agreement with Pages et al [221], I found that VEGF increased ERK 1/2 phosphorylation (Figure 8). Western blot and densitometric analyses showed that VEGF increased ERK 1/2 phosphorylation by 50% and 61% in PA-1 and SW 626 ovarian cancer cells, respectively (Figure 8, inset). Treatment with U0126 suppressed ERK 1/2 phosphorylation and completely abrogated VEGF-mediated ERK 1/2 phosphorylation (Figure 8, inset).
Likewise, inhibition of ERK 1/2 by U0126 abrogated VEGF-mediated stimulation of telomerase activity to below endogenous levels (Figure 8) suggesting a role for MAPK in VEGF-mediated activation/regulation of telomerase activity. Treatments with U0126 diluent, methanol, did not alter VEGF induction of telomerase activity or VEGF-mediated ERK 1/2 phosphorylation. Telomerase-negative FHIOSE 118 cells remained telomerase negative and were unaffected by ERK 1/2 inhibition thereby serving as a negative control.

Figure 8  VEGF Induction of Telomerase Activity is ERK 1/2-Dependent
Cultures of FHIOSE 118, PA-1, and SW 626 were treated with ± 10µM U0126 and ± 50 ng/mL VEGF for 4 hours. Samples were also treated with U0126 diluent methanol serving as control. Cells were then harvested and analyzed for telomerase activity by PCR-ELISA. Results were expressed as mean optical density at 490 nm for triplicate samples ± S.E. p values indicate the statistical significance between VEGF treated cells vs. untreated controls (*) and VEGF + U0126 treated cells vs. VEGF treated cells (**). Western blot analysis (Inset) was performed to confirm VEGF-mediated phosphorylation of ERK 1/2 and U0126 inhibition of ERK 1/2 phosphorylation. β-actin was used as the loading control.
Proximal 976- to 378-bp regions are required for induction of hTERT promoter activity by VEGF

Since VEGF increased hTERT mRNA, using full length and deletion reporter constructs, reporter assays were performed to identify the promoter region(s) responsive to VEGF (Figure 9). Compared to endogenous promoter activity (Figure 9), the full length promoter (PGL3-1375) was induced by VEGF by 42% (Figure 9). However, VEGF induced maximal activity between 378-bp and 976-bp hTERT promoter regions, which were at least 4 times greater than that of the full length (PGL3-1375) hTERT promoter (Figure 9). In contrast, VEGF failed to induce activity at the 1175-bp and 378-bp hTERT promoter regions. Interestingly, VEGF suppressed endogenous core promoter (PGL3-181) activity by 136%. This suggests that VEGF positively targets transcription binding sequences within the 976- to 378-bp regions of the hTERT promoter.

To confirm specificity of VEGF induction of the 378-bp to 976-bp hTERT promoter regions, reporter assays were also performed on SW 626 cells treated with 50 ng/mL VEGF and either 1.3 μM VEGF receptor inhibitor CBO-P11 or 10 μg/mL ERK 1/2 inhibitor U0126 (Figure 9). VEGF failed to induce activity of the 1375-bp, 976-bp, 776-bp, and 578-bp regions of the hTERT promoter when co-treated with CBO-P11 by 67%, 42%, 92%, and 78%, respectively (Figure 9). In addition, U0126 significantly suppressed VEGF-mediated induction of luciferase activity of the 1375-bp, 976-bp, 776-bp, and 578-bp regions of the hTERT promoter by 65%, 25%, 91%, and 44%, respectively (Figure 9). Furthermore, co-treatments with VEGF and either CBO-P11 or
U0126 alleviated VEGF-mediated suppression of 181-bp core promoter region by approximately 50% (Figure 9).

**LPA stimulates telomerase activity in ovarian cancer cells in a VEGF-dependent manner**

VEGF ELISA was performed on conditioned media from the ovarian cancer cell line, SW 626, and the IOSE cell line, FHIOSE 118 (Figure 10, inset). In agreement with Hu *et al* [215], it was also observed that LPA increased VEGF secretion at least 1.5-fold in the ovarian cancer cell line, SW 626 and that LPA failed to promote VEGF production in the normal cell line, FHIOSE 118 (Figure 10, inset).

To determine, then, whether telomerase activity was also a molecular target of LPA as an upstream regulator of VEGF, the effect of LPA on telomerase in ovarian cell lines FHIOSE 118, PA-1, and SW 626 was examined (Figure 11). LPA (20 µg/mL) induced significant increases in telomerase activity in ovarian cancer cell lines PA-1 (41%) and SW 626 (41%) (Figure 10). However, LPA failed to stimulate telomerase activity in telomerase-negative FHIOSE 118 ovarian cells serving as control (Figure 10). Treatment with CBO-P11 suppressed 29% and 21% of endogenous telomerase activity in PA-1 and SW 626 ovarian cancer cell lines, respectively (Figure 10). Furthermore, CBO-P11 significantly suppressed LPA activation of telomerase activity in PA-1 (53%) and SW 626 (42%) cancer cell lines (Figure 10) suggesting that LPA-induced telomerase activity is VEGF-dependent.
Figure 9  976- to 378-bp hTERT Promoter Regions are required for VEGF Induction of Telomerase Activity

SW 626 cells were transfected with full length and deleted hTERT promoter reporter constructs as well as a pCMV-β-galactosidase expression vector. Cells were serum-starved for at least 24 hours and treated with ± 50 ng/mL VEGF, ± 1.3 µM CBO-P11, and ± 10 µM/mL U0126. After 48 hours of transfection, luciferase and β-galactosidase assays were performed. Reporter activity was normalized by dividing luciferase activity with β-galactosidase. GFP was used to monitor transfection efficiency. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples and p values indicate statistical differences between luciferase assays in SW 626 cells treated with VEGF vs. untreated controls (*), VEGF treated cells vs. VEGF + VEGF receptor inhibitor CBO-P11 treated cells (^) and VEGF treated cells vs. VEGF + ERK 1/2 inhibitor U0126 treated cells (`).
Figure 10  LPA Induction of Telomerase Activity is VEGF-Dependent

FHIOSE 118, PA-1, and SW 626 cells were treated with 20µg/mL LPA and examined for LPA stimulation of telomerase activity at 24 hours. In addition, ovarian cancer cells were treated with ± 20 µg/mL LPA, ± 1.3 µM CBO-P11, harvested, and examined for telomerase activity by PCR-ELISA. Results were expressed as mean optical density at 490 nm for triplicate samples ± S.E. p values indicate the statistical significance of LPA treated cells vs. untreated controls (*) and LPA + CBO-P11 treated cells vs. LPA treated cells (^). (Inset) Enzyme-linked immunosorbent assay analysis of secreted VEGF protein concentrations in conditioned media of FHIOSE 118 and SW 626 cell lines treated with ± 20 µg/mL LPA for 24 hours. p values indicate the statistical significance of LPA treated SW 626 cells vs. untreated controls (*).
LPA-mediated telomerase activity is ERK 1/2-dependent

In accordance to Kumagai et al [222], it was found that LPA increased ERK 1/2 phosphorylation (Figure 11, inset). Western blot and densitometric analyses found that LPA increased ERK 1/2 phosphorylation by 63% and 33% in PA-1 and SW 626 cancer cells, respectively. Treatment with U0126 suppressed ERK 1/2 phosphorylation and abrogated LPA-mediated ERK 1/2 phosphorylation (Figure 11, inset). Similarly, LPA increased telomerase activity by 37% and 29% in PA-1 and SW 626 cancer cells, respectively. As VEGF, inhibition of ERK 1/2 by U0126 abrogated LPA stimulation of telomerase activity to below endogenous levels (Figure 11) suggesting a role of ERK 1/2 in LPA-mediated induction of telomerase activity.
Figure 11 LPA Induces Telomerase Activity via the ERK 1/2 Pathway

FHIOSE 118, PA-1, and SW 626 cells were treated with ± 20 µg/mL LPA and ± 10 µM U0126 for 24 hours. Cells were then harvested and analyzed for ERK 1/2 protein phosphorylation by Western blot (Inset) and telomerase activity by PCR-ELISA. For Western blot analysis, β-actin was used as loading control. p values indicate the statistical significance of LPA treated cells vs. untreated controls (*) and LPA + CBO-P11 treated cells vs. LPA treated cells (**). For telomerase analysis, results were expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Proximal 976- to 378-bp regions are required for induction of hTERT promoter activity by LPA

Since VEGF positively targeted transcription binding sequences within the 976- to 378-bp regions of the hTERT promoter to induce telomerase activity, additional reporter assays were performed to identify the promoter region responsive to LPA (Figures 12A and B). Compared to endogenous promoter activity, full length promoter (PGL3-1375) was induced by LPA by 42% (Figure 12A). Like VEGF, LPA induced maximal activity between 976- and 378-bp hTERT promoter regions, which were 2 times greater than that of the full length (PGL3-1375) hTERT promoter (Figures 12A and B). Interestingly, like VEGF, LPA also suppressed endogenous core promoter (PGL3-181) activity by 3-fold (Figure 12B).

To confirm that LPA induction of telomerase was ERK 1/2- and VEGF-dependent, reporter assays were performed on SW 626 cells treated with 20 µg/mL LPA and either 1.3 µM VEGF receptor inhibitor CBO-P11 or 10 µg/mL ERK 1/2 inhibitor U0126 (Figures 12A and B). LPA failed to induce activity of the 1375-bp, 976-bp, 776-bp, and 578-bp regions of the hTERT promoter when co-treated with CBO-P11 or U0126 (Figure 12A) suggesting LPA induction of hTERT promoter activity is also ERK 1/2-dependent (Figures 12A and B). Furthermore, like VEGF, co-treatments with LPA and either CBO-P11 or U0126 alleviated LPA-mediated suppression of 181-bp core promoter region (Figures 12A and B).
Figure 12  Proximal hTERT 976- to 378-bp Promoter Regions are Responsive to LPA-Induced Telomerase Activity

SW 626 cells were transfected with full length and deleted hTERT promoter reporter constructs as well as a pCMV-β-galactosidase expression vector. Cells were serum-starved for at least 24 hours and treated with ± 20 µg/mL LPA, ± 1.3 µM CBO-P11, and ± 10 µM/mL U0126. After 48 hours of transfection, luciferase and β-galactosidase assays were performed. Reporter activity was normalized by dividing luciferase activity with β-galactosidase. GFP was used to monitor transfection efficiency. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples and p values indicate statistical differences between luciferase assays in SW 626 treated with LPA vs. untreated controls (*), LPA treated cells vs. LPA + VEGF receptor inhibitor CBO-P11 treated cells (^), and LPA treated cells vs. LPA + ERK 1/2 inhibitor U0126 treated cells (`).
Figure 13 VEGF- and LPA-Induced Telomerase Activity is Sp1-Dependent

SW 626 cells were transfected with 1 µM Sp1 siRNA and treated with ± 50 ng/mL VEGF for 4 hours or ± 20 µg/mL LPA for 24 hours prior to collection. Cells were then harvested and analyzed for Sp1 expression by Western blot (A, B insets) and telomerase activity by PCR-ELISA (A, B). For Western blot analyses, β-actin was used as loading control. p values indicate the statistical significance of Sp1 transfected cells vs. untreated controls (*), VEGF or LPA treated cells vs. untreated controls (~), and Sp1 transfected + VEGF or LPA treated cells vs. VEGF or LPA treated cells (^). For telomerase analysis, results were expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Figure 14  VEGF and LPA Target Sp1 Binding Sites in the hTERT Promoter

(A, B) SW 626 cells were transfected with full length, deletion hTERT promoter luciferase constructs, and Sp1 siRNA along with pCMV-β-galactosidase expression vector and treated with ± 50 ng/mL VEGF for 4 hours (A) or ± 20 µg/mL LPA for 24 hours (B) prior to collection. Luciferase reporter activity was normalized by β-galactosidase activity. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples and p values indicate statistical differences between luciferase assays in Sp1 siRNA transfected cells vs. untreated controls (*), VEGF or LPA treated cells vs. untreated controls (~) and Sp1 siRNA transfected + VEGF or LPA treated cells vs. VEGF or LPA treated cells (^).
VEGF- and LPA-induced hTERT promoter activation is Sp1-dependent

To identify whether Sp1 is a major transcription factor involved in VEGF- and LPA-induced hTERT activation, Western blot analyses (Figure 13), telomerase PCR-ELISA analyses (Figure 13), and reporter assays (Figure 14) were performed using Sp1 siRNA. SW 626 cells were transfected with ± 1 µM Sp1 siRNA and treated with ± 50 ng/mL VEGF or ± 20 µg/mL LPA. Sp1 siRNA reduced endogenous Sp1 protein levels in SW 626 cells transfected with Sp1 siRNA (Figures 13A and B, insets, lane 2) and abolished VEGF- and LPA-mediated increased Sp1 levels (Figures 13A and B, insets, lane 4) compared to Sp1 siRNA untransfected controls (Figures 13A and B, insets, lanes 1 and 3) as determined by Western blot analyses. In addition, siRNA directed at Sp1 was sufficient to decrease endogenous telomerase activity by approximately 42% and decreased VEGF- and LPA-induced telomerase activity by 56% (Figures 13A and B). Furthermore, reporter assays using hTERT full length and deletion reporter constructs confirmed a role for Sp1 in VEGF- and LPA-induced telomerase activity (Figures 14A and B). Compared to the endogenous promoter activity, the full length hTERT promoter was inhibited by Sp1 siRNA by 69% and VEGF- and LPA-induced full length hTERT promoter activities were inhibited by 42% and 45%, respectively (Figures 14A and B). Sp1 siRNA inhibited endogenous 976-bp hTERT promoter activity by 63% and VEGF- and LPA-induced promoter activity was reduced by 80% and 69%, respectively. In contrast, the 776- to 378-bp hTERT promoter regions were not significantly affected by Sp1 siRNA (Figures 14A and B).
Discussion

Telomerase regulation is complex involving transcriptional regulation [138], alternate mRNA splicing [223], and post-translational regulation by hTERT phosphorylation [175]. Recent studies have also shown that growth factors are crucially involved in regulating telomerase activity and hTERT reverse transcriptase gene expression. For instance, in prostate cancer cells, insulin-like growth factor 1 (IGF-1) stimulates baseline telomerase activity up to 10-fold through hTERT phosphorylation and up-regulation of hTERT mRNA expression [184].

The present experiments assessed telomerase as a potential molecular target of VEGF and its upstream regulator, LPA, in ovarian cancer cells. Results show that both VEGF[165] and LPA increased telomerase activity in telomerase-positive ovarian cancer cells, but not in telomerase-negative IOSE cells. Inhibition of VEGF with either neutralizing antibodies or VEGF receptor inactivation suppressed VEGF- and LPA-mediated induction of telomerase activity. Therefore, these data demonstrate that VEGF and LPA are not limited to regulation of endothelial cell growth and survival, but also appears to regulate telomerase activity in ovarian cancer cells, thereby providing a plausible mechanism for the presence of their receptors in non-endothelial cells. Both, VEGF and LPA, directly activated telomerase through up-regulation of hTERT transcription via the ERK 1/2 pathway and positively targeted transcription binding sequences within the 976- to 378-bp regions of the hTERT promoter. Maximal baseline hTERT promoter activity was noted at 976- and 181-bp regions, possibly due to
endogenous growth factors and transcription factors present in the cells. In contrast, maximal exogenous VEGF- and LPA-mediated transcriptional activity targeted to the 976- to 378-bp regions of the hTERT promoter which contains putative binding sites for several transcription factors including Sp1 [224]. Sp1 silencing suggested that Sp1 is one major transcription factor involved in the VEGF- and LPA-induced transactivation of hTERT in ovarian cancer cells. Since Sp1 silencing was not sufficient to completely abrogate VEGF- and LPA-induced telomerase activity, it is likely that additional transcription factors, possibly such as AP1 and AP2, binding within the 976- to 378-bp regions of the hTERT promoter also promote of VEGF- and LPA-mediated telomerase activity in ovarian cancer cells.

Of interest binding sites for AP1 and AP2 are present in the 976- to 378-bp regions of the hTERT promoter. In addition, p42/p44 MAP kinases activate the VEGF promoter at its -88 to -66 region where Sp1 and AP2 binding sites reside in endothelial cells [225]. Likewise, TGF-α also induces VEGF synthesis by AP2-mediated transcriptional activation in cervical cancer cells [226, 227]. Similarly, it has been suggested that LPA stimulation of ovarian tumor growth via VEGF occurs through activation of AP1 and Sp1 [215]. Since both Sp1 and AP2 play important roles in LPA and VEGF regulation, AP2 in conjunction with Sp1 may be important in LPA- and VEGF-mediated telomerase activity in ovarian cancer.

While maximal telomerase induction was noted within the 976- to 378-bp regions of the hTERT promoter, VEGF and LPA suppressed activity of the core promoter region
(PGL3-181) of the hTERT promoter. The core promoter region contains two E box binding factors and putative protein binding sites for transcription factors Sp1 and Ets [138]. However, E box binding proteins are known to heterodimerize with a variety of transcription factors with helix-loop-helix domains including Myc-related family members and Max-related family members [228]. c-Myc may form dimers with Max, which then binds to specific E-box binding sites to transactivate the hTERT promoter. However, c-Myc can also dimerize with Mad1 at the same binding site to suppress hTERT transcription [229]. Since the pro-angiogenic growth factor, transforming growth factor β1 (TGF-β1), has been shown to upregulate Mad1 expression resulting in a repression the hTERT promoter and telomerase activity [230], it is possible that VEGF and/or LPA, likewise, could favor c-Myc and Mad1 dimerization contributing to suppression in the hTERT core promoter. Therefore, additional studies are needed to determine whether VEGF and LPA favor dimerization of Myc and Mad1 in this region of the promoter as mechanisms for core hTERT promoter repression. However, net activation of the hTERT promoter by VEGF and LPA favor upregulation of telomerase activity.

Most studies examining the relationship of VEGF-mediated telomerase activity have focused in endothelial cells [231-233]. However, little is known about the regulation of hTERT by VEGF in non-endothelial cells. Zaccagnini et al showed that VEGF increased expression of mouse TERT and telomerase activity in skeletal muscles, satellite and endothelial cells contributing to tissue regeneration following hind limb
ischemia [234]. Several groups have reported a correlation between VEGF and hTERT mRNA expressions in breast carcinomas potentially contributing to the aggressive behavior of these tumors [235, 236]. My data, then, are the first to demonstrate not only the ability of VEGF and LPA to regulate telomerase in non-endothelial cells, but that telomerase may be a unique cellular target of both VEGF and LPA functions in ovarian cancer cells (Figure 15). Consequently, the effectiveness of anti-angiogenic therapies may be two-fold, suppression of new blood vessel growth from local endothelial cells and inhibition of telomerase activity within tumor cells, and, therefore, may have important implications for the clinical management of tumors. In summary, these data represent a paradigm shift in our previously held views of VEGF solely as a regulator of endothelial cell growth and survival and identify telomerase as a novel molecular target of LPA.
Figure 15 Proposed VEGF/LPA Regulation of Telomerase Activity in Ovarian Cancer Cells

Delineation of a possible signaling pathway for induction of telomerase activity by VEGF as well as by its upstream regulator, LPA. LPA, a potential biomarker for ovarian cancer, promotes VEGF production and secretion by ovarian cancer cells. In turn, VEGF can act in a paracrine or autocrine manner to promote angiogenesis and telomerase activity, respectively. The latter occurs in an ERK 1/2-dependent signaling manner targeting transcription factors, including Sp1, to the -976 to -378 hTERT promoter regions to enhance hTERT transcription and, subsequently, telomerase activity, essential for cancer cell survival.
Chapter III

Vitamin E Suppresses LPA-Mediated Induction of Telomerase Activity

in Ovarian Cancer Cells

Abstract

Dietary factors influence tumor formation and progression. Vitamin E is a dietary anti-oxidant capable of eliminating free radical damage, inducing apoptosis, and decreasing oncogene expression suggesting that vitamin E may be a strong candidate for cancer prevention and/or chemotherapeutic intervention. In addition, vitamin E is known to degrade and suppress LPA activity. Since I previously showed that lysophosphatidic acid (LPA) induces telomerase activity in human ovarian cancer cells, I studied whether vitamin E could indirectly suppress telomerase activity by abrogating LPA activity. My data show that (1) vitamin E consistently abolished LPA-stimulated telomerase activity; (2) vitamin E suppressed endogenous telomerase activity in ovarian cancer cells; (3) vitamin E reduced hTERT mRNA transcript levels; (4) vitamin E reduced hTERT promoter activity maximally targeting the –976 to –578 bp promoter regions; and (5) vitamin E improved cisplatin-mediated cytotoxicity as evidenced by reduced cancer cell growth and increased caspase 3 activity. These data suggest that, by suppressing
telomerase activity, vitamin E may be an important protective agent against ovarian cancer cell growth as well as potentially effective therapeutic adjuvant.

**Introduction**

Ovarian cancer is the leading cause of gynecologic cancer death in women in the US. It is the fourth leading cause of cancer death among women after lung, breast, and colorectal cancer [1] and is associated with a 1.7% lifetime risk [2]. It is estimated that 22,220 new cases are diagnosed in the US annually and that 16,210 women die annually from this disease [1]. The one-year survival rate for ovarian cancer can be as high as 79% and when diagnosed in an early stage the 5-year survival rate is almost 95% [1]. However, when diagnosed at a later stage, the 5-year survival rate is generally no better than 35% [1]. Neither the survival rate nor the treatment for ovarian cancer has changed significantly for 30 years. Optimal cytoreduction followed by platinum-based chemotherapy remains the mainstay of therapy in the management of advanced epithelial ovarian cancers [237]. However, while the response rate to primary chemotherapy can be as high as 76%, response rate is dramatically reduced after relapse of the disease [46]. Platinum resistance, defined as disease recurrence less than six months from completion of therapy is an important prognosis predictor. Patients with platinum-resistant tumors have a response rate of less than 10% when retreated with platinum compounds [46]. Alternative options also have poor responses rates of 18-30% [238-240].

The majority of ovarian cancers are sporadic in origin, but about 10% of all epithelial ovarian carcinomas are associated with a hereditary predisposition and are
characterized by an increased incidence and earlier onset of disease [198]. Epidemiological studies suggest that, besides race [241] and familial history of breast or ovarian cancer [198], events associated with OSE traumatization may result in aberrant OSE growth leading to ovarian epithelial carcinogenesis [242]. Specifically, it has been suggested that incessant ovulation [243] causes rapid cycles of OSE division leading to errors in DNA replication and repair resulting in the inactivation and overexpression of tumor suppressor genes and oncogenes, respectively [244]. Thus, increased age, reproductive history (nulliparity), early menarche, late menopause, and fertility drug use increase the risk for ovarian cancer. In contrast, suppression of ovulation by pregnancy, lactation or oral contraceptive use decreases the risk for ovarian cancer [245, 246]. Lifestyle factors including dietary fat intake and smoking may also increase the risk for ovarian cancer while dietary intake of vitamins A, C, D, and E may protect against ovarian cancer [247, 248].

Though a number of genetic abnormalities have been identified [197, 198], no single genetic alteration is common to all ovarian cancers. Telomerase, a ribonucleoprotein that elongates telomeric (TTAGGG)$_m$ DNA repeats \textit{de novo} [89], is expressed in over 90% of ovarian tumors [249] and plays an important role in immortalization and carcinogenesis [136]. In the ovary, telomerase activity is absent in normal OSE and pre-malignant lesions, while tumors cells from both ascites fluid and ovarian carcinomas express telomerase activity [250, 251]. Though telomerase
regulation is complex [138, 175, 223, 252, 253], telomerase activity in ovarian cancer also correlates with clinical stage and tumor aggressiveness [249, 250].

While the primary function of telomerase is the maintenance of structural integrity at the linear chromosome ends, recent studies have shown an association between telomerase activity and increased chemotherapeutic resistance consistent with poor prognosis in ovarian cancers [254]. Telomerase appears to mediate its protective effect by conferring resistance to apoptosis [116, 120]. Likewise, I have shown that telomerase re-expression is associated with reduced caspase-mediated apoptosis and increased Bcl-2 expression in OSE cell lines [118].

LPA, the simplest glycerophospholipid, is present at high levels in plasma and ascites fluid from ovarian cancer patients and is thought to be a potential diagnostic marker for ovarian cancer [255]. Reduced levels of lipid phosphate phosphatase (LPP) and the production of aberrant forms of LPA may contribute to elevated LPA levels associated with ovarian cancer [256, 257]. LPA appears to assist ovarian cancer cell growth and invasion by numerous mechanisms including activation of cyclin D1 and metalloproteinases [258, 259]. In addition, ovarian cancer cells express LPA receptors, LPA1 (Edg2) and LPA2 (Edg4) [215].

Previously, I have shown that LPA induces telomerase activity in human ovarian cancer cells. Since it has been shown that vitamin E degrades and suppresses LPA activity, I sought to determine whether vitamin E inhibits LPA-mediated induction of telomerase activity in human ovarian cancer cells.
Materials and Methods

Cell Culture

The human ovarian cancer cell lines, C13, OV2008, PA-1, SW 626, A2780s, A2780cp, the telomerase-negative SV-40 large T antigen transfected normal ovarian surface epithelial (OSE) [217] cell lines FHIOSE 1816-575, FHIOSE 118, IOSE 80, and the telomerase-positive SV-40 large T antigen/hTERT transfected IOSE 80 + hTERT cell line [118, 260] maintained in our laboratory were used. Cells were maintained in Medium 199/MDCB 105 (1:1) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 µg/mL gentamicin (GIBCO BRL, Grand Island, NY) in a humidified 5% CO₂/95% air atmosphere. Cells were treated with 0-100 IU d-alpha tocopheryl acetate (vitamin E) ± 20 µg/mL Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA) ± 25 µM of cisplatin (CDDP) (Sigma-Aldrich, St. Louis, MO) for 0 to 72 hours and then collected to perform the analyses described below.

PCR-ELISA Telomerase Assay

Telomerase activity was measured using the telomerase polymerase chain reaction enzyme-linked immunosorbent (PCR-ELISA) assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions and as performed previously [252, 253]. This assay has been demonstrated to be as sensitive as the radioactive telomere repeat amplification protocol (TRAP) assay [261]. Briefly, cells were washed with DPBS, trypsinized, counted, and centrifuged at 500 g for 5 minutes.
Pellets were washed twice in DPBS, then resuspended in 200 µL of lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) and kept on ice for 30 minutes, after which time the lysates were centrifuged at 100,000 g for 60 minutes at 4°C. Lysates were then assayed using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) for the determination of protein concentration, according to the manufacturer’s instructions. In order to perform the telomerase PCR-ELISA within a linear range, cell extracts equivalent to 3 µg of protein were used. Following PCR-ELISA, telomerase activity was detected using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and recorded as absorbance units from triplicate samples ± S.E.

**RT-PCR**

To determine the contribution of vitamin E for transcriptional regulation of telomerase, RT-PCR studies were performed as described previously [252, 253]. Total RNA was collected using Trizol reagent (GIBCO BRL, Grand Island, NY). One microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample. To ensure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with the duplicate preparation lacking reverse transcriptase. The cDNA samples were amplified using the Applied Biosystems GeneAmp kit (Foster City, CA). The hTERT primers used were hTERT-S (CGGAAGAGTGTCTGGAGCAA) and hTERT-AS (GGATGAAGCGGAGTCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) with β-actin primers actin-S
(GGGAATTCAAAACTGGAACGGTGAAGG) and actin-AS (GGAAGCTTATCAAAGTCCTCGGCCACA) for an internal control. PCR was performed for 33 cycles of 95°C for 20 seconds, 68°C for 40 seconds, and 72°C for 30 seconds. β-actin primers were added at cycle 16. The amplified products were then separated by gel electrophoresis, stained with 1X SybrGreen (Cambrex Bioscience Rockland, Inc., Rockland, ME), and analyzed with the Kodak EDAS 120 Digital Analysis System. Net hTERT mRNA intensities from triplicate treated samples were normalized to their corresponding β-actin mRNA levels and expressed as the average percent of control hTERT mRNA ± S.E.

VEGF ELISA

To measure VEGF production by cultured cells, equal amounts of conditioned media from cell lines treated with ± 20 µg/mL LPA ± 100 IU vitamin E for 24 hours were collected, centrifuged at 500 g for 5 minutes at room temperature to remove any cellular debris and the supernatants were frozen at -80°C until assayed using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions for VEGF\textsubscript{165} levels. The enzymatic reaction was detected at 450 nm using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and the results expressed as ρg/mL VEGF of triplicate experiments ± S.E.
SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blot analyses were performed as described previously [252, 253]. Adherent cell populations were trypsinized, pelleted for 5 minutes at 500 g, and lysed in ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 30 minutes at 4°C. Lysates were then centrifuged at 100,000 g for 1 hour at 4°C. Protein concentrations of the lysates were determined using the DC Protein Assay (Biorad, Hercules, CA) according to the manufacturer’s instructions. Twenty micrograms of protein were added to 4X loading buffer (250 mM Tris pH 6.8, 8% SDS, 20% glycerol, 0.012% bromophenol blue, 4% β-mercaptoethanol), heated to 95°C for 5 minutes, electrophoresed in 12.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane (Amersham Piscataway, NJ). All membranes were blocked for 1 hour with 5% non-fat milk Tris Buffered Saline plus 0.1% Tween-20 (T-TBS) and incubated at least overnight at 4°C in primary antibody. Polyclonal antibody to procaspase 3 (35 kDa) (1:1000), cleaved caspase-3 (19/17 kDa) (1:1000), and cleaved DFF45 (12 kDa) (1:1000) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Membranes were incubated and developed according to Enhanced Chemiluminescent Protocol, according to manufacturer’s instructions (Amersham Piscataway, NJ). After initial blotting, membranes were reprobed for β-actin (Sigma-Aldrich, St. Louis, MO) to ensure even loading. Blots were scanned and analyzed with Life Sciences GE Healthcare ImageQuant image analysis software (GE Healthcare Bio-Sciences Corp., Piscataway,
Values reported for target proteins were normalized to the blots' respective β-actin levels. Triplicate samples were expressed as average ± S.E.

**Luciferase Reporter Assay**

To measure hTERT promoter activity, the full-length (pGL3-1375), and deleted hTERT promoter-luciferase constructs (pGL3-1175, -976, -578) were used as previously described [220]. SW 626 cells were serum starved for 24 hours and then transiently co-transfected with 3 µg of DNA and 1U of β-galactosidase cDNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) in serum- and antibiotic-free media. Cultures were replenished with serum 1 hour after transfection. Expression of luciferase was measured 48 hours after transfection using the Luciferase Assay System (Promega, Madison, WI) according to manufacturer’s instructions. At the time of collection, cells were microscopically observed to ensure cell viability with no signs of apoptosis and parallel cultures were transfected with green fluorescent protein (GFP) to ensure high transfection efficiency. Transcriptional activity results from three independent studies were expressed as relative luciferase activity ± S.E., after normalization with β-galactosidase activity.

**MTS Assay for Cell Viability**

Cell growth was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay which is based on the soluble formazan production by dehydrogenase enzymes found in metabolically active cells (Promega, Madison, USA). Cells were trypsinized and 2 X 10³ cells were seeded in 96-well plates in 0.2 mL of growth media ± vitamin E (100 IU) ±
CDDP (25 µM). Prior to collection, MTS/phenazine methosulfate (PMS) solution was added to each well and incubated at 37°C for 1 hour, at which time the absorbance at 490 nm was determined using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and the results expressed as mean absorbance of triplicate experiments ± S.E.

Statistical Analysis

Samples for telomerase PCR-ELISA, RT-PCR, luciferase, Western blot, and MTS assays were run in triplicate, and the data were subjected to the Student’s t test for determination of statistical significance between control and treated samples.

Results

Vitamin E suppresses telomerase activity

To evaluate the effect of vitamin E on telomerase activity, the concentration- and time-dependent effects of vitamin E on telomerase activity in SW 626 ovarian cancer cells were initially determined. PCR-ELISA showed vitamin E clearly decreased telomerase activity in human ovarian cancer cells. Incubation of SW 626 cells with increasing concentrations of vitamin E (0-100 IU) for 24 hours resulted in a concentration-dependent down-regulation of telomerase activity with maximal telomerase inhibition (3-fold suppression of activity) when treated with 100 IU vitamin E (Figure 16A). Likewise, vitamin E-mediated inhibition of telomerase was also time-dependent. Greatest suppression of telomerase by vitamin E occurred at 24 hours when
telomerase activity decreased by 55% (Figure 16B). Therefore, cells were subsequently treated for 24 hours with 100 IU vitamin E in ensuing experiments. A panel of additional ovarian cancer cell lines, PA-1, C13, OV2008, A2780s, and A2780cp, were treated with 100 IU vitamin E for 24 hours to confirm vitamin E-mediated suppression of telomerase. Though there was no consistent vitamin E-mediated suppression of telomerase between drug sensitive parental cell lines (OV2008, A2780s) with their drug-resistant daughter cell lines (C13, A2780cp), each cell line showed a significant decrease in telomerase activity compared to their respective controls (C13 21%, OV2008 24%, A2780s 54%, and A2780cp 32%) (Figure 17), thereby substantiating that vitamin E suppresses telomerase activity in ovarian cancer cells. In contrast, telomerase remained undetectable in telomerase-negative ovarian cell lines, FHIOSE 1816-575 and 118, when treated with vitamin E (Figure 17).
Figure 16  Vitamin E Suppression of Telomerase Activity is Dose- and Time-Dependent

Telomerase activity was assessed by PCR-ELISA in cultured human ovarian cancer SW626 cells treated with 0 to 100 IU vitamin E (A) for 0 to 24 hours (B). * indicates the statistical significance of levels of telomerase activity in SW626 cells treated with 100 IU vitamin E for 24 hours compared to untreated controls. Data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E.
Figure 17 Vitamin E Suppresses Telomerase Activity in Human Ovarian Cancer Cells

FHIOSE 1816-575, FHIOSE 118, SW 626, PA-1, OV2008, C13, A2780s, and A2780cp cells were treated ± 100 IU vitamin E for 24 hours, collected, and examined for telomerase activity by PCR-ELISA. p values indicate statistically significant differences in telomerase activity between vitamin E treated cell lines and their untreated counterparts. Data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E.

Vitamin E decreases hTERT RNA expression

Since vitamin E significantly suppressed telomerase in SW 626 ovarian cancer cells, SW 626 ovarian cancer cells were used in subsequent experiments to determine the potential role of vitamin E on telomerase transcription. RT-PCR was performed following incubation of SW 626 cells with 100 IU vitamin E for 0, 3, 18, and 24 hours to
determine whether vitamin E-mediated suppression of telomerase was transcription dependent (Figure 18). Vitamin E steadily decreased hTERT mRNA levels with greatest vitamin E-mediated suppression resulting in almost 30% decreased hTERT mRNA expression at 18 hours (Figure 18) which preceded maximal inhibition of telomerase activity at 24 hours (Figure 17). Actin mRNA levels remained unchanged throughout vitamin E treatments (Figure 18).

**Figure 18** Vitamin E-Mediated Suppression of Telomerase is Transcription-Dependent

SW 626 cells were incubated ± 100 IU vitamin E for 0 to 24 hours, examined by RT-PCR for hTERT mRNA and expressed as net % control mRNA normalized to corresponding β-actin mRNA levels. * indicates the statistical significance of hTERT mRNA levels in SW 626 cells treated with 100 IU vitamin E compared to untreated controls.
Vitamin E targets the -976 to -578 responsive regions of the hTERT promoter

Reporter assays using full length and deleted hTERT reporter constructs were performed with SW 626 cells to identify the vitamin E-responsive elements in the hTERT promoter responsible for telomerase suppression. Though vitamin E suppressed hTERT luciferase reporter activity in the full length (-1375) and the -1175 bp regions up to 47%, figure 19 shows the largest decrease in vitamin E-mediated luciferase reporter activity occurred within -976 to -578 bp hTERT promoter regions, with 89% and 64% inhibition, respectively.

Figure 19 The 976- to 578-bp hTERT Promoter Regions are Required for Vitamin E Suppression of Telomerase Activity

SW 626 cells were transfected with full length and deletion hTERT promoter luciferase constructs PGL3-1375, PGL3-1175, PGL3-976, PGL3-578 [220] as indicated along with pCMV-β-galactosidase expression vector and treated ± 100 IU vitamin E for 24 hours. Luciferase reporter activity was normalized by dividing luciferase activity with β-galactosidase. Results are expressed as the mean luciferase activity ± S.E. from triplicate
samples and p values indicate statistical differences between luciferase assays in SW 626 cells with and without vitamin E treatment.

**Vitamin E suppresses LPA-induced telomerase activity in ovarian cancer cells**

Vitamin E has been shown to degrade and suppress LPA activity [262]. While I did not directly measure LPA levels following vitamin E treatment, VEGF ELISA was performed on conditioned media from SW 626 cancer cells treated with ± vitamin E and ± LPA to indirectly demonstrate the ability of vitamin E to suppress LPA activation of its downstream target, VEGF (Figure 20A). LPA increased VEGF secretion at least 1.5-fold in the SW 626 cells (Figure 20A). In contrast, vitamin E suppressed VEGF endogenous levels by 22%. In addition, vitamin E abrogated LPA-increased VEGF secretion by 62% (Figure 20A). To study whether vitamin E could similarly suppress telomerase activity by abrogating LPA-mediated telomerase activity, ovarian cell lines FHIOSE 118, PA-1, SW 626, cisplatin-sensitive OV2008, cisplatin-resistant daughter cell line C13, cisplatin-sensitive A2780s, and cisplatin-resistant daughter cell line A2780cp were treated with ± 20 µg/mL LPA and ± 100 IU vitamin E for 24 hours, and examined for telomerase activity (Figure 20B). When ovarian cancer cells were treated with vitamin E alone, vitamin E suppressed endogenous telomerase activity in all ovarian cancer cell lines tested (PA-1 42%, SW 626 38%, OV2008 13%, C13 34%, A2780s 34%, A2780CP 38%) (Figure 20B). In addition, when co-treated with LPA, vitamin E consistently abolished LPA induction of telomerase activity in all ovarian cancer cell lines tested (PA-1 63%, SW 626 60%, OV2008 59%, C13 58%, A2780s 66%, A2780CP 64%) (Figure 20B). In contrast, LPA and vitamin E failed to either induce or suppress telomerase activity in
telomerase-negative ovarian cell line, FHIOSE 118 serving as control (Figure 20B). These findings show vitamin E suppresses LPA-mediated telomerase activity in human ovarian cancer cells.

To confirm vitamin E abrogated LPA-mediated induction of telomerase activity, reporter assays were performed on SW 626 cells treated with 20 µg/mL LPA and 100 IU vitamin E. Figure 21 shows that LPA failed to induce activity of the 976-bp and 578-bp of the hTERT promoter when co-treated with vitamin E (Figure 21).
Figure 20  
Vitamin E Suppresses LPA-Mediated Telomerase Activity

(A) Enzyme-linked immunosorbent assay analysis of secreted VEGF protein concentrations in conditioned media of SW 626 cells treated with ± 20 µg/mL LPA ± 100 IU vitamin E for 24 hours. (B) Ovarian cell lines, FHIOSE 118, PA-1, SW 626, OV2008, C13, A2780s, and A2780cp were treated with 0.1% FBS, ± 100 IU vitamin E and ± 20 µg/mL LPA for 24 hours and assessed for telomerase activity by PCR-ELISA. p values indicate statistically significant differences in telomerase activity between LPA treated cell lines vs. vitamin E + LPA treated cell lines. Data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E. p values indicate the statistical significance of LPA treated cells vs. untreated control (*), vitamin E treated cells vs. untreated control (~), and LPA + vitamin E treated cells vs. LPA treated cells (^).
Figure 21  Vitamin E Negatively Regulates LPA-Mediated hTERT Promoter Activity

SW 626 cells were transfected with full length and deletion hTERT promoter luciferase constructs PGL3-1375, PGL3-1175, PGL3-976, PGL3-578 [220] as indicated along with pCMV-β-galactosidase expression vector and treated ± 100 IU vitamin E ± 20 µg/mL LPA for 24 hours. Luciferase reporter activity was normalized by dividing luciferase activity with β-galactosidase. Results are expressed as the mean luciferase activity ± S.E. from triplicate samples and p values indicate statistical differences between luciferase assays in SW 626 cells treated with LPA vs. cells treated with vitamin E + LPA.
Figure 22 Vitamin E Increases Chemotherapeutic Sensitivity in Ovarian Cancer Cells

Cisplatin-sensitive, OV2008 (A, B, C) and its daughter cisplatin-resistant, C13 (D, E, F), ovarian cancer cells were treated ± 100 IU vitamin E ± 25 µM CDDP and were subjected to MTS assays (A,D), Western immunoblot (B, E), and PCR ELISA for telomerase (C, F). A, D) Measurement of cell growth by MTS was expressed as average absorbance.
Figure 22  (Continued) Vitamin E Increases Chemotherapeutic Sensitivity in Ovarian Cancer Cells
from triplicate samples at 490 nm ± S.E.  *, ** and *** indicate statistical differences between control vs. CDDP treated cells, control vs. vitamin E and CDDP treated cells, and CDDP treated cells vs. vitamin E and CDDP treated cells at 24 hours, respectively.  B, E) Parallel OV2008 and C13 cultures were treated ± 100 IU vitamin E ± 25 µM CDDP for 24 hours and subjected to SDS-PAGE and Western immunoblot for caspase 3 cleavage. Untreated controls (lane 1), cells treated with CDDP (lane 2), cells treated with vitamin E (lane 3), and cells treated with both vitamin E and CDDP (lane 4) are indicated. Actin served as a loading control for Western immunoblot analyses. Telomerase activity was measured by PCR-ELISA following treatment ± 100 IU vitamin E ± 25 µM CDDP for 24 hours (B, E) and telomerase activity was expressed as mean absorbance from triplicate samples at 490 nm ± S.E. with p values indicating differences between vitamin E treated and untreated control samples.

Vitamin E enhances cisplatin-mediated cytotoxicity in ovarian cancer cells

Anderson et al have shown that a vitamin E derivative, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)-chroman-6-yloxyacetic acid (α-TEA) in combination with cisplatin reduced tumor burden of cisplatin-resistant human ovarian cancer cells xenografted into immune compromised nude mice. To determine the potential therapeutic benefit of vitamin E-mediated telomerase inhibition for platinum-mediated cytotoxicity, MTS, Western immunoblot, and telomerase PCR-ELISA assays were performed in the cisplatin-sensitive cancer cell line, OV2008, and its cisplatin-resistant daughter cell line, C13, following treatment ± 25 µM CDDP ± 100 IU vitamin E for up to 72 hours (Figure 22). Significant cisplatin-induced growth inhibition occurred by 24 hours when OV2008 cell growth decreased 60% in CDDP treated OV2008 cells compared to the untreated controls (Figure 22A). OV2008 cell growth decreased by 68% by 24 hours when treated with CDDP + vitamin E suggesting that vitamin E increased platinum-mediated cytotoxicity by 8%. To determine whether apoptosis was induced,
SDS-PAGE analyses for procaspase 3, cleaved caspase 3, and cleaved DFF45 in OV2008 cells treated ± vitamin E ± CDDP for 24 hours were performed (Figure 22B). Treatments with CDDP + vitamin E stimulated caspase 3 activation by 40% when compared to cells treated with CDDP alone as seen by the reduction of procaspase 3 (35 kDa) expression and an increase in caspase 3 activation (19/17 kDa) as determined by Western blot and densitometry analyses (Figure 22B). Caspase 3 activation leads to DNA fragmentation factor (DFF) 45 cleavage into 24- and 12-kDa fragments releasing DFF40 allowing translocation to the nucleus and cleaving genomic DNA, a key step in apoptosis [263, 264]. Western blot analyses against cleaved DFF45 (12 kDa) showed an increase in DFF45 activation in CDDP treated cells and CDDP + vitamin E treated by 72% and 76%, respectively, as determined by densitometry analyses. However, additional MTS assays in which OV2008 and C13 cells were treated with 20, 40, 60, 100, and 200 IU vitamin E ± CDDP for 0 to 72 hours did not show a significant additive effect of vitamin E for CDDP-mediated cytotoxicity (data not shown). In addition, vitamin E significantly suppressed telomerase activity compared to untreated controls regardless of the presence of CDDP and CDDP alone did not dramatically alter telomerase levels (Figure 22C).

As expected for drug resistant cells, treatment of C13 ovarian cancer cells with CDDP alone suppressed cell growth to a lesser extent (33% at 24 hours) than OV2008 cells (Figure 22D). C13 cell growth decreased by 52% at 24 hours when treated with CDDP + vitamin E suggesting that vitamin E increased platinum-mediated cytotoxicity by approximately 20%. To determine whether apoptosis was induced, SDS-PAGE
analyses for procaspase 3, cleaved caspase 3, and cleaved DFF45 in C13 cells treated ± vitamin E ± CDDP for 24 hours were performed (Figure 22E). Procaspase 3 activity was decreased in CDDP and CDDP + vitamin E treated cells by 43% and 34%, respectively, compared to untreated cells as determined by densitometry. Decrease in procaspase 3 activity lead to increase in cleaved caspase 3 in CDDP and CDDP + vitamin E treated cells by 76% and 74%, respectively, compared to untreated cells. Cleaved DFF45 activity was also elevated in CDDP and CDDP + vitamin E treated cells by 69% and 70%, respectively, compared to untreated cells. Similarly, vitamin E significantly suppressed telomerase activity in C13 cells compared to untreated controls regardless of the presence of CDDP. In addition, CDDP alone did not dramatically alter telomerase levels (Figure 22F).

To determine if vitamin E targeted telomerase for increased platinum-mediated cytotoxicity, telomerase-negative IOSE 80 and telomerase-positive IOSE 80 + hTERT cells were analyzed by MTS, Western immunoblot, and telomerase PCR-ELISA assays as above following treatment ± 25 µM CDDP ± vitamin E for up to 72 hours (Figure 23). Maximal cisplatin-induced growth inhibition occurred by 24 hours when cell growth decreased 66% in CDDP treated IOSE 80 compared to their untreated controls (Figure 23A). IOSE 80 cell growth only decreased by 48% by 24 hours in IOSE 80 treated with CDDP + vitamin E for 24 hours suggesting that vitamin E protected telomerase-negative cells against cisplatin-induced cytotoxicity by approximately 20%. To determine whether apoptosis was induced, SDS-PAGE analyses for procaspase 3, cleaved caspase
3, and cleaved DFF45 in C13 cells treated ± vitamin E ± CDDP for 24 hours were performed (Figure 23B). Procaspase 3 activity was decreased in CDDP and CDDP + vitamin E treated cells by 43% and 34%, respectively, compared to untreated cells as determined by densitometry. Decrease in procaspase 3 activity lead to increase in cleaved caspase 3 in CDDP and CDDP + vitamin E treated cells by 75%, compared to untreated cells. Cleaved DFF45 activity was also elevated in CDDP and CDDP + vitamin E treated cells by 70% and 69%, respectively, compared to untreated cells. Lastly, IOSE 80 cells remained telomerase negative regardless of vitamin E or CDDP treatment (Figure 23C).

In contrast, cell growth decreased 43% in IOSE 80 + hTERT cells treated with CDDP and additionally to 54% when cells were treated with CDDP + vitamin E for 24 hours (Figure 23D). Similarly, IOSE 80 + hTERT cells treated with CDDP showed a marked increase in caspase 3 activation compared to their controls (Figure 23E). Unlike IOSE 80 cells, however, IOSE 80 + hTERT cells treated with the combination of CDDP + vitamin E demonstrated a higher increase in activation of caspase 3 (80%) as determined by densitometry compared to the IOSE 80 + hTERT cell treated with CDDP alone (69%) (Figure 23E). In addition, cleaved DFF45 activity was higher in CDDP + vitamin E treated cells (78%) than CDDP treated cells alone (74%). Lastly, vitamin E reduced telomerase activity 3-fold in IOSE 80 + hTERT cells while a 4-fold decrease in telomerase activity was observed when IOSE 80 + hTERT cells were treated with CDDP
+ vitamin E (Figure 23F) corroborating the effects of these two agents on telomerase-positive ovarian cell lines.
Figure 23  Vitamin E May Protect Telomerase-Negative Cells from Cisplatin-Mediated Cytotoxicity

Telomerase-negative, IOSE 80 (A, B, C) and telomerase-positive, IOSE 80 + hTERT (D, E, F) cells were treated ± 100 IU vitamin E ± 25 µM CDDP and were subjected to MTS assays (A, D),
Figure 23  (Continued) Vitamin E May Protect Telomerase-Negative Cells from Cisplatin-Mediated Cytotoxicity

Western immunoblot (B, E) and PCR-ELISA for telomerase (C, F) as described in the Materials and Methods section. Measurement of cell growth by MTS was expressed as average absorbance from triplicate samples at 490 nm ± S.E. *, **, and *** indicate statistical differences between control vs. CDDP treated cells, control vs. vitamin E and CDDP treated cells, and CDDP treated cells vs. vitamin E and CDDP treated cells at 24 hours, respectively. Parallel IOSE 80 and IOSE 80 + hTERT cultures were subjected to SDS-PAGE and Western immunoblot for caspase 3 cleavage with untreated controls (lane 1), cells treated with CDDP (lane 2), cells treated with vitamin E (lane 3) and cells treated with both vitamin E and CDDP (lane 4) are indicated. Actin served as a loading control for Western immunoblot analyses. Telomerase activity was measured by PCR-ELISA (C, F) following treatment ± 100 IU vitamin E ± 25 µM CDDP for 24 hours (B, E). Telomerase activity was expressed as mean absorbance from triplicate samples at 490 nm ± S.E. with p values indicating differences between vitamin E treated and untreated control samples.
Discussion

Vitamin E, the common term for compounds that demonstrate tocopherol and tocotrienol activities, has long been recognized as a dietary anti-oxidant. However, in addition to eliminating free radical damage, vitamin E functions in cell signaling pathways, regulation of oncogene expression, and cancer prevention [265]. Regarding the latter, epidemiological studies show that diets rich in vitamin E or dietary supplementation of vitamin E are associated with a reduced risk of ovarian cancer [266-269]. Clinically, vitamin E can alleviate chemotherapy-induced side effects and improve chemotherapeutic efficacy. Argyriou et al demonstrated that dietary vitamin E supplementation significantly reduced cisplatin and paclitaxel-induced neurotoxicity [270]. Further, dietary administration of vitamin E to ovarian cancer patients between periods of chemotherapeutic treatment reduced cathepsin activity, thereby reducing extracellular matrix proteolysis important for tumor invasion and metastasis [271]. Preliminary case reports also indicate that high dose oral vitamin E therapy during first-line platinum/paclitaxel chemotherapy results in normalization of CA125 levels after first cycle chemotherapy [272]. Likewise, dietary vitamin E has been associated with increased survival in patients with ovarian cancer [273]. Lastly, Hahn et al [274] observed a 4.8 fold reduction of lung tumor metastasis in mice model of mammary cancer when the mice were fed mouse food with alpha-TEA, a synthetic vitamin E analog.
There is growing evidence that the physiological function of vitamin E in cancer extends beyond an anti-oxidant to include regulation of gene transcription and enzymatic activities [275]. Notably, vitamin E can induce apoptosis by inhibition of protein kinase C activity [275] or increased sensitivity to TRAIL [276]. In addition, vitamin E inhibits cancer cell invasiveness by decreasing levels of matrix metalloproteinases and can inhibit cell cycle progression by downregulation of cyclins D1 and E [277]. Since telomerase plays an important role in cellular immortalization and tumor progression, these experiments focused on the potential for vitamin E to alter telomerase activity. Novel findings are reported that vitamin E not only inhibited endogenous telomerase activity in telomerase-positive OSE or ovarian cancer cells while telomerase-negative OSE cells remain unaffected, but it also abrogated LPA-mediated increase in telomerase activity in ovarian cancer cells. Further, vitamin E-mediated suppression of telomerase was transcription dependent as noted by reduced hTERT mRNA, but not actin mRNA levels following treatment with vitamin E suggesting that vitamin E did not decrease gene expression globally. The ability of vitamin E to suppress only 30% of hTERT mRNA levels during 18 hours of vitamin E treatment may reflect the characteristically long half-life of hTERT mRNA though maximal vitamin E-mediated suppression of hTERT mRNA levels preceded greatest inhibition of telomerase activity.

Suppression of hTERT transcription by vitamin E appeared to maximally target the -976 to -578 bp regions of the hTERT promoter. This finding is in agreement with Fujimoto et al who identified MZF-2 as a negative regulator of hTERT transcription.
within the -776 and -378 bp hTERT promoter regions resulting in transcriptional hTERT silencing [278]. In addition, p73, CTCF and USF have also been shown to negatively regulate telomerase transcriptional activity [279-281]. Interestingly, binding sites for MZF-2 and CTFC are present in the -976 and -578 hTERT promoter regions where vitamin E appeared to maximally suppress hTERT transcription in the present study. Given that the hTERT promoter has been cloned and several potential transcription factor binding sites noted [137], the relatively small suppression of hTERT promoter activity found in both the full length 1375 and 1175 deleted promoter constructs following vitamin E treatments may reflect the presence of both negative and positive hTERT transcription regulators present within these regions of the hTERT promoter excluded from the -976 to -578 bp regions. Clearly, then, further studies are warranted to identify the transcription factors that bind to the 976- to 578-bp regions of the hTERT promoter responsible for vitamin E-mediated inhibition of telomerase.

Of clinical significance, these experiments found that vitamin E suppresses LPA-mediated telomerase activity in ovarian cancer cells (Figure 24). Since LPA is found in very high levels in plasma and ascites of ovarian cancer patients [211] and it seems to facilitate ovarian cancer cell growth and invasion [212, 213], it is important to know that vitamin E could be an agent that inhibits LPA and its downstream targets. In agreement with others [282], these experiments found that vitamin E increased cisplatin-mediated cytotoxicity in ovarian cancer cells in culture. Though prevailing clinical thought opposes the use of anti-oxidants fearing that vitamin E may protect cancer cells from free
radical damage, Jha et al showed that a number of cancer cell lines, including ovarian cancer, treated with vitamin E and X-irradiation were sufficiently growth arrested to warrant consideration of vitamin E for potential therapeutic benefit [283]. In agreement, these experiments show that vitamin E decreased telomerase activity and increased cisplatin-mediated cytotoxicity in telomerase-positive ovarian cells. In addition, it was shown that vitamin E may act as a protective agent in normal, telomerase-negative, ovarian cells against cisplatin-mediated cytotoxicity which may help alleviate the apprehension for using vitamin E therapeutically. Though in vivo testing and clinical studies need to be performed to further evaluate the clinical efficacy of vitamin E for chemotherapeutic efficacy, results show that, by targeting telomerase, supplemental vitamin E may be a useful adjuvant for prevention of ovarian cancer and/or improved chemosensitivity, which could reduce the mortality of a disease that kills thousands of women annually.
Chapters 2 and 3 show that VEGF and LPA induce telomerase activity by ERK 1/2-dependent transcriptional activation within the -976 to the -378 bp hTERT telomerase promoter regions. In addition, the current experiments show that vitamin E consistently abrogates LPA-mediated telomerase activity through transcriptional inhibition of the hTERT -976 to -578 bp promoter regions.
Chapter IV

EGF-Mediated Telomerase Activity is Pyk2-Dependent

in Ovarian Cancer Cells

Abstract

Telomerase is present in over 90% of human cancers suggesting that this enzyme is required for tumor cell proliferation and malignant progression. Several growth factors including epidermal growth factor (EGF) have been identified as regulators of telomerase activity, but the exact molecular mechanisms of this regulation are unknown. Since EGF can stimulate growth of normal ovarian surface epithelial (OSE) as well as ovarian cancer cells, and EGF receptors are present on both normal and malignant ovarian cells, these experiments sought to investigate the potential role for EGF to regulate telomerase activity in ovarian cancer cells. The results of my experiments show that: (1) while none of the ovarian cancer cell lines examined secreted significant amounts of EGF, exogenous EGF directly activated telomerase activity; (2) EGF up-regulated hTERT transcription; (3) EGF-mediated telomerase activity was ERK 1/2-dependent; (4) EGF positively targeted the core, 181 bp, promoter of the hTERT promoter where Sp1 and c-Myc are major transcription factors involved in EGF-mediated telomerase activity in ovarian cancers; and (5) these experiments also identified proline-rich tyrosine kinase 2
(Pyk2) as a novel key mediator of EGF-mediated telomerase activity. Therefore, these experiments suggest that EGF stimulation of telomerase activity in ovarian cancer cells is mediated by Sp1 and c-Myc transcription factors within the hTERT core promoter in an ERK 1/2/Pyk2-dependent manner.

**Introduction**

Telomerase is a ribonucleoprotein responsible for DNA telomeric maintenance at the ends of chromosomes. Telomerase synthesizes and adds the six-base motif (TTAGGG)$_n$ in humans to the ends of linear chromosomes maintaining telomere length stability that otherwise would gradually erode after each cell replication [89]. Telomerase activity has been detected in over 90% of solid tumor cells. It is also activated in embryonic and germline cells, but not in normal somatic cells, with the exception of some stem cells that are involved, for example, in tissue renewal [89]. Since telomerase plays a vital role in tumor cell survival and proliferation, telomerase is a potentially important therapeutic target.

Transcriptional activation of the hTERT gene is a critical, initial rate-limiting step in hTERT function and telomerase activity. Multiple binding sites for transcriptional regulation reflect the complex regulation of the hTERT gene. Growth factors are known to regulate telomerase activity through transcriptional activation in several cancers. One of these growth factors is epidermal growth factor (EGF) [176, 178]. EGF is one of the EGF receptor ligands known to participate in various cellular functions such as proliferation, migration, differentiation, and survival [284]. EGF is a 53 amino acid
polypeptide with a molecular weight of 6045 Da [285]. It acts by binding to both low affinity (KD = 1–2 nM) and high affinity (KD = 10–50 pM) sites on cells that express epidermal growth factor receptor (EGFR) [286] and stimulating the intrinsic protein-tyrosine kinase activity of the receptor. Growth-related responses to EGF include the induction of nuclear proto-oncogene expression, such as Fos, Jun, and Myc.

EGFR belongs to the EGF/ERbB family of type I receptor tyrosine kinases that participate in various cellular functions. They are composed of four members, EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4 [284]. These receptors are single chain transmembrane polypeptide proteins possessing three different domains: (1) the extracellular domain which allows ligand binding to receptors, (2) the transmembrane domain involved in dimerization interaction between receptors, and (3) the intracellular tyrosine kinase domain that phosphorylates tyrosine residues on substrate proteins. The cytoplasmic domain also consists of a carboxy-terminal tail containing tyrosine autophosphorylation sites linking these receptors to proteins containing Src homology 2 and phosphotyrosine-binding domains. These proteins allow activation of several signal transduction molecules such as Pyk2 and ERK 1/2 [284].

The other members of the EGFR ligand family consist of heparin binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), epiregulin, amphiregulin, epigen, β-cellulin (BTC), and four neuregulins (NRG-1, 2, 3, and 4) [284, 287, 288]. Each ligand binds to the extracellular domain of ErbB receptors inducing the formation of homo- and heterodimers of the receptors, which allows the intrinsic tyrosine
kinase domain to be activated, resulting in a phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptor [284]. These autophosphorylated residues serve as docking sites for a variety of signaling molecules, whose recruitment leads to the activation of intracellular signaling pathways [284].

Several G protein-coupled receptors (GPCRs) have been shown to transactivate EGFR through GPCR agonists including thrombin, ANG II, endothelin-1, carbachol, and LPA [289]. This transactivation mediates several critical downstream signals functions, such as ERK activation, c-fos induction, and cell proliferation [289-291]. In order for this transactivation to occur, second messengers directly and/or signal transduction pathways operated by second messengers, such as elevation of intracellular Ca\(^{2+}\) [292, 293], activation of protein kinase C (PKC) [293], and generation of reactive oxygen species (ROS) [294-296] are required. In addition, metalloproteases are also required for EGFR transactivation by several GPCR agonists [297, 298]. Lastly, cytosolic nonreceptors tyrosine kinase such as Src and Pyk2 are also required in the EGFR transactivation [299, 300].

Pyk2 is a cytoplasmic non-receptor tyrosine kinase related to focal adhesion kinase that can be activated by several extracellular signals including inflammatory cytokines, UV radiation, tumor necrosis factor-\(\alpha\), and changes in calcium levels [301]. Overexpression or altered activity of Pyk2 has been linked to changes in cell adhesion, as well as invasion and metastasis of various malignancies such as prostate cancer [302]. Pyk2 can activate phosphatidylinositol triphosphate kinase (PI-3-K) [303], which, in turn,
can phosphorylate jun N-terminal kinase (JNK) [304] as well as the mitogen-activated protein kinase (MAPK) signaling pathway [305]. Therefore, Pyk2 appears to be a key mediator of intracellular signaling linking a variety of extracellular stimuli to the MAPK, PI-3-K, and JNK pathways depending on the context of bound effector systems [306-308]. For instance, our laboratory found that Pyk2 mediates exogenous stress-mediated regulation of telomerase through PI-3-K/JNK signaling pathways in human ovarian cancer cells [253].

Since gene amplification leading to EGFR overexpression and EGFR transactivation are found in carcinomas of the breast, lung, and ovaries [309-311], and Pyk2 is important in EGFR transactivation and telomerase activity, the purpose of these experiments was to determine whether EGF could regulate telomerase activity in ovarian cancer cells in a Pyk2-dependent manner. The data show that exogenous EGF directly activated telomerase through an up-regulation of hTERT transcription via the ERK 1/2 pathway and positively targeted transcription binding sequences within the hTERT core promoter where transcription factors Sp1 and c-Myc appear to be major mediators of EGF-induced telomerase activity of ovarian cancer cells. In addition, when ovarian cancer cells were transfected with wild type Pyk2, EGF-mediated telomerase activity was markedly increased. In contrast, transfection with kinase deficient Pyk2 abolished EGF-mediated induction of telomerase activity. In summary, these experiments suggest that EGF stimulation of telomerase activity in ovarian cancer cells is mediated by Sp1 and c-
Myc transcription factors within the hTERT core promoter in an ERK 1/2 /Pyk2-dependent manner.

Materials and Methods

Cell Lines and Tissue Culture

Telomerase-positive ovarian carcinoma cell lines SKOV3, ES-2, C-13, PA-1, SW626, and one non-tumorigenic SV40 large-T antigen-transfected human ovarian surface epithelial (IOSE) cell line, FHIOSE 118, derived from normal ovarian surface epithelium were used [217]. Cells were cultured in Medium 199/MDCB 105 (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 µg/mL Gentamicin (GIBCO, NY). The cells were incubated at 37°C with 5% CO₂ / 95% air.

EGF, HGF, VEGF ELISA

To measure EGF, HGF, and VEGF production by cultured cells, equal amounts of conditioned media from cell lines were collected, centrifuged at 500 g for 5 minutes at room temperature to remove any cellular debris and the supernatants were frozen at -80°C until assayed using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions for EGF, HGF, and VEGF levels, respectively. The enzymatic reactions were detected at 450 nm using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and the results expressed as µg/mL of triplicate experiments ± S.E.
Treatment with EGF, EGF Receptor Inhibitor, and ERK 1/2 Inhibitor

Cells were grown in 0.1% FBS media for at least 18 hours prior to treatment with 2 ng/mL of EGF dissolved in 0.1% BSA (Sigma, St. Louis, MO), 50 nM of AG 1478 EGF receptor-specific inhibitor tyrphostin dissolved in DMSO (BioSource, Camarillo, CA) or 10 µg/mL U0126 ERK 1/2 inhibitor dissolved in methanol (Cell Signaling, Beverly, MA) in 0.1% FBS media for 0 to 24 hours and then collected to perform the necessary analyses.

Telomerase Assay

To quantitatively detect changes in telomerase activity levels, cells were assayed for telomerase activity using the telomerase polymerase chain reaction-sandwich enzyme-linked immunosorbent assay (PCR-ELISA) following manufacturer’s protocol (Roche, Indianapolis, IN) and as previously described [218]. This assay has been demonstrated to be as sensitive as the radioactive telomere repeat amplification protocol (TRAP) assay [261]. Briefly, cells were washed with DPBS, trypsinized, and centrifuged at 500 g for five minutes. Pellets were washed twice in DPBS, then resuspended in 150 µL of lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The cell pellets were kept on ice for 30 minutes and then centrifuged at 100,000 g for 60 minutes at 4°C. Supernates were then assayed using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) to determine protein concentration following detergent solubilization according to manufacturer’s instructions. To perform the telomerase PCR-ELISA within a linear
range, all of the cells extracts equivalent to 3 µg of protein were used. Following PCR-ELISA, telomerase activity was detected using the ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and recorded as optical density ± S.E.

**RT-PCR**

To examine the contribution of transcriptional control of telomerase regulation by EGF, RT-PCR was performed as previously described [218]. Total RNA was collected using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample. The cDNA samples were amplified using the Perkin-Elmer (Palo Alto, CA) GeneAmp kit. The hTERT primers used were hTERT-S (CGGAAGAGTGTCTGGAGCCAA) and hTERT-AS (GGATGAAGCGGAG TCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) and with β-actin primers Actin-S (CAGGTCATCACCATTGGCAATGAGC) and Actin-AS (GATGTCCACGT CACACTTCATGA) for an internal control. The amplified products were then separated by electrophoresis on a 1% agarose gel, stained with 1X SyberGreen (FMC Bioproducts, Rocklan, ME) and analyzed with the Kodak EDAS 120 Digital Analysis System. Net hTERT mRNA intensities from treated samples were normalized to their corresponding β-actin mRNA levels.

**Western Blot Analysis**

For Western blot analyses, cell lysates were prepared in CHAPS lysis buffer as previously described [219]. Protein concentrations of cell lysates were determined using
the Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA) in accordance to the manufacturer’s instructions. Protein extracts (20 µg) were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) and separated via 12.5% or 7.5% SDS-PAGE and electroblotted onto Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ) by wet transfer. Immunoblotting was performed using antibodies against ERK 1/2 (1:1000), MAPK phosho-ERK 1/2 (1:1000), anti-Myc (1:1000; Cell Signaling, Beverly, MA), anti-Sp1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated and unphosphorylated Pyk2 (1:1000; Upstate Biotechnology, Lake Placid, NY). β-actin (1:5000, Sigma Aldrich, St. Louis, MO) was used as loading control. Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Blots were scanned and analyzed with Life Sciences GE Healthcare ImageQuant image analysis software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Values reported for target proteins were normalized to the blots' respective β-actin levels.

**Luciferase Reporter Assay**

To measure hTERT promoter activity, the full-length (pGL3-1375), and deleted hTERT promoter-luciferase constructs (pGL3-1175, -976, -776, -578, -378, -181) and mutated hTERT core promoter constructs (hTERT/-181E1/m, hTERT/-181E1, 2/m, hTERT/-181 Sp1/m) were used as previously described [220]. To determine whether Pyk2 mediated EGF induction of telomerase, Myc-tagged wild-type (WT) Pyk2 or Myc-
tagged kinase-deficient (KD) Pyk2 were used as previously described [253]. To examine the roles of c-Myc and Sp1 in EGF-mediated telomerase activity, cells were transfected with 0.5 µM c-Myc siRNA or ± 1 µM Sp1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA). SW 626 cells were incubated in 0.1% FBS for 24 h and then transiently co-transfected with 3 µg of DNA and 1U of β-galactosidase cDNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) in serum- and antibiotic-free media. Cultures were replenished with serum 1 h after transfection. Expression of luciferase was measured 48 h after transfection using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturers’ instructions. At time of collection, cells were microscopically observed to ensure cell viability with no signs of apoptosis using parallel cultures transfected with green fluorescent protein (GFP). Transcriptional activity was expressed as relative luciferase activity ± S.E., after normalization with β-galactosidase activity.

**Statistical Analysis**

Samples for ELISA, telomerase PCR-ELISA, RT-PCR, and reporter assays were run in triplicate, and the data were subjected to the Student’s t test for determination of statistical significance.
Results

**EGF induces telomerase activity**

To determine whether EGF stimulated telomerase activity in ovarian cancer cells, time-dependent and dose-dependent assays were performed (Figures 25A and B). Telomerase activity was measured by PCR-ELISA for 0 to 24 hours following treatment with 0 to 25 ng/mL of EGF, respectively. Cells were incubated in media containing 0.1% FBS for 24 hours prior to culturing them in the presence or absence of EGF. Figure 25A shows that at one hour, EGF elicited greatest increase in telomerase activity in ovarian cancer cells PA-1 (40%) and SW 626 (63%). Treatments with 2 ng/mL EGF resulted in a two-fold increase in telomerase activity in ovarian cancer cells PA-1 and SW 626 (Figure 25B). A panel of additional ovarian cancer cell lines, SKOV3, C13, PA-1, ES-2, and SW 626 were treated with 2 ng/mL EGF for an hour to confirm EGF-mediated stimulation of telomerase activity (Figure 26). While none of the ovarian cancer cell lines examined produced significant EGF (Figure 26, inset), exogenous EGF increased telomerase activity. Each cell line showed a significant increase in telomerase activity compared to their respective controls (SKOV3 38%, C13 28%, PA-1 44%, ES-2 48%, and SW 626 49%) (Figure 26). Different dosages of EGF or treatment intervals did not affect telomerase activity in telomerase negative FHIOSE 118 cell line serving as control (Figures 25 and 26).

To demonstrate specificity of EGF for telomerase activity in ovarian cancer cells, SW 626 cancer cells were treated with the EGF receptor-specific inhibitor tyrphostin AG
While AG 1478 did not significantly suppress endogenous telomerase activity in SW 626, AG 1478 inhibited EGF-stimulated telomerase activity by 300% (Figure 27). In contrast, telomerase-negative FHIOSE 118 cell were unaffected by EGF inhibition serving as control. Treatments with AG 1478 diluent, DMSO, did not alter EGF stimulation of telomerase activity.

**EGF-stimulated telomerase activity is transcription-dependent**

RT-PCR was performed to determine the relative levels of hTERT mRNA in PA-1 and SW 626 cells following EGF stimulation (Figure 28). PA-1 and SW 626 cells showed an increase of 123% and 127%, respectively, in relative levels of hTERT mRNA transcript after 1h EGF treatment when normalized to β-actin (Figure 28).
Figure 25  EGF Regulation of Telomerase Activity is Time- and Dose-Dependent

Telomerase activity was measured by PCR-ELISA in cultured human ovarian FHIOSE 118, PA-1, and SW 626 cells treated with 2 ng/mL EGF for 0 to 24 hours (A) and 0 to 25 ng/mL EGF for 1 hour (B) in 0.1% FBS.  * indicates the statistical significance of levels of telomerase activity in PA-1 and SW 626 cancer cells treated compared to untreated controls.  Data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E.
Figure 26 EGF Stimulates Telomerase Activity in Human Ovarian Cancer Cells
SKOV3, C13, PA-1, ES-2, SW 626, and FHOSE 118 ovarian cell lines were treated with ± 2 ng/mL for 1 hour in 0.1% FBS, collected and examined for telomerase activity by PCR-ELISA. For telomerase analysis, data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E. * indicates statistically significant differences in telomerase activity between EGF treated cell lines and their untreated counterparts. (Inset) Enzyme-linked immunosorbent assay analyses of secreted EGF, HGF, and VEGF protein concentrations in conditioned media of SKOV3, C13, PA-1, ES-2, SW 626, and FHOSE 118 cell lines.
Figure 27   EGF is Specific to Stimulate Telomerase Activity

To show specificity of EGF to stimulate telomerase activity, FHIOSE 118 and SW 626 cells were treated with ± 2 ng/mL EGF for 1 hour ± 50 nM EGF receptor inhibitor, AG 1478 in 0.1% FBS. Samples were also treated with AG 1478 diluent, DMSO, serving as control. p values indicate statistical differences between telomerase activity in SW 626 cancer cells treated with EGF vs. untreated control (*), AG 1478 treated SW 626 cells vs. untreated control (~), EGF + DMSO treated SW 626 cells vs. untreated control (^), and EGF + AG 1478 treated SW 626 cells vs. EGF treated SW 626 cells (**). Data from three independent experiments were quantified and expressed as mean absorbance at 490 nm ± S.E.
EGF Stimulation of Telomerase Activity is Transcription-Dependent

PA-1 and SW 626 cells were incubated ± 2 ng/mL EGF for 0, 30, 45 minutes, 1 and 2 hours in triplicate and examined by RT-PCR for hTERT mRNA. Results are expressed as mRNA expression normalized to corresponding β-actin mRNA levels. * indicates the statistical significance of hTERT mRNA levels in PA-1 and SW 626 cells treated with 2 ng/mL EGF compared to untreated controls. Graphical representations provided below of respective blots showing net intensities of hTERT in PA-1 and SW 626 cancer cells relative to β-actin mRNAs.

EGF-induced telomerase activity is Pyk2-dependent

To determine whether Pyk2 was a mediator in EGF-induced telomerase activity in human ovarian cancer cells, SW 626 cancer cells were transfected with c-Myc tagged wild type Pyk2 (Pyk2 WT) or c-Myc tagged kinase deficient Pyk2 (Pyk2 KD) and treated cells with 2 ng/mL EGF and ± 50 ng/mL EGFR inhibitor, AG 1478, an hour prior to collection. After 48 hours, protein lysates were obtained and tested for transfection
efficiency via Western blot analysis for Pyk2 protein levels (Figure 29, inset). To study the effect of Pyk2 on telomerase activity, PCR-ELISA was performed. Pyk2 KD reduced EGF-mediated induction of telomerase activity by 47% compared to EGF treated SW 626 cells (Figure 29). In contrast, SW 626 cells transfected with Pyk2 WT demonstrated increased telomerase activity by 54%. Furthermore, SW 626 cells transfected with Pyk2 WT and treated with 2 ng/mL EGF showed an increase in telomerase activity by 64% compared to EGF treated SW 626 cells (Figure 29).

**EGF-stimulated telomerase activity is ERK 1/2 dependent**

In agreement with Maida *et al* [176], EGF increased ERK 1/2 phosphorylation in ovarian cancer cells (Figure 30). Western blot and densitometric analyses showed that EGF increased ERK 1/2 phosphorylation by 34% and 52% in PA-1 and SW 626 ovarian cancer cells, respectively (Figure 30, inset). Cells treated with U0126 showed a significant decrease in EGF-mediated ERK 1/2 phosphorylation by 71% and 39% in PA-1 and SW 626 ovarian cancer cells, respectively (Figure 30, inset). In agreement with results obtained in Figure 26, EGF stimulated telomerase activity by 47% in PA-1 and SW 626 cells. Inhibition of ERK 1/2 by U0126 abrogated EGF-stimulated telomerase activity by 84% and 76% in PA-1 and SW 626 cells, respectively (Figure 30), confirming a role for ERK 1/2 in EGF-stimulated telomerase activity in ovarian cancer cells.

To determine whether Pyk2 induction of telomerase was also ERK 1/2-dependent, Western blot analyses were performed (Figure 31, inset). ERK1/2 phosphorylation increased in EGF-treated SW 626 cells when compared to untreated controls. ERK 1/2
phosphorylation was also increased in all Pyk2 WT transfected SW 626 cells. In contrast, ERK 1/2 phosphorylation decreased in all Pyk2 KD transfected SW 626 cells (Figure 31, inset).

Figure 29    EGF Stimulation of Telomerase Activity is Pyk2-Dependent
SW 626 cells were transfected with Myc-tagged Pyk2-KD or Pyk2-WT and treated with ± 2 ng/mL EGF and ± 50 nM EGFR inhibitor AG 1478 and assayed for telomerase activity by PCR-ELISA. Results were expressed as mean optical density at 490 nm for triplicate samples ± S.E.  p values indicate the statistical significance of EGF treated cells vs. untreated control (*), Pyk2 KD transfected cells vs. untreated control (~), Pyk2 KD + EGF vs. EGF treated cells (~~), Pyk2 KD + EGF + AG 1478 vs. EGF treated cells (~~~), Pyk2 WT vs. untreated control (^), Pyk2 WT + EGF vs. EGF treated cells (^^), Pyk2 WT + EGF + AG 1478 vs. EGF treated cells (^^^). (A, inset) Cell lysates were analyzed for Pyk2 expression and ERK 1/2 phosphorylation by Western blot. β-actin was used as loading control. For telomerase analysis, results were expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Figure 30 EGF Stimulation of Telomerase Activity is Via the ERK 1/2 Signaling Pathway

Cultures of PA-1 and SW 626 cells were treated with ± 10µM U0126 and ± 2 ng/mL EGF for 1 hour in 0.1% FBS. Cells were then harvested and analyzed for telomerase activity. Results were expressed as mean optical density at 490 nm for triplicate samples ± S.E. p values indicate the statistical significance between EGF treated cells vs. untreated controls (*), U0126 treated cells vs. untreated controls (~), and EGF + U0126 treated cells vs. EGF treated cells (`). (A, inset) Western blot analysis was performed to confirm U0126 inhibition of ERK 1/2 phosphorylation. β-actin was used as the loading control.

hTERT core promoter region is required for EGF stimulation of hTERT promoter activity

 Reporter assays were performed using full length, deletion promoter constructs, and deletion core promoter constructs to identify the hTERT promoter region responsive to EGF (Figure 31). The full length hTERT promoter (PGL3-1375) was induced by EGF
by 61% compared to endogenous promoter activity (Figure 31). However, EGF stimulated maximal activity at the core hTERT promoter regions (PGL3-181) by 67% compared to endogenous core promoter activity (Figure 31). The hTERT core promoter contains two c-Myc binding sites and five Sp1 binding sites known to be activators of hTERT transcription [312]. To further evaluate the binding sites responsive to EGF-mediated induction of telomerase within the core promoter, hTERT luciferase reporters containing mutations of E-box 1 (hTERT/-181E1/m), E-box 2 (hTERT/-181E1, 2/m), and all Sp1 sites (hTERT/-181Sp1/m) were performed [220]. Luciferase assays showed that EGF-induced hTERT promoter activity was abrogated by mutation of Sp1 sites. In contrast, mutation of E-box 1 did not affect EGF-induced hTERT promoter activity. Interestingly, mutation of E-box 1 and 2 showed a decrease, but not complete abrogation, in core promoter transactivation when compared to the full core promoter activity. My results suggest that Sp1 binding sites within the hTERT core promoter are essential and that E-box 2 might be important in EGF-induced telomerase activity in human ovarian cancer cells.

To confirm specificity of EGF induction of the hTERT core promoter, reporter assays were also performed on SW 626 cells treated with 2 ng/mL EGF and either 50 nM EGF receptor inhibitor AG 1478, 10 µg/mL ERK 1/2 inhibitor U0126 (Figure 31). EGF failed to induce activity of the 1375-bp and 181-bp regions of the hTERT promoter when co-treated with AG 1478 (Figure 31). Co-treatments with EGFR inhibitor, AG 1478, also confirmed the importance of Sp1 and E-box 2 in EGF-mediated transactivation of the
hTERT core promoter. In addition, U0126 significantly suppressed EGF-mediated induction of luciferase activity of the 1375-bp and 181-bp regions of the hTERT promoter (Figure 31).

To confirm EGF-stimulated telomerase activity was Pyk2-dependent in ovarian cancer cells, reporter assays were also performed on SW 626 cells transfected with hTERT core promoter or hTERT core promoter mutations, Pyk2 WT or Pyk2 DN and treated with ± 2 ng/mL EGF (Figure 32). Overexpression of wild type Pyk2 significantly enhanced EGF-mediated hTERT core promoter transactivation, while the kinase deficient Pyk2 significantly blocked EGF-mediated transactivation of the core promoter (Figure 32). These findings suggest that Pyk2 is an important mediator of EGF-mediated stimulated telomerase in ovarian cancer cells.
Figure 31  

EGF Drives hTERT Core Promoter Activity

Sw 626 cells were transfected with full length and deletion hTERT promoter luciferase constructs PGL3-1375, PGL3-1175, PGL3-976, PGL3-776, PGL3-578, PGL3-378, PGL3-181, and hTERT core promoter deletion constructs PGL3-181 Sp1/m, PGL3-181 E1/m, and PGL3-181 E1,2/m as indicated [220] along with pCMV-β-galactosidase expression vector. Cells were serum-starved for at least 24 hours and treated with ± 2 ng/mL EGF, ± 50 nM AG 1478, and ± 10 µM/mL U0126. After 48 hours of transfection, luciferase and β-galactosidase assays were performed. Reporter activity was normalized by dividing luciferase activity with β-galactosidase. GFP was used to monitor transfection efficiency. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples. p values indicate statistical differences between luciferase assays in SW 626 treated with EGF vs. untreated controls (*), EGF treated cells vs. EGF + EGF receptor inhibitor AG 1478 treated cells (′), and EGF treated cells vs. EGF + ERK 1/2 inhibitor U0126 treated cells (−).
Figure 32   Pyk2 Drives hTERT Core Promoter Activity

SW 626 cells were transfected with hTERT core promoter and deletion hTERT core promoter luciferase constructs along with pCMV-β-galactosidase expression vector and co-transfected with ± c-Myc-tagged Pyk2-KD and ± Pyk2-WT. An hour prior to collection, cells were treated with ± 2 ng/mL EGF. GFP was used to monitor transfection efficiency. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples. p values indicate statistical differences between luciferase assays in EGF treated cells vs. untreated control (*), Pyk2 DN + EGF treated cells vs. Pyk2 DN treated cells (~), and Pyk2 WT + EGF treated cells vs. Pyk2 WT treated (^).

EGF-Mediated Telomerase Activity is c-Myc- and Sp1-Dependent

Since binding sites for transcription factors, c-Myc and Sp1, are present within the hTERT core promoter, Western blot (Figure 33, inset), PCR-ELISA (Figure 33), and reporter analyses (Figure 34) were performed using c-Myc and Sp1 siRNAs to confirm whether these transcription factors are important in EGF-mediated telomerase activity. SW 626 cells were transfected with ± 0.5 µM c-Myc siRNA ± 1 µM Sp1 siRNA and treated with ± 2 ng/mL EGF. c-Myc and Sp1 siRNAs reduced endogenous c-Myc and Sp1 protein levels in SW 626 cells when transfected with their respective siRNAs and
abolished EGF-mediated increased c-Myc and Sp1 levels compared to c-Myc and Sp1 siRNAs untransfected controls as determined by Western blot analyses (Figure 33, inset). In addition, c-Myc and Sp1 siRNAs were sufficient to decrease endogenous telomerase activity by 50% and 65%, and decreased EGF-mediated telomerase activity by 47% and 46%, respectively (Figure 33). Furthermore, reporter assays using hTERT full length, core promoter, and core promoter mutated constructs were performed to confirm that c-Myc and Sp1 are important transcription factors in EGF-mediated telomerase activity. Compared to endogenous promoter activity, the full length hTERT promoter was inhibited by c-Myc and Sp1 siRNAs by 67% and 80%, respectively, and EGF-induced full length hTERT promoter activity was inhibited by 63% and 50%, respectively (Figure 34A). When both siRNAs were transfected into the cells, EGF-mediated telomerase activity was reduced by 75% (Figure 34A). In regards to the hTERT core promoter, endogenous hTERT core promoter activity was inhibited by Sp1 and c-Myc siRNAs by 67% and 33%, respectively (Figure 34A). EGF-induced hTERT core promoter activity was suppressed by 67% and 44% in the cells transfected with Sp1 and c-Myc siRNAs, respectively (Figure 34A). When both Sp1 and c-Myc siRNAs were combined, EGF-mediated hTERT core promoter activity was inhibited by 78% (Figure 34A). To confirm that EGF-mediated telomerase activity in ovarian cancer cells require Sp1 and c-Myc transcription factors, reporter assays using mutant core promoter reporter plasmids and Sp1 and c-Myc siRNAs were performed (Figure 34B). The results revealed that abrogation of Sp1 sites significantly reduced responsiveness to EGF (Figure 34B). Even though, abrogation of the E-boxes by substitution mutations still allowed for some EGF-
induced hTERT activation, indicates that c-Myc along with Sp1 may play a role in EGF-mediated telomerase activity in ovarian cancer cells (Figure 34B).

Figure 33  EGF-Mediated Telomerase Activity is c-Myc- and Sp1-Dependent
SW 626 cells were transfected with ± 0.5 µM c-Myc siRNA ± 1 µM Sp1 siRNA and treated with ± 2 ng/mL EGF for an hour prior to collection. Cells were then harvested and analyzed for c-Myc and Sp1 expression by Western blot (Inset) and telomerase activity by PCR-ELISA. For Western blot analyses, β-actin was used as loading control. p values indicate the statistical significance of EGF treated cells vs. untreated controls (*), c-Myc, Sp1 transfected cells vs. untreated controls (~, ~~), c-Myc, Sp1 transfected + EGF treated cells vs. EGF treated cells (^, ^^), and c-Myc and Sp1 transfected + EGF treated cells vs. EGF treated cells (**). For telomerase analyses, results were expressed as mean optical density at 490 nm for triplicate samples ± S.E.
Figure 34  EGF Targets c-Myc and Sp1 Binding Sites in the hTERT Core Promoter
Figure 34  (Continued) EGF Targets c-Myc and Sp1 Binding Sites in the hTERT Core Promoter

SW 626 cells were transfected with full length hTERT promoter, hTERT core promoter, mutated hTERT core promoter luciferase constructs, c-Myc and/or Sp1 siRNAs along with pCMV-β-galactosidase expression vector and treated with ± 2 ng/mL EGF for an hour prior to collection. Luciferase reporter activity was normalized by β-galactosidase activity. Results were expressed as the mean luciferase activity ± S.E. from triplicate samples and p values indicate statistical differences between luciferase assays in EGF treated cells vs. untreated controls (*), c-Myc transfected cells vs. untransfected counterparts (~), c-Myc transfected + EGF treated cells vs. EGF treated cells (^), Sp1 transfected cells vs. untransfected counterparts (~~), Sp1 transfected + EGF treated cells vs. EGF treated cells (^^), and c-Myc + Sp1 transfected cells vs. EGF treated cells (**).
Discussion

The EGFR is overexpressed in over 70% of primary ovarian cancers [313] and has been directly associated with poor postoperative prognosis due to, in part, chemotherapy resistance [314-316]. Furthermore, the activation of the EGFR signaling pathway in cancer cells is associated with increased cell proliferation, angiogenesis, metastasis, and decreased apoptosis [317]. Since EGF receptors are constitutively activated in ovarian cancer and EGF, an EGFR ligand, promotes OSE cell growth, the potential contribution of EGF in the regulation of telomerase activity was examined. A better understanding of this signaling pathway, then, could provide insight on developing effective chemotherapeutic drugs. These experiments showed EGF stimulates telomerase activity in a transcription-dependent manner by activating the Sp1 and c-Myc binding sites within the hTERT core promoter via the Pyk2/ERK 1/2 signaling pathway (Figure 35).

The core promoter region contains an E box binding factor and putative protein binding sites for transcription factors Sp1 and AP2 [138]. E box binding proteins are known to heterodimerize with a variety of transcription factors with helix-loop-helix domains including Myc-related family members and Max-related family members [228]. c-Myc may form dimers with Max which then bind to specific E-box binding sites to transactivate the hTERT promoter. However, c-Myc can also dimerize with Mad1 at the same binding site to suppress hTERT transcription [229]. Interestingly, Budiyanto et al showed a correlation between c-Myc and Sp1 transcription factors and EGFR when squamous cell carcinoma cell line treated with EGFR inhibitor, AG 1478, significantly
reduced the expression of c-Myc and Sp1. In addition, they showed that AG 1478 increased the level of Mad-1 protein expression [178]. These results and my luciferase reporter assays results are consistent with the notion that Sp1 and c-Myc cooperate to activate hTERT transcription and telomerase activity [138, 146, 318].

However, previous studies have also shown that EGF can regulate telomerase in vulvar cancer and squamous cell carcinoma through Ets-mediated transactivation of TERT via the MAPK signaling pathway [176, 178]. In contrast, my results show that Sp1 and c-Myc transcription factors appear to be the major transcription factors involved in EGF-mediated stimulation of telomerase in ovarian cancer cells. Yet, neither c-Myc and Sp1 siRNAs transfections nor E-box and Sp1 mutated binding sites in the hTERT core promoter did not completely abolish all promoter activity in my studies. Therefore, since Ets binding sites in the hTERT core promoter were intact in all of my experiments, Ets may account for non-c-Myc and/or Sp1-mediated hTERT activity.

Of particular interest, these experiments provide novel findings that Pyk2 kinase activity was essential for EGF-mediated telomerase activity in ovarian cancer cells (Figure 35). So, Pyk2-mediated telomerase activity may represent a novel molecular mechanism by which Pyk2 regulates tumor cell proliferation and/or apoptosis to confer increased tumor cell survival. Pyk2 along with other nonreceptor tyrosine kinases such as focal adhesion kinase (FAK), are important signaling effectors linking integrin and growth factor receptor signaling to cell proliferation, migration, survival, and apoptosis in many cell types [304, 319]. FAK is the prototypical signaling effector coupling integrin-matrix interactions to intracellular signaling events [320]. It is rapidly activated
following integrin engagement and exerts downstream effects by serving both as an effector kinase as well as a scaffold protein for various molecules including Src and PI-3-K [321]. A variety of extracellular signals that elevate intracellular calcium concentrations and stress signals mediated by tumor necrosis factor (TNF-α) or ultraviolet (UV) irradiation lead to activation of Pyk2 [304, 319, 321]. Pyk2 has been implicated in the regulation of migration and invasion of glioma, endothelial, smooth muscle cells, and intestinal cells [322-325]. In tumors, Pyk2 expression decreases with increasing tumor grade in prostatic adenocarcinoma [301]. Likewise, Meyer et al showed that hyperactivation of receptor tyrosine kinase, FGFR3, in multiple myeloma cells recruited Pyk2 leading to activation of proliferative and/or anti-apoptotic signaling pathways [326].

Consequently, the clinical relevance of my research in current developmental efforts of monoclonal antibodies and receptor tyrosine kinase inhibitors (TKIs) where binding of EGF growth or active active site of the kinase are blocked is two fold. Since EGF increases telomerase activity and Pyk2 plays an important role in signal transduction of EGF/EGFR interactions, further studies pursuing either monoclonal antibody to EGF or TKIs for EGFR may lead to more effective and/or improved drugs development for ovarian cancer.
The data of this chapter show that EGF stimulation of telomerase activity was mediated by Sp1 and c-Myc transcription factors within the hTERT core promoter in an ERK 1/2 /Pyk2-dependent manner.
Chapter V

Conclusions

Re-expression of telomerase in cancers maintains genomic stability of critically shortened telomeres allowing for continuous cell proliferation and immortalization [327]. As a clinical consequence, however, the re-expression of telomerase in cancers suggests that telomerase may be an important target for chemotherapeutic intervention [328, 329]. By specifically targeting telomerase, it may be possible to restore normal telomeric attrition resulting in cellular senescence and apoptosis. Therefore, it is important to identify positive and negative regulators of telomerase activity in order to develop novel therapies against telomerase.

Novel findings herein indicated that VEGF is not limited to regulation of endothelial cell growth and survival, but also appears to regulate telomerase activity in ovarian cancer cells via an ERK 1/2 transcription-dependent activation of the Sp1 binding sites within the 976- to 378-bp hTERT promoter region. These findings provide a plausible mechanism for the presence of VEGF receptors in non-endothelial cells. Consequently, the effectiveness of anti-angiogenic therapies may function to suppress new blood vessel growth from local endothelial cells to ovarian tumors as well as to inhibit telomerase activity within the tumor cells.

This study also identified telomerase as a novel molecular target of LPA. As a positive upstream regulator of VEGF, LPA-mediated telomerase activity in ovarian
cancer was also found to occur in an ERK 1/2-dependent manner to activate the Sp1 binding sites within the 976- to 378-bp hTERT promoter regions. Therefore, while most clinical trials focus on LPA as a potential biomarker for ovarian cancer, this study provides novel information on the function of LPA that may contribute to the malignant phenotype of ovarian cancer.

Furthermore, this study reported that the anti-tumorigenic role of vitamin E role in ovarian cancer is two fold. Vitamin E suppressed endogenous telomerase activity and abrogated LPA-induced telomerase activity in ovarian cancer cells. Though in vivo testing and clinical studies need to be performed to further evaluate the clinical usefulness of vitamin E for chemotherapeutic efficacy, the present study shows that, by targeting telomerase, supplemental vitamin E may be a valuable adjuvant for prevention of ovarian cancer and/or improved sensitivity to standard chemotherapeutic agents.

Lastly, this study demonstrated that EGF-induced telomerase activity in ovarian cancer cells is ERK 1/2- and Pyk2-dependent. EGF-mediated telomerase activity activated c-Myc and Sp1 binding sites within the hTERT core promoter region. Since overexpression of EGFR correlates with increased metastasis, decreased survival, and poor prognosis, further studies on the inhibition of EGFR-associated tyrosine kinases currently in clinical trials for the treatment of solid tumors, including ovarian cancer are warranted as they may block major signaling pathways for telomerase activation as a mechanism of antitumor activity of these drugs.

Interestingly, VEGF, LPA, and EGF all transduced activation of telomerase in ovarian cancer cells through the activation of ERK 1/2. These findings are consistent
with the notion of MAPK as a cellular central hub controlling the effects of growth factors and stresses on cellular proliferation and differentiation [330, 331].

In this study, VEGF-, LPA-, and EGF-elicited transcriptional effects on the hTERT gene were mediated by Sp1 and c-Myc transcription factors with VEGF and LPA targeting Sp1 binding sites within the 976- to 378-bp hTERT promoter regions and EGF targeting Sp1 and c-Myc binding sites within the hTERT core promoter. Consequently, c-Myc and Sp1 appear to be major transcription factors associated with hTERT transcription and it is not surprising, then, that they are overexpressed with malignant transformation [229, 318]. The ability of multiple growth factors to positively regulate telomerase using redundant pathways in ovarian cancer indicates both the importance of telomerase activation to maintain the malignant phenotype as well as the complexity of telomerase regulation.

In summary, chemosensitivity is a crucial prognostic factor for ovarian cancer and levels of or changes in telomerase may predict therapeutic outcome. Understanding the mechanisms that positively and negatively regulate telomerase, especially those that involve VEGF/LPA, EGF, and vitamin E, could enhance cellular sensitivity to chemotherapeutic agents resulting in an improved therapeutic efficacy and clinical outcome.
References


About the Author

Yira Bermudez received a Bachelor of Science in Chemistry from the University of Tampa. After graduation, she worked at Bausch & Lomb, Inc. as an Analytical Research Chemist and Quality Control Chemist for over 5 years. While working at Bausch & Lomb, she completed a Master of Business Administration degree from the University of Phoenix. She entered the Ph.D. in Medical Sciences program at the University of South Florida’s College of Medicine, Department of Pathology and Cell Biology in 2003.

While in the Ph.D. program, Ms. Bermudez has received the Superior Presentation Award and the Women in Women’s Health Science Research Award at the University of South Florida College of Medicine 2005 Research Day, and the 2006 American Association for Cancer Research Minority Scholar Award for presentation of her work. Ms. Bermudez’s extracurricular activities included being Honor Council Representative and College of Medicine Committee on Research Graduate Student Representative.