TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS INTO PRIMARY INVASIVE DUCTAL BREAST CARCINOMA CELL LINES AND SUBSEQUENT EFFECTS ON GENE TRANSCRIPTION

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TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS

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EFFECTS ON GENE TRANSCRIPTION

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE

Introduction 12
Breast Cancer Review 13
Role of Genetic Mutations in Breast Cancer 15
Review of the E2F-1 Transcription Factor 17
Aberrant Gene Expression in Breast Cancer- Telomerase 22
Review of Tumor Suppressor Genes and Breast Cancer 30
p53 Tumor Suppressor Protein 30
p73 Tumor Suppressor Protein 31
$p21^{WAF1/CIP1}$ Tumor Suppressor Protein 33
BRCA1 Tumor Suppressor Protein 35
Protein Transduction Review 36
Overview of Proposed Research 44
Literature Cited 53

III. PRODUCTION, PURIFICATION AND TRANSDUCTION OF E2F-1/TATHA AND E132/TATHA RECOMBINANT PROTEINS

Abstract 64
Introduction 65
Materials and Methods 67
Results 79
Discussion 85
Literature Cited 105

IV. TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS REPRESSES EXPRESSION OF HTERT IN PRIMARY DUCTAL BREAST CARCINOMA CELL LINES

Abstract 108
Introduction 109
Materials and Methods 112
Results 122
Discussion 124
Literature Cited 132

V. TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS ACTIVATES THE P73 AND $P21^{WAF1/CIP1}$ TUMOR SUPPRESSOR PATHWAYS IN P53-MUTATED BREAST CARCINOMA CELLS

Abstract 135
Introduction 136
Materials and Methods 139
Results 150
Discussion 154
Literature Cited 164

VI. GENERAL DISCUSSION AND CONCLUSION 168

LITERATURE CITED 182

LIST OF TABLES

Tables Page

CHAPTER II

1.1. Staging of Breast Cancer ... 46

1.2. Comparison of Breast Cancer Cell Lines ... 47

1.3. Familial Cancer Syndromes Associated with the Development of Breast Cancer ... 48

LIST OF FIGURES

Figures Page

CHAPTER II

2.1. E2F-1 signaling pathways involved in cell cycle control and apoptosis 49

2.2. Effects of telomerase inhibition on telomere length over time 50

2.3. Model of p53/p73 pathways and induction of cell cycle arrest and/or apoptosis 51

2.4. Mechanism of p53-independent apoptosis 52

CHAPTER III

3.1. Plasmid map illustrating construction of E2F-1/TatHA vector........................ 90

3.2. Restriction enzyme digestion of E2F-1/TatHA DNA..................................... 91

3.3. 5' Site-directed mutagenesis of pCMV E132 via PCR amplification 92

3.4. Plasmid map illustrating construction of E132/TatHA vector 93

3.5. Restriction enzyme digestion of E132/TatHA DNA 94

3.6. Western blot verifying production of E2F-1/TatHA and E132/TatHA recombinant proteins 95

3.7. InVision™ His-tag stained SDS-PAGE gels to verify expression of 6-histidine tag in E2F-1/TatHA and E132/TatHA proteins 96

3.8. FPLC purification protocol for E2F-1/TatHA and E132/TatHA 97

3.9. FPLC chromatogram representing protein purification of E2F-1/TatHA 98

3.10. FPLC chromatogram representing protein purification of E132/TatHA 99

3.11. FPLC purification of E2F-1/TatHA recombinant proteins and analysis with SDS-PAGE gel electrophoresis 100

3.12. FPLC purification of E132/TatHA recombinant proteins and analysis with SDS-PAGE gel electrophoresis 101

3.13. Brightfield images of infiltrating ductal breast cancer cell lines 102

3.14. Transduction of E2F-1/TatHA and E132/TatHA proteins into HCC1937 cells 103

3.15. Transduction of E2F-1/TatHA and E132/TatHA proteins into MCF-7 cells 104

CHAPTER IV

4.1. SDS-PAGE gels of recombinant E2F-1/TatHA and E132/TatHA proteins ... 128

4.2. Immunocytochemistry images of HCC1937 cells 129

4.3. Fold repression of hTERT gene expression in breast cancer cell lines-TatHA control 130

4.4. Fold repression of hTERT gene expression in breast cancer cell lines-E132/TatHA control 131

CHAPTER V

5.1. Construction of expression vectors and specific domains within E2F-1 Tat fusion proteins 157

5.2. Western blot analysis of E2F-1/TatHA and E132/TatHA proteins 158

5.3. Fold change in p73 gene expression in HCC1599 cells 159

5.4. Fold change in p73 gene expression in HCC1937 cells 160

5.5. Fold change in $p21^{WAF1/CIP1}$ gene expression in HCC1599 and HCC1937 cells 161

5.6. Apoptotic activity in HCC1937 cells following 24 hours of Tat fusion protein treatment 162

5.7. Immunocytochemistry of apoptotic activity in HCC1599 and HCC1937 cells following 24 hours of treatment with Tat fusion proteins 163

ABBREVIATIONS

5-FU	5-Fluorouracil
Amp	Ampicillin
APAF1	Apoptotic Protease Activating Factor 1
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-Related Protein
BAX	BCL2-Associated X Protein
BCA	Bicinchoninic Acid
bp	Basepairs
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA2	Breast Cancer Susceptibility Gene 2
BSA	Bovine Serum Albumin
CBP	CREB Binding Protein
CCD	Charge-Coupled Device
cDNA	Complementary Deoxyribonucleic Acid
CDKS	Cyclin Dependent Kinases
CHK2	Checkpoint Kinase 2
CKI	Casein Kinase 1
CRP	Covance Research Products
dH_2O	Distilled Water
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Dependent Protein Kinase
Dnase	Deoxynuclease
dNTPs	Deoxynucleotide Triphosphates
DP-1	DRTF1-Polypeptide 1
DRTF-1	Differentiation Regulated Transcription Factor Protein
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
ETOH	Ethanol
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FPLC	Fast Protein Liquid Chromatography
GADD45	Growth Arrest and DNA-Damage-Inducible Protein 45

H_2O	Water
HA	Hemagglutinin
HCl	Hydrochloric Acid
HEPES	N-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid
HRP	Horse Radish Peroxidase
HS	Heparan Sulfate
HSV-1	Herpes Simplex Virus-Type 1
hTERT	Human Telomerase Reverse Transcriptase
hTR	Human Telomerase Ribonucleic Acid
IGF-1	Insulin-Like Growth Factor 1
IPTG	Isopropyl-Beta-D-Thiogalactopyranoside
IU	International Units
kb	Kilobases
KKL	Klenow-Kinase-Ligase
LB	Luria Broth
M	Molar
MCS	Multiple Cloning Site
MDM2	Mouse Double Minute 2
MEME	Minimal Essential Medium, Eagle's
mM	Millimolar
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
NF-kappaβ	Nuclear Factor-kappa Beta
p53AIP1	p53-Regulated Apoptosis Inducing Protein 1
PBS	Phosphate Buffered Saline
P/CAF	p300/CBP-Associated Factor
pCMV	Plasmid Cytomegalovirus
PCR	Polymerase Chain Reaction
PIDD	p53-Induced Protein with a Death Domain
PIG3	p53-Induced Protein 3
PTD(s)	Protein Transduction Domain(s)
PTEN	Phosphatase and Tensin Homolog
PUMA	p53 Up-Regulated Modulator of Apoptosis
RNA	Ribonucleic Acid
Rnase	Ribonuclease
rpm	Revolutions Per Minute
RPMI	Rosswell Park Memorial Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SCC25	Squamous Cell Carcinoma 25
SCCHN	Squamous Cell Carcinoma Head and Neck
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SYBR	Synergy Brands, Inc
Tat	Transactivator of Transcription

TatHA	Transactivator of Transcription with Hemagglutinin Tag
TBS-T	Tris Buffered Saline-Tween 20
TFPs	Tat Fusion Proteins
TMR	Texas Methyl Red
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor alpha
TUNEL	Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
U	Units
μg	Micrograms
μl	Microliters
μM	Micromolar
XPC	Xeroderma Pigmentosum, Complementation Group C

CHAPTER I

INTRODUCTION

Breast cancer affects 1 in 8 women worldwide. The American Cancer Society estimates that in 2006, approximately 212,920 women will be diagnosed with invasive breast cancer, and an estimated 40,970 women will die from this disease (1). As the medical world revolutionizes diagnostic equipment and medical therapies for the early detection and treatment of breast cancer, the underlying cause of breast cancer must be addressed and alternative therapies investigated. Simply stated, breast cancer is a result of genetic mutation. Mutations leading to altered gene expression spearhead the development and progression of breast cancer. Our research focuses on several key genetic alterations in primary breast carcinoma cells, and via our proposed cancer therapy, we intend to decrease cancer cell division and/or induce cancer cell death.

Breast carcinoma cells reach their immortalized state via several routes of deregulated gene expression and genetic mutations. This research focuses on the several important genetic alterations detected in breast cancer. First, the majority of cancer cells overexpress telomerase which promotes restoration of the telomeric DNA on chromosomal ends during cell division. The catalytic subunit of telomerase, hTERT (human telomerase reverse transcriptase), is the rate-limiting enzyme responsible for synthesizing telomeric DNA. Disruption of telomerase has been found to impede the

recapping of telomeres, thus triggering pathways involved in cell senescence and/or apoptosis. Second, in addition to overexpression of telomerase, at least 40-50% of breast cancers have mutations in the p53 tumor suppressor gene. The p53 protein is the primary "gatekeeper" of the cell cycle; therefore, mutations in the p53 gene results in impaired cell cycle control thus promoting tumorigenesis. Importantly, the E2F-1 transcription factor is a known repressor of hTERT transcription and transcriptional activator of p53 and p53-homologue proteins. In the presence of p53 mutations, the p53-homologue, the p73 tumor suppressor protein, can assume the responsibilities of wildtype p53. To explore these two genetic alterations in breast carcinomas, we will be utilizing the E2F-1 transcription factor. Previously, the E2F-1 transcription factor has only been investigated using viral-based methodologies (i.e. adenoviruses and adeno-associated viruses) to introduce the E2F-1 transcription factor into cancer cells to study their impact on gene expression. However, viral-based methodologies are often irreversible, do not affect 100% of treated cells and the viral host vector could elicit an immune response. We propose protein therapy with the E2F-1 transcription factor to impact all targeted cancer cells in a reversible manner. We will be investigating an alternative protein therapy with the E2F-1 transcription factor to modulate expression of telomerase and induce expression of p73 and $p21^{WAF1/CIP1}$ tumor suppressor genes in p53-mutated breast carcinoma cell lines. Several p53-mutated cell lines will be investigated in our research due to the high prevalence of p53-mutations in breast cancer.

This research uses an alternative method of introducing the E2F-1 transcription factor into breast carcinoma cells by employing Tat-mediated protein transduction. Proteins attached to the HIV-1 Tat transduction domain have been found to transduce

100% of target cells (2, 3). Once Tat fusion proteins enter cells, they have the opportunity to elicit their biological effect followed by degradation of the recombinant Tat proteins. Importantly, transduction of the E2F-1 transcription factor into all the targeted cancer cells will facilitate a cellular response to our protein therapy. It is anticipated that biologically active E2F-1 proteins will completely repress the expression of telomerase and effectively active p73 cell pathways in p53 mutated cell lines. We anticipate greater levels of repression and/or activation of target genes versus viral-based methodologies due to the more effective introduction of active proteins versus viral-based methods that depend on the carcinoma cells to produce active proteins. In addition, the proposed E2F-1 Tat fusion proteins would function as reversible therapeutic agents because once protein treatment is discontinued, the remaining proteins are degraded via native ubiquitin-proteosome pathways based on the half-lives of the proteins (2-4).

To investigate the biological effect of E2F-1 Tat fusion peptide treatment on breast cancer cells, it was necessary to produce protein expressing constructs and develop an efficient protocol for purifying the recombinant proteins. The primary goal was to develop a successful technique to produce and purify recombinant E2F-1 Tat fusion proteins with greater than 95% transduction potentials. The E2F-1 Tat constructs contained the HIV transduction domain, a hemagglutinin (HA) tag, 6-histidine tag, and the wildtype or mutant E2F-1 gene. Therefore, the proteins translated from the mRNA transcribed from the E2F-1 construct cDNA would produce the following: an E2F-1 protein attached to the HIV-1 Tat transduction domain for efficient transduction of active proteins, a HA tag for detection via immunocytochemistry and a 6-histidine tag for purification via FPLC. In general, the purification of Tat fusion proteins is highly

dependent on the solubility and stability of the proteins under specific physiological parameters (i.e. soluble proteins at final a pH range of 7.4-7.5) required for in vitro testing of cell lines. To briefly summarize this phase of the research, it was necessary to generate constructs with the HIV-1 Tat transduction domain attached to E2F transcription factors in order to test their transduction potential and biological efficacy in carcinoma cell lines. Expression vectors containing the wildtype E2F-1 gene and a mutant E2F-1 gene, E132 (point mutation in DNA-binding domain), were obtained in order to clone the cDNA into the multiple cloning site (MCS) of the pTatHA expression vector to generate E2F-1/TatHA and E132/TatHA constructs. The pTatHA vector already contained the HA tag and 6-histidine tag necessary for detecting and purifying the recombinant E2F-1 proteins. A bacterial expression system was utilized to produce the recombinant proteins, and our purification protocol was optimized by combining affinity chromatography, strong denaturants and FPLC to yield a large quantity of E2F-1 Tat fusion proteins. Therefore, we optimized a methodology to produce biologically active E2F-1 Tat fusion proteins to impact gene expression in breast cancer cell lines. The following objectives summarize the goals of the first phase of this research:

A. Develop an effective methodology to generate constructs with the HIV-1 Tat transduction domain attached to E2F-1 transcription factors.

B. Produce large quantities of purified, biologically active E2F-1/TatHA and E132/TatHA proteins using a bacterial expression system followed by FPLC purification and dialysis.

C. Verify transduction of the E2F-1/TatHA and E132/TatHA fusion proteins into breast carcinoma cell lines.

The second phase of the proposed research investigates repression of telomerase activity in primary ductal breast cancer cells through transcriptional regulation of the catalytic subunit hTERT. We hypothesize that inhibition of telomerase expression can be achieved via Tat mediated protein transduction of the repressor protein E2F-1. Telomerase, a ribonucleoprotein, synthesizes tandem repeats of the DNA sequence TTAGGG at the terminal ends of chromosomes permitting continuous genomic replication and cell division. Telomerase includes an RNA component (hTR) that serves as the template for telomeric DNA and a protein catalytic subunit (hTERT) with reverse transcriptase activity (5). Telomerase activity is regulated by transcription of hTERT which is detectable in 80-90% of malignancies and absent in most normal somatic cells (6). Importantly, fine-needle aspirations of early stage malignant breast tumors revealed that 81% were positive for telomerase (7), and Hiyama et al. (2000) also reported that 95% of advanced stage breast cancers express telomerase (8).

We expect that efficient transduction of the E2F-1/TatHA fusion proteins will effectively repress hTERT expression in the primary ductal breast cancer cell lines HCC1937 and HCC1599. E2F-1 has been identified as a transcriptional repressor of the hTERT gene via binding two separate sites (-174 bp and -98 bp) of the proximal hTERT promoter (9). Efficient transduction of the E2F-1 protein via Tat mediated transduction should facilitate complete repression of hTERT gene expression. Our in vitro experiments with breast cancer cell lines were designed to permit a thorough evaluation

of the overall effects of the E2F-1 repressor protein and its role in transcriptional repression of the hTERT gene. It was hypothesized that complete repression of telomerase activity could be achieved in vitro via protein transduction with E2F-1 to ultimately decrease cell division and/or induce apoptosis. This proposed study was also expected to provide a greater understanding of E2F-1 repressor proteins functioning as transcriptional repressors with a potential role in cancer therapeutics. In summation, the primary objectives of the second phase of this research are as follows:

A. Determine if the E2F-1 transcription factor can repress telomerase activity in primary ductal breast cancer cells through transcriptional regulation of the catalytic subunit hTERT.

B. Achieve complete repression of hTERT in breast cancer cell lines following transduction of wildtype E2F-1/TatHA proteins versus the control E132/TatHA protein using real-time RT-qPCR methodologies.

The third phase of this research focuses on activation of the p73 tumor suppressor gene in p53-mutated breast cancer cell lines. Common mutations that lead to diminished cell-cycle control in breast cancer include alterations in the p53 tumor suppressor gene. Approximately 50% of cancers, including breast cancer, harbor mutations of the p53 tumor suppressor gene (12). However, the p53-homologue, p73, is rarely mutated in carcinoma cells (13). In cancer cells harboring a p53 mutation, multiple studies suggest that overexpression of the p73 tumor suppressor gene can induce apoptosis (14, 15). In

the absence of p53, it is imperative that the proposed cancer therapy stimulates intact cell cycle pathways to inhibit cancer cell division or induce cancer cell death. The p73 pathway provides an alternate route through which apoptosis of cancer cells can be achieved since compelling evidence supports that p73 induction is associated with inducing downstream target genes controlling cell cycle arrest and apoptosis.

An important regulator of p73 expression, the E2F-1 transcription factor, has been found to activate p73 expression and induce cancer cell death (16, 17). Activation of the p73 tumor suppressor pathway has been observed in adenoviral and retroviral studies transfecting the E2F-1 gene into carcinoma cells (16, 17). Additionally, upregulation of p53-responsive genes has been observed in cells upon activation of p73 (18, 19). Stimulation of the p73 tumor suppressor gene has also induced $p21^{WAF1/CIP1}$ expression leading to cell growth arrest and apoptosis (19, 20). We hypothesized that our alternative, reversible methodology to induce p73 expression in breast carcinoma cells, utilizing protein transduction with E2F-1 Tat fusion proteins (TFPs), would effectively target and kill breast cancer cells. We expected the E2F-1 Tat fusion proteins to effectively transduce the targeted breast carcinoma cells, activate transcription of p73 and induce apoptosis.

The $p21^{WAF1/CIP1}$ gene functions as an inhibitor of cyclin-dependent kinases and is directly induced by the p53 tumor suppressor protein and its homologues. The $p21^{WAF1/CIP1}$ gene is an uncommon target for mutational inactivation in breast cancer (21); however, in the absence of p53, its innate role in controlling the cell cycle may be compromised. Wildtype p53 directly induces expression of $p21^{WAF1/CIP1}$ resulting in cell cycle arrest; therefore, in the absence of p53, this pathway controlling the cell cycle is

unstimulated. Importantly, the induction of the p53-homologue p73 can stimulate activation of $p21^{WAF1/CIP1}$ promoting cell cycle arrest in cancer cell division. In combination to p73, $p21^{WAF1/CIP1}$ gene expression in cells is also reportedly affected by the BRCA1 tumor suppressor protein (22). Our study investigates the induction of $p21^{WAF1/CIP1}$ via p73 overexpression using real-time RT-qPCR, and it is anticipated that our experiments will distinguish possible differences in BRCA1 (wildtype versus mutated) on $p21^{WAF1/CIP1}$ expression and subsequent apoptotic activity. We propose that the decreased expression of BRCA1 in the HCC1937 cell line (homozygous 5382C mutation) could impact the ability of p73 overexpression to activate $p21^{WAF1/CIP1}$. Overall, we expect to observe increased fold induction of p73 in all cell lines tested with possible variation in $p21^{WAF1/CIP1}$ expression based on the BRCA1 status of tumor cells.

The following points summarize our objectives for the final phase of this research.

A. Following treatment with E2F-1/TatHA, E132/TatHA and TatHA control proteins, monitor fold changes in gene expression of the p73 tumor suppressor gene in breast cancer cell lines via real-time RT-qPCR.

B. Determine if overexpression of the p73 tumor suppressor gene can activate expression of the p53-responsive gene, $p21^{WAF1/CIP1}$, in p53-mutated breast cancer cell lines.

C. Following induction of p73 gene expression, distinguish differences in $p21^{WAF1/CIP1}$ expression in breast cancer cell lines with and without BRCA1 mutations.

D. Assess apoptotic activity in breast cancer cells following E2F-1/TatHA, E132/TatHA, and TatHA fusion peptide treatment.

In summary, the proposed research investigates the contributory role of specific genetic alterations in breast cancer research and possible avenues for therapeutic intervention. In the following chapters, an effective methodology to produce and purify E2F-1 Tat fusion proteins is outlined. The transduction of Tat fusion proteins into breast cancer cell lines will be demonstrated to illustrate the efficient delivery of biologically active proteins. Chapter IV will investigate our hypothesis that E2F-1 transcription factor proteins can effectively repress hTERT in telomerase expressing breast cancer cell lines. Chapter V will investigate if Tat-mediated transduction of E2F-1 proteins into cells can also induce expression of the p73 tumor suppressor gene in p53-mutated cell lines. We will also determine if the p53-responsive gene, $p21^{WAF1/CIP1}$, can be upregulated via overexpression the p53-homologue, p73.

Overall, the effects of E2F-1 Tat fusion protein treatment on breast cancer cell growth and apoptosis will be assessed in breast cancer cell lines. It is anticipated that the objectives outlined above will lead to a greater understanding of the therapeutic potential of E2F-1 Tat fusion protein treatment as an adjuvant to current chemotherapeutic treatments of breast cancer.

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CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Breast cancer will affect 1 in 8 women worldwide in their lifetime and 1 in 33 will die from this disease (1). In 2006, the American Cancer Society estimated that 212,920 women were diagnosed with invasive breast tumors and approximately 40,970 women died from breast cancer (1). The incidence of breast cancer appears to be increasing worldwide, in part because of better ability for diagnosis due to significant advancements in mammography and the advent of magnetic resonance imaging (MRI) as a screening tool for breast tumors (1-5). However, despite advancements in the early detection and treatment of breast cancer, limitations in therapeutic options still exist because of drug side effects and poor tumor response to treatment. Most chemotherapeutic and radioactive treatments have deleterious systemic effects in women undergoing therapy for breast cancer. Conventional treatments for breast cancer target the uncontrolled growth of cancer cells while a primary source of disease, genetic mutation, is not understood enough to serve as a central target of cancer treatment plans. The future of breast cancer treatment depends on the exploration of therapies targeting specific genetic alterations associated with the development and progression of breast cancer. Therefore, our

research will explore a novel therapy targeting specific genetic alterations in breast carcinomas to impact gene expression and induce cancer cell death.

BREAST CANCER REVIEW

Detection, Diagnosis and Staging of Breast Cancer

Breast cancer originates at the cellular level from genetic alterations. As tumor cells proliferate, their presence manifests into a detectable form. Detection usually occurs when women or their physician incidentally palpate a breast mass or a suspicious finding is identified via screening mammography. The definitive diagnosis of breast cancer then depends on the pathology of a breast tumor (i.e. fine-needle aspiration of a breast tumor or evaluation of tissue from a lumpectomy) followed by surgical cancer staging. Breast cancer staging requires an additional dissection of mammary lymph nodes to identify the sentinel lymph node and assess lymphatic spread of tumor cells. Metastatic disease is suspected based on the extent of lymphatic involvement in combination to physical diagnosis and testing for tumors in the lungs, liver, bones and brain. Overall, breast cancer staging offers valuable information regarding disease activity and the most beneficial therapeutic intervention. Table 1.1 outlines breast cancer staging as published in Harrison's Principles of Internal Medicine, 16th edition (6). The grading of breast cancer is performed according to the Scarff-Bloom-Richardson tumor grading system, and the histological grade of the tumor cells correlates with important prognostic factors. Grade 1 indicates the breast tumor is well-differentiated, cells appear normal without rapid growth and cancer cells are arranged in small tubules (7). Grade 1

breast cancers have a 95% 5-year survival rate (7, 8). Grade 2 breast cancer refers to moderately-differentiated breast cancer cells with mixed characteristics between grades 1 and 3 (7). Grade 2 breast cancers are associated with a 75% 5-year survival rate in patients (7, 8). Grade 3 breast cancer indicates the tumor cells are poorly-differentiated, cells do not appear normal and they tend to grow and spread more aggressively. Grade 3 breast cancer is associated with a 50% 5-year survival rate (7, 8).

The staging and grading of breast cancer is applicable to our research when considering the tumor stage of the cell lines utilized for in vitro studies. Table 1.2 outlines the specific characteristics of the breast cancer cell lines evaluated in our studies. Importantly, 80% of breast cancers are classified as primary infiltrating or invasive ductal breast carcinomas. The two primary infiltrating ductal breast cancer cell lines investigated by this research are HCC1937 and HCC1599. The HCC1937 cell line is derived from a TNM stage IIB, grade 3 primary ductal breast carcinoma, and the HCC1599 cell line originates from a TNM IIIA, grade 3 primary ductal carcinoma. Additional metastatic breast cancer cell lines (stage IV) were examined in our work including MCF-7, MDA-MB-231 and MDA-MB-435S cells. Several specific mutations, oncogenes, and staging for each of the breast cancer cell lines are listed in Table 1.2 (9-17).

Risk Factors Associated with Breast Cancer

Although breast cancer is a result of genetic alterations, multiple known risk factors are attributed to the increased risk of developing breast cancer. Women have an increased breast cancer risk if they are Caucasian or if they have a positive family history

of breast cancer (18). Increased risk of developing breast cancer is also associated with increasing age, and/or a previous medical history of endometrial cancer, mammary dysplasia or contralateral breast cancer (19). Additionally, a greater incidence of breast cancer is associated with menarche before the age of 12 and late menopause after the age of 50 which are phenomena leading to increased total hormonal exposure of breast tissue (19). Advanced age at first pregnancy and nulliparity are also attributed to increased development of breast cancer in women (19). Oral contraceptive use is associated with a slightly increased breast cancer risk while postmenopausal hormone replacement therapy is attributed to a higher incidence of breast cancer (19). Women who are determined to be at greater risk of developing breast cancer should be screened on a regular basis by their physician to ensure early detection and intervention of precancerous and cancerous breast lesions. Genetic testing to detect predisposition to developing breast cancer in women with known risk factors may also become an accepted medical intervention in the near future to better develop treatment plans for these women. Likewise, genetic screening for known mutations or aberrant gene expression associated with breast cancer could decrease mortality rates by allowing early detection and serve as a justification for gene therapy.

ROLE OF GENETIC MUTATIONS IN BREAST CANCER

Breast cancer is a result of genetic mutation. The source of genetic mutation is variable and can be associated with germline mutations in known tumor suppressor genes or sporadic mutations due to radiation exposure, carcinogens, environmental factors

and/or other unknown causes. Hereditary breast cancer is linked to mutations in tumor suppressor genes, and these women have an increased lifetime risk of developing breast cancer. Knudson's two-hit hypothesis explains this predisposition to cancer and the occurrence of familial cancer syndromes (refer to Table 1.3 for specific examples). Knudson's hypothesis states that the first 'hit' or genetic mutation is inherited due to a germline mutation in a cancer susceptibility gene, and the second 'hit' occurs somatically in the other allele of the same gene (20). Following the second hit, the person is predisposed to developing cancer due to the functional inactivation of a tumor suppressor gene or a gene involved in regulation of the cell cycle. Table 1.3 demonstrates autosomal dominant and recessive hereditary cancer syndromes and their association with breast cancer, other malignancies and their respective genetic mutations (20-23).

In breast cancer, mutations in the p53 tumor suppressor gene are present in 40-50% of carcinomas (24, 25). The p53 tumor suppressor gene is regarded as the "gatekeeper" of the cell cycle, and mutations can result in deregulated cellular growth resulting in cancer. Gene mutations in breast cancer susceptibility genes 1 and 2 (BRCA1 and BRCA2) are attributed to 3-8% of all breast cancer cases and 30-40% of familial breast cancer cases (26). BRCA1 and BRCA2 also function as tumor suppressor proteins and numerous mutations in their genes have been detected and attributed to missense, truncating and silent mutations (27). Mutations in the retinoblastoma (Rb) tumor suppressor gene are also linked to progression of breast cancer, and approximately 20-30% of breast carcinomas are positive for a deletion or mutations of the Rb gene (28). The important role of tumor suppressor genes in breast cancer development will be discussed in greater detail in subsequent sections of this review. In addition, the E2F-1

transcription factor and its association with tumor suppressor genes and its potential role in cancer therapeutics will be described. We will also explore an alternative route of introducing the E2F-1 transcription factor into cells via protein transduction and discuss the therapeutic implications of our proposed methods.

REVIEW OF THE E2F-1 TRANSCRIPTION FACTOR

Family of E2F Transcription Factors

The E2F transcription factor family consists of three groups with six different E2F proteins. Overall, the E2F family of transcription factors is multifaceted in its ability to both activate and repress target genes and reportedly promote and inhibit cellular proliferation (29). Group one contains E2F-1, E2F-2, E2F-3; group two includes E2F-4 and E2F-5; and group three only contains the E2F-6 protein. Expression of group one E2F members is controlled by cell growth with significant accumulation at the G1/S phase boundary of the cell cycle. The E2F-4 and E2F-5 transcription factors bind the Retinoblastoma (Rb) protein family members, but function as transcriptional repressors in conjunction with the p130 protein in G0 and G1 phase of the cell cycle. The E2F-6 transcription factor is unique because it does not contain the domains involved in transactivation of E2F target genes nor the domains that bind the Rb protein.

Effects of E2F-1 on the Cell Cycle

The E2F-1 gene is located on chromosome 20q11.2 and the translated protein is approximately 47 kilodaltons in size. The E2F-1 transcription factor is found within the

cell nucleus and plays an integral role in the G1- to S-phase transition in the cell cycle. E2F-1 forms a heterodimer complex with DP-1 (DRTF-1 polypeptide-1) to form the DRTF1/E2F-1 transcription complex which functions to bind the hypophosphorylated Rb proteins (30). In mid to late G1 phase, Rb is phosphorylated and the DRTF1/E2F-1 complex detaches from Rb, and E2F-1 is released to transactivate its target genes. The transcriptional activity of native E2F- 1 is stimulated via phosphorylation by CDK2 and cyclin A-CDK2 in S-phase (31). Following activation, E2F-1 binds specific promoter sequences in target genes to activate transcription, ultimately functioning to mediate both cell proliferation and apoptosis.

The level of native E2F-1 protein fluctuates in the cell cycle. E2F-1 levels begin to rise in G1/S phase, and increase as cells progress into S-phase (32). The S-phase of the cell cycle comprises the complete and accurate duplication of all genes in a cell, and excess E2F1 can activate an S-phase checkpoint (32). It is important to control E2F-1 levels throughout S-phase because it is apoptotic in excess. In contrast, low levels of E2F-1 expression delay S-phase entry, decrease activation of E2F-1 responsive genes and diminish apoptotic activity in cells (32, 33). In normal cells, feedback control, i.e. intact Rb tumor suppressor gene, exists to prevent overexpression of E2F-1 from inducing apoptosis. However, it has been reported that virtually all human tumors exhibit alteration in the Rb pathway (32); thus the effect of E2F-1 overexpression on cell senescence and cell death warrants further investigation.

E2F-1 is the primary E2F family member directly involved in apoptosis. Apoptosis typically occurs following the synthesis of S-phase genes which subsequently activate apoptosis-related genes. Numerous studies transfecting the E2F-1 gene into cells

revealed that apoptosis followed synthesis of S-phase enzymes and DNA after overexpression of E2F-1 (32). Three different pathways have been identified to describe E2F-1-mediated apoptosis: 1) p53-dependent apoptosis via activation of the CDKN2A transcript $p14^{ARF}$, 2) inhibition of survival signals thus making cells more susceptible to apoptotic signals such as TNF-alpha, 3) induction of p73 expression leading to activation of p53-responsive gene targets and programmed cell death (34). Figure 2.1 illustrates several of the E2F-1 signaling pathways involved in cell cycle progression, cell cycle arrest and apoptosis (35). Additionally, microarray analysis of E2F-1 induced apoptosis has revealed upregulation and downregulation of several key proteins involved in signal transduction, cell cycle regulation and apoptosis (36). Jamshida-Parsian et al. (2005) performed microarray analysis of the gene expression associated with adenovirus-mediated overexpression of E2F-1 in melanoma cells (36). They reported at least 5.5-fold induction of apoptosis-related genes BCL2L11 and CDKN1A ($p21^{WAF1/CIP1}$) and 7.2-fold repression of the MYC oncogene (36). Other CDK inhibitor genes involved in regulation of cell proliferation were also induced following E2F-1 overexpression including CDKN2C (p18 CDK4 inhibitor), CDKN1B (p27, Kip1), CDKN1C (p57, Kip2), CDKN1C (p57, Kip2) and CDKN2A (p16, cell cycle checkpoint gene) (36). Microarray analysis also revealed a 6.3-fold upregulation in MAP3K5 (induces apoptosis by extracellular signals) and a 22.6-fold increase in the FGFR3 (fibroblast growth factor receptor 3) gene which is associated with cell growth and oncogenesis (36). Overall, this study provided data supporting novel pathways to explain the mechanism of E2F-1 mediated apoptosis thus supporting the therapeutic potential of the E2F-1 transcription factor.

Inherent Controls of E2F-1 Level in Cells

The level of E2F-1 expression in cells is both positively and negatively controlled by multiple factors. Activation of E2F-1 has been observed via IGF-1 stimulation and positive regulation by cyclins D and E. The expression levels of E2F-1 also increase in the presence of the c-myc oncogene and self-induction also occurs following stimulation of other S-phase genes. Conversely, E2F-1 is inactivated by binding hypophosphorylated Rb protein and negatively regulated by cdk inhibitors $p21^{WAF1/CIP1}$, p27, INK and TNF-alpha. In addition to complex regulation of E2F-1 levels, the E2F-1 protein is controlled via proteosomal degradation. The stability of E2F-1 is modified by covalent phosphorylation, and the exact mechanism of degradation depends on kinases such as cyclin A/cdk2. Regulation of E2F-1 protein levels ultimately occurs by the ubiquination-dependent proteasome degradation pathway (37).

The E2F-1 transcription factor can activate or repress transcription of target genes, but its activity is dependent on a number of factors. When E2F-1 interacts with the Rb protein, its transcriptional potential is negatively affected by recruiting a histone deacetylase complex and by contacting promoter-bound proteins (37). Additionally, phosphorylation regulates the E2F-1/Rb interaction which is ultimately controlled by cyclin D/cdk4 at the G1/S transition of the cell cycle (37). E2F-1 forms a heterodimeric complex with DP1 (DRTF proteins) proteins, and it binds the promoters of genes that contain E2F-responsive elements in order to activate transcription.

Activity of native E2F-1 is dependent on post-translational modification to increase activity of the protein. E2F-1 is subject to modification by acetylation, and the acetylation domains are in close proximity to the DNA-binding domain (38). P/CAF is a

p300/CBP-associated factor that functions to acetylate E2F-1 and affect its activity in several ways. It increases the DNA-binding ability, activation potential and the protein half-life (38). P/CAF and p300/CBP are more commonly known to acetylate histones associated with transcriptionally active DNA, but also function to acetylate other transcription factors such as p53. Acetylation of E2F-1 by P/CAF results in an increased transcriptional activation capacity (38). It is speculated that E2F-1 is not acetylated when bound to the Rb protein; however, when E2F-1 is unbound, acetylation serves to protect it from degradation as it functions as a transcriptional activator. Overall, it appears that acetylation of E2F-1 by either P/CAF or p300/CBP can regulate the transcription activity of E2F-1(38). Ianari et al. (2004) reported that the formation of E2F-1-P/CAF complexes in response to DNA damage supports a role for P/CAF HAT in E2F-1-dependent apoptosis because the protein is stabilized by acetylation (37). Stabilization of E2F-1 occurs in response to genotoxic stress via phosphorylation by ATM kinase, RAD3-related (ATR) kinase, and checkpoint kinase 2 (37). DNA damage also results in the stabilization of E2F-1 via acetylation which boosts its ability to activate downstream targets (37).

A potential drawback to producing the E2F-1 proteins in a prokaryotic system is the possibility of proteins requiring post-translational modifications to elicit their biological actions. It is possible that complete repression or activation of target genes would require the E2F-1 transcription factor to be acetylated. If this proves to be the case, a eukaryotic expression system could be utilized to express the proteins. It has also been proposed that acetylation protects E2F-1 from a ubiquitin-mediated degradation and/or proteolytic cleavage (37). The biological activity of our proposed E2F-1 Tat

fusion proteins may require modifications such as acetylation to increase transactivation of target genes and to extend the protein half-lives. Currently, the wildtype E2F-1 protein half-life is approximately 70 minutes (increases to 3 hours if degradation pathways are inhibited) (32); however, it is anticipated that the half-life of our E2F-1 fusion proteins would be significantly extended due to altered cellular trafficking and our alternative introduction of this transcription factor.

ABERRANT GENE EXPRESSION IN BREAST CANCER- TELOMERASE

The unique ability of immortalized cells to bypass normal controls of cellular proliferation is a phenomenon that is heavily investigated by physicians and scientists. A part of this phenomenon is the increased expression of telomerase in tumor cells. In human cells, telomeres are protein-DNA complexes that cap the ends of chromosomes. During cell divisions, the telomeric DNA shortens progressively until the Hayflick limit of the cell is reached (approximately 50-80 cell divisions), and cells enter a senescent state (39, 40). Telomerase, a ribonucleoprotein, synthesizes tandem repeats of the DNA sequence TTAGGG at the terminal ends of chromosomes permitting continuous genomic replication and cell division. Telomerase includes three primary components: an RNA component (hTR) serving as the template for telomeric DNA, a protein catalytic subunit (hTERT- human telomerase reverse transcriptase) with reverse transcriptase activity and telomerase associated proteins (41). While telomerase is repressed in most human somatic cells, the majority of cancer cells exhibit reactivation of telomerase to maintain the length of telomeric DNA and protect chromosomal DNA during unlimited cell

divisions (42, 43). The expression of telomerase is tightly regulated by expression of the hTERT gene; therefore, complete hTERT repression is the objective of potential cancer therapies. The development of an effective telomerase inhibitor via hTERT repression would result in efficient telomeric DNA shortening and induction of cellular pathways triggering cell senescence and/or apoptosis.

A significant proportion of breast cancers express telomerase which contributes to their immortalized state of growth. Telomerase activity is detectable in 80-90% of malignancies and is absent in most normal somatic cells (44). Fine needle aspirations of malignant breast tumors revealed that 81% were positive for telomerase (45). Hiyama et al. (42) also reported that 95% of advanced stage breast cancers were positive for telomerase. In addition, telomerase is currently being evaluated as a tumor marker for breast cancer and other solid malignancies (46). Due to the importance of telomerase maintaining the immortalized cell state, the development of effective telomerase inhibitors is an important area of cancer research. Therefore, the second phase of our research focuses on the development of a novel telomerase inhibitor, the E2F-1 protein fused to the transduction domain of HIV-1, which should transduce target cells and inhibit expression of hTERT in multiple stages of breast cancer cell lines.

Role of E2F-1 in Telomerase Expression

Because telomerase activity is regulated by the transcription of hTERT, the cloning and characterization of the hTERT-promoter has permitted examination of elements controlling transcriptional activation and repression of hTERT (47). The transcriptional effects of the E2F family of transcription factors on the hTERT promoter

have been investigated by several groups. The E2F family of transcription factors mediates cell cycle progression, and they are released upon phosphorylation of Rb family proteins (48-50). While most of these factors are associated with inducing transcription of genes, E2F-1 has been identified as the primary E2F transcriptional repressor of the hTERT gene (48). Won et al. recently reported that E2F-1, E2F-2 and E2F-3, but not E2F-4 and E2F-5, repress the hTERT promoter in human tumor cells (29). In 2001, Crowe et al. reported the E2F-1 protein binds two sites (-174 bp and -98 bp) of the proximal hTERT promoter and functions as a transcriptional repressor (48). In addition, a study that examined the apoptotic function of E2F-1 found that cell death generated by ectopic expression of E2F-1 was independent of the p53 regulatory pathway (49). Therefore, even in cells with intact p53 tumor suppressor gene function, overexpression of E2F-1 should inhibit transcription of the hTERT gene and potentially induce apoptosis.

Several studies have investigated the role of telomerase inhibition resulting in cell death. Saretzki et al. reported that ribozyme-mediated telomerase inhibition induced immediate cell death via a newly proposed "fast-track" mechanism (51). Theoretically, inhibition of telomerase will result in apoptosis following sufficient cell divisions to shorten telomeres to a length that triggers inherent apoptotic pathways (see Figure 2.2). Saretzki et al. proposed two different mechanisms through which telomerase inhibition could induce cell death: 1) "fast track" mechanism not requiring telomere shortening via interfering with "capping" function of telomerase, and 2) "classical" mechanism of apoptosis triggered by slow telomere shortening in each cell division (51). This proposed "fast-track" mechanism of apoptosis in ovarian cancer cells was observed with their

ribozyme-mediated telomerase inhibitor treatment 72 hours post transduction (51). This study reported a 75% reduction in telomerase activity as detected via semiquantitative analysis and massive cell death following 3 days of treatment with their telomerase inhibitor (51). These data support a novel route of apoptosis induced by a telomerase inhibitor associated with uncapped telomeres triggering arrest/apoptosis pathways (51).

As mentioned previously, telomerase is undetected in most normal somatic cells. A treatment targeting expression of telomerase would therefore have a negligible effect on somatic cells. Germ cells (i.e. sperm) and stem cells do express telomerase; however, as Figure 2.2 illustrates, the long term effects of a telomerase inhibitor on hTERT would have a marginal impact, and the telomeric DNA of these cell types would most likely be regenerated following removal of the telomerase inhibitor (52). Cancer cells have telomeres that appear shorter than normal cells due to telomere shortening during tumor development (52), thus the cells are predisposed to apoptosis following additional telomeric shortening in the presence of a telomerase inhibitor. As demonstrated in Figure 2.2, during a defined treatment time period with a telomerase inhibitor, such as our proposed hTERT repressor, E2F-1/TatHA, the germ and stem cells would recover following treatment, but the cancer cells would undergo apoptosis or cell senescence.

E2F-1 Transcription Factor and Apoptosis

The impact of the E2F-1 transcription factor in cancer cells is a developing area of research. Several groups have reported induction of apoptosis in cancer cells following E2F-1 overexpression. In 1999, Dong et al. first reported that E2F-1 overexpression at high doses (MOI= 100) induced apoptosis in melanoma cells (53). In 2002, Dong et al.

reported that adenoviral-mediated overexpression of E2F-1 in combination to chemotherapeutic drugs in the melanoma cell lines SK-MEL-28 and SK-MEL-2 induced apoptosis (53). The transfection of E2F-1 resulted in greater chemosensitivity of melanoma cells to the drugs roscovitine, etoposide and Adriamycin but not 5-FU, cyclohexamide and cisplatin (54). Overall, they concluded a new chemosensitization therapy for melanoma was possible via adenovirus-mediated E2F-1 gene transfer in combination to Topoisomerase II poisons and other chemotherapeutic agents (54).

The role of E2F-1 in cancer cell death has been explored by multiple groups. Henderson et al. (2000) revealed that E2F-1 suppressed cancer cell growth while decreasing telomerase activity in the Tu-167 SCCHN (squamous cell carcinoma head and neck) cell line (55). They examined the effects of wildtype p16, $p21^{WAF1/CIP1}$, E2F-1 and p53 transfection in 11 different SCCHN cell lines and concluded that E2F-1, $p21^{WAF1/CIP1}$ and p53 overexpression repressed telomerase expression (55). Their work supports a role for E2F-1 in deregulated telomerase expression and cell senescence, but they did not propose a mechanism of action or quantitate the degree of telomerase repression. Adenoviral-mediated overexpression of E2F-1 also induced apoptosis in human breast and ovarian carcinoma cell lines (56). Hunt et al. reported morphological changes consistent with apoptosis in four out of five cell lines tested within 48 hours of exposure to high concentrations of E2F-1 (56). Apoptosis was also confirmed with DNA fragmentation analysis and FACs analysis (56). Apoptotic changes were present in cell lines with mutant p53, thus supporting the role of E2F-1 induced cell death independently of p53, but no specific mechanism of action was suggested (56).

Although the above studies support a role for E2F-1 in cancer cell death, none of these adenoviral-mediated studies directly examined the repressive effects of E2F-1 overexpression on hTERT transcription. The correlation between decreasing telomerase activity via E2F-1 repression and the induction of tumor cell apoptosis has not yet been fully examined. In addition, the mechanism by which E2F-1 induced apoptosis in the above studies has not yet been clearly deduced; therefore, the repressive effects of E2F-1 on telomerase and induction of apoptosis must be examined to fully characterize the in vitro effects of E2F-1 (53, 55, 56).

E2F-1 Functions as a Transcriptional Repressor

The characterization of E2F-1 as a transcriptional repressor is a new role for this factor because it has traditionally been denoted as a transactivator of various genes. Hsieh et al. (57) reported that transcriptional repression rather than the transactivation function of E2F-1 is involved in the induction of apoptosis. Zhang et al. (58) provided the first direct evidence that telomerase is required for maintaining the vitality of both human tumor and immortal cells. Zhang et al. also reported that cell death in immortal cells was telomere-length dependent, and upon inhibiting telomerase in cells with short telomeres, the resulting damage to the chromosomes triggered apoptotic cell death (58). Interestingly, Yamasaki et al., (59) determined that E2F-1 null mutant mice developed a variety of malignant tumors, and Field et al. (60) indicated that E2F-1 functions in mice to promote apoptosis and suppress cell proliferation. Tumor formation in the absence of E2F-1 was also suggested by Crowe et al. (48). This study utilized E2F-1 expression vectors and liposomal transfection into SCC25 cells resulting in transcriptional repression

of hTERT via consensus binding in the hTERT-promoter at putative E2F-1 sites (48). Importantly, Crowe et al. reported that transfection of the E2F-1 constructs reduced endogenous hTERT mRNA levels by 4-fold, and the reduced hTERT mRNA levels were accompanied by decreasing telomerase activity (48). This study was also one of the first to correlate the novel transcriptional repressive effects of E2F-1 on the hTERT gene promoter while monitoring subsequent telomerase activity (48). However, the repressive effects of E2F-1 on hTERT must be further examined. The liposome-mediated transfection of E2F-1 via the Lipofectamine kit (Invitrogen Life Technologies, Carlsbad, CA) as described in Crowe et al. does not guarantee transfection of 100% of the cancer cells (48), nor do any of the adenoviral-mediated studies previously mentioned (53, 55, 56). Therefore, to determine if E2F-1 can completely repress hTERT transcription, thus decreasing telomerase activity and inducing apoptosis, the E2F-1 protein must be effectively transduced into 100% of the cancer cells. Our proposed methodology utilizing protein transduction with E2F-1 Tat fusion peptides will lead to the development of a transduction technique to completely transduce the cancer cells of interest. The proposed research will also potentially generate a method to completely inhibit telomerase thus inducing cell senescence and/or apoptosis in cancer cells.

Association between BRCA1, E2F-1 and Telomerase Expression

In 2001, results published by Ho et al. (61) determined that E2F-1 and E2F-4 protein levels were decreased in primary breast carcinomas, and 70% of the tumors expressed a low level of E2F-1. Additionally, the metastatic nodal tissue examined revealed that 100% of the tissue samples had significantly low levels of E2F-1 as

compared to normal breast tissue (61). Ho et al. also suggested that E2Fs act as tumor suppressors in breast cancer and their down-regulation may be important in the development of metastases (61). Therefore, it would be important to determine the repressive effects of E2F-1 on hTERT transcription in tumor cells with and without the normal BRCA1 protein to determine any variance between BRCA1 status and inhibition of telomerase and induced apoptosis. Recently, Wang et al. (9) determined that the BRCA1 promoter was transactivated in a dose-dependent manner by E2F-1 and served as a target for E2F-dependent transcriptional regulation. Other studies have also demonstrated that BRCA1 is a potent inducer of apoptosis by binding directly to p53 and therefore stimulating transcriptional activation of pro-apoptotic genes such as BAX (58, 62).

Our research will utilize primary infiltrating ductal carcinoma cells, HCC1937 and HCC1599, both with and without a BRCA1 gene mutation. As described in Table 1.2, the HCC1937 cell line tested in our studies has a BRCA1 mutation consisting of a homozygous mutation of the 5382C allele. Therefore, telomerase inhibition and apoptosis in primary infiltrating ductal carcinoma cell lines with a BRCA1 gene mutation (HCC1937) will be compared to cancer cell lines without a BRCA1 gene mutation (HCC1599).

REVIEW OF TUMOR SUPPRESSOR GENES AND BREAST CANCER

Review of the p53 Tumor Suppressor Gene and Breast Cancer

The p53 tumor suppressor protein serves as a cell cycle gatekeeper that functions to suppress cell growth by either cell cycle arrest or induction of apoptosis. It also plays a crucial role in DNA repair, angiogenesis and cellular senescence. However, in 50% of all cancers, including breast cancer, the p53 protein is mutated and unable to perform its normal biological actions (25, 28). The p53 protein is encoded by the p53 gene located on chromosome 17p13 and contains 11 exons (22). The p53 protein contains three functionally distinct regions which are the N-terminal region, a central DNA-binding core region and a basic C-terminal region (22). The p53 tumor suppressor gene mutations that inactivate the p53 protein are commonly missense mutations or nonsense mutations in one allele (25, 28, 63). In the presence of p53 mutations, the functional absence of p53 can be compensated for by p53 homologue proteins such as p73 and p63. The p53 protein has an integral role in cell cycle arrest and apoptosis. The p53 protein is commonly activated following a variety of cell stresses, and via upstream kinases, the p53 protein is phosphorylated and activated (25). The cell cycle is inhibited following activation of targets such as $p21^{WAF1/CIP1}$, 14-3-3σ (maintains G2 cell cycle arrest) and GADD45 (25). Apoptosis is triggered following activation of various apoptotic targets such as BAX, APAF1, PUMA, p53AIP1, PIDD and Noxa (25). Alternatively, activation of p53 can result in activation of genes involved in DNA repair (such as p53R2) and inhibit metastasis and angiogenesis (25). Figure 2.3 demonstrates a simplified model of

p53 and a p53 homologue, p73, in cell signaling resulting in apoptosis and/or cell cycle arrest.

Review of the p73 Tumor Suppressor Gene and Breast Cancer

In 1997, two p53 homologues were identified- p73 and p63. The p73 protein, located on the human chromosome 1p36.3, shares 63% of the DNA binding region of p53, 38% identity with the tetramerization domain, and 29% identity with the transactivation domain (64). The significant homology between p73 and p53 suggests that the p73 protein can function to regulate cell growth and apoptosis in the absence of p53. As previously mentioned, it is estimated that 50% of cancers harbor mutations of the p53 tumor suppressor gene (25); however, p73 is rarely mutated in carcinoma cells (64). In breast cancer, loss of heterozygosity of the p73 gene region has only been described at a rate of 13% (65). Mutations in p73 are uncommon in breast cancer with overexpression being the most common abnormality which correlates with an advanced tumor stage and a poor prognosis (25, 66). However, a subset of advanced stage breast cancers (lymph node metastasis, vascular invasion, and high-grade malignancy) have been found to overexpress the p73 protein (25, 65).

The p73 gene encodes a complex number of isoforms produced by alternative splicing of the C-terminus. At least 6 different isoforms have been identified (α, β, γ, δ, ε, and ζ) that share an N-terminal transactivation domain, DNA-binding domain and the oligomerization domain (65). Although the isoforms differ in their C-terminal ends, each of the transcriptionally active isoforms of p73 functions to activate transcription of target genes. The p73α and p73β isoforms are primarily associated with apoptosis and cellular

senescence and are the isoforms of particular interest in our research. The p73α isoform is longer than the p73β isoform by 137 residues on the C-terminus of the protein (67). The p73β isoform also slightly differs from p73α in the last 5 residues of the C-terminus (67). Some studies suggest the p73β isoform is a more potent transcriptional inducer of target genes and more effective at inducing apoptosis (68, 69), but p73α is also credited as an effective activator of transcription and apoptosis (70). In this proposed research, our primers determine the expression of transcriptionally active p73, including isoforms p73α and p73β (71). In addition to the transcriptionally active isoforms of p73, transcriptionally inactive truncated isoforms of the p73 protein (ΔNp73) can be produced via a different transcriptional start site (intron 3) (72). Importantly, these inactive isoforms can exhibit a dominant negative effect toward p73 and wild-type p53 (72). Negative feedback regulation of p73 by ΔNp73 has been reported which appears to control cell survival and apoptosis (72). Despite the association of p73 and ΔNp73, our research only focused on detection of the transcriptionally active isoforms of p73. The primers utilized in our studies to quantitate expression of p73 are specific for the transcriptionally active isoforms, including p73α and p73β, to detect the impact of our proposed E2F-1 Tat fusion protein treatment on cancer cells.

Activation of the p73 tumor suppressor pathway has been observed in adenoviral and retroviral studies transfecting the E2F-1 gene into carcinoma cells (73, 74). Seelen et al. identified E2F-1 binding sites of the p73 promoter and suggested that regulation of p73 expression by E2F-1 occurs primarily at sites -155 and -132 of the proximal p73 promoter (75). Upregulation of p53-responsive genes has also been observed in cells upon activation of p73 (34, 76). Specifically, stimulation of the p73 tumor suppressor

gene has induced p21$^{WAF1/CIP1}$ expression leading to cell growth arrest and apoptosis (76, 77). In 2003, Marabese et al. reported p73 activation (~2.5-fold increases) following treatment of cells with DNA damaging agents such as doxorubicin (71). Their study also indicated an important role for E2F-1 in binding the p73 promoter and activation of transcription which triggered downstream pathways that induced apoptosis (71). The cell signaling pathways involving p73 are comparable to the pathways activated by p53, and the downstream target genes of p53 can be activated by p73. The similarities in transcriptional activators, modifiers and targets of p73 and p53 are outlined in Figures 2.3 and 2.4. Importantly, Figure 2.4 illustrates the important role of p73 expression in apoptosis. Overexpression of p73 results in activation of pro-apoptotic genes such as BAX (BCL2-associated X protein), PUMA (p53 upregulated modulator of apoptosis) and Noxa (Latin for "damage") (78). Activation of Noxa results in the release of cytochrome c to the cytosol thus triggering apoptosis (78). Upregulation of PUMA provokes translocation of BAX to the mitochondria which also results in release of cytochrome c. Ultimately, activation of effector caspases resulting from mitochondrial dysfunction forces the cells down a pathway of destruction. The apoptotic pathway is a primary target for therapies inducing p73 overexpression.

Review of the p21$^{WAF1/CIP1}$ Gene and Breast Cancer

The p21$^{WAF1/CIP1}$ gene is located on chromosome 6p21.2 and belongs to the family of Cip/Kip cyclin dependent kinase (cdks) inhibitors. It functions to prevent phosphorylation of cdk substrates and thus impedes cell cycle progression (25). Specifically, activation of p21$^{WAF1/CIP1}$ results in cell cycle arrest in G1 or G2 following

DNA damage (79). The p21$^{WAF1/CIP1}$ gene is a primary transcriptional target of the p53 tumor suppressor protein. The p21$^{WAF1/CIP1}$ gene is not a common target for mutational inactivation in breast cancer; in fact, mutations in p21 $^{WAF1/CIP1}$ are extremely rare in most cancers (80). However, in the absence of p53, its innate role in controlling the cell cycle may be compromised. Downregulation of p21$^{WAF1/CIP1}$ is a common occurrence following inhibition or mutation of p53. Multiple other factors also repress expression of p21$^{WAF1/CIP1}$ including the c-Myc proto-oncogene, the human papillomavirus, hepatitis C virus, E-box binding protein p47ING1a (a member of the inhibitor-of-growth family) and the CCAAT displacement protein/cut homeodomain protein (CDP/cut) (81). Interestingly, an isoform of the CDP/cut protein, p75, is often expressed at high levels in primary breast tumors and breast cancer cell lines versus low levels of expression in normal breast tissue (81). This suggests that p75 repression of p21$^{WAF1/CIP1}$ may contribute to tumorigenesis via inhibition of cell cycle arrest (81). In tumors with p53 mutations, a p53-independent pathway has the potential to activate expression of p21$^{WAF1/CIP1}$. The p73 protein is a known upstream regulator of the p21$^{WAF1/CIP1}$ gene (82). In 2005, Schmelz et al. reported induction of p21$^{WAF1/CIP1}$ expression in myeloid leukemia cells following induction of p73 with 5-Aza-2'-deoxycytidine (5-Aza-CdR) (82). Schmelz et al. also determined that exogenous induction of p73 isoforms p73α and p73β upregulated p21$^{WAF1/CIP1}$ expression resulting in chemosensitization of epithelial cells to 5-Aza-CdR (82). Thus, induction of p21$^{WAF1/CIP1}$ by p73 overexpression resulting from our proposed E2F-1 Tat fusion protein therapy may also serve to chemosensitize breast carcinoma cells to standard chemotherapeutic agents.

Review of BRCA1 Tumor Suppressor Gene and Breast Cancer

Mutations in Breast Cancer Susceptibility genes 1 and 2 (BRCA1 and BRCA2) occur in only 5-10% of all breast cancers. Overexpression of wildtype BRCA1 in ovarian and breast cancer cell lines has been found to reduce cellular proliferation (28). Our research is particularly interested in BRCA1 mutations since one of the primary ductal carcinoma cells lines investigated by our research harbors a homozygous 5832C BRCA1 mutation. The BRCA1 gene is located on chromosome 17q21.31 and mutational inactivation is generally the result of missense, truncation and silent mutations (27). The BRCA1 protein normally functions to regulate p53 and $p21^{WAF1/CIP1}$ levels in cells. Recent studies support the role of BRCA1 in the transcriptional regulation of $p21^{WAF1/CIP1}$, GADD45, Cyclin B1, DBB2, XPC, and multiple other targets (83). BRCA1 binds to DNA without any sequence specificity which may be an important part of its role in DNA repair and transcription. BRCA1 has a direct role in cell cycle checkpoints and inhibits cell cycle progression into S-phase. In studies that overexpressed the BRCA1 protein, $p21^{WAF1/CIP1}$ was activated in a p53-independent manner (83). In cell lines lacking $p21^{WAF1/CIP1}$, overexpression of BRCA1 did not lead to G1 arrest, thus supporting the concept that the arrest of the cell cycle in G1 phase is dependent on the expression of $p21^{WAF1/CIP1}$ (83). These findings support a role for the BRCA1 protein in regulating the $p21^{WAF1/CIP1}$ promoter suggesting that the BRCA1 protein is required for $p21^{WAF1/CIP1}$ activation. This research will investigate possible differences in $p21^{WAF1/CIP1}$ expression in wildtype BRCA1 versus mutant BRCA1 expressing cell lines following transactivation of the $p21^{WAF1/CIP1}$ gene.

PROTEIN TRANSDUCTION REVIEW

Discovery of HIV-1 Tat Protein:

This research uses an alternative method of introducing the E2F-1 transcription factor into breast carcinoma cells by employing Tat-mediated protein transduction. In 1988, HIV-1 Tat-mediated protein transduction was independently reported by Green (84) and Frankel (85) after experimental data indicated the 86 amino acid transactivator protein, Tat (transactivator of transcription), could be internalized by cells with subsequent activation of the HIV-1 promoter. Their work prompted intense investigations that explored the implications of Tat-mediated protein transduction as a novel therapeutic intervention to treat cancer and other diseases. In 1994, Fawell et al. reported that various cargo molecules complexed to the Tat protein could be internalized by tissue culture cells (86). They chemically cross-linked Tat peptides (amino acid residues 1-72 or 37-72) to a variety of cargoes such as β-galactosidase, RNase A and horseradish peroxidase (86). In vitro, their work revealed that Tat chimeras were effective on all cell types, and in vivo, the Tat proteins were primarily internalized by endothelial cells, Kupffer cells, and/or splenic macrophages because high levels of Tat-β-galactosidase was detectable in mouse hearts, livers, and spleens (86).

In 1998, Nagahara et al. introduced an in-frame Tat bacterial expression vector with a hemaglutinin tag referred to as pTatHA (87). The vector also contains an N-terminal 6-histidine tag, an 11-amino acid protein transduction domain (residues 47-57) flanked by glycine residues to permit free bond rotation and a polylinker serving as a multiple cloning site (87). Nagahara et al. reported transduction of denatured Tat

proteins into approximately 100% of primary or transformed cells using a protein misfolding purification protocol that employed a bacterial expression system (87). The pTatHA vector has been utilized by numerous groups to produce in-frame genetic fusion proteins with the Tat transduction domain to study their subsequent effects both in vitro and in vivo. Recent advances in basic science and pre-clinical/clinical gene therapy fields have led to the investigation of cell permeable peptides as tools for gene and protein transduction. The efficient delivery of therapeutic or toxic agents to carcinoma cells has been investigated with viral and non-viral gene therapy.

Comparison of Tat Therapy to Viral-based Methodologies

Viral delivery of anti-cancer agents has been a key area of investigation targeting cellular delivery of therapeutic agents to arrest cell growth or induce apoptosis. There are a number of disadvantages associated with the use of viruses in cancer therapy. Viral vectors only have a limited DNA carrying capacity and the large-scale manufacturing of virus can be cost prohibitive. Additionally, viral gene therapy has encountered problems in immunogenicity and the undesirable insertion of genetic material into the host's genome. However, recombinant viral vectors are very effective at delivering the genetic material across cell membranes to transport the DNA into the cell cytoplasm and to its nuclear target (88). A similar effect can be achieved by employing protein transduction domains. Importantly, several modes of transducing recombinant proteins have been investigated as alternative vehicles for anti-cancer agents to affect cellular transcription and gene expression. Protein transduction serves as an alternate modality to introduce

therapeutic agents without the common drawbacks associated with viral-based methodologies.

Protein Therapy with Protein Transduction Domains (PTD)

Protein therapy is dependent on the direct delivery of proteins through the plasma membrane and into specific cell compartments. Small cationic peptides, commonly referred to as protein transduction domains (PTDs), have the ability to deliver large proteins (up to 120 kDa) into mammalian cells in vitro and in vivo (84, 85). The general modes of transducing cellular proteins include protein transduction domains such as the homeodomain of Antennapedia, the Herpes simplex virus type 1 (HSV-1) structural protein VP22 and the Tat transduction domain of HIV-1. In addition, transportan and polyarginine have also been investigated as cell-permeable peptides (CPP).

Antennapedia Protein Transduction Domain

The homeodomain of the Antennapedia protein, Antp, has been extensively studied and was the first PTD to carry a cargo protein across cell membranes (89). In general, the Antennapedia PTD is efficient at transporting protein less than 100 amino acids in length but transduction can be unpredictable and structurally dependent (90). High concentrations of $Antp_{43\text{-}58}$ peptide (greater than a few μM) have been found to induce cell lysis in vitro, but this toxic effect appears to be cell type specific (91). The DNA-binding region of the Antp transcription factor has been extensively studied and is sufficient to transduce cells. This PTD is referred to as penetratin-1 and is one of the most common protein transduction domains researched in combination to Tat (92). In

2006, Letoha et al. reported that treatment with penetratin alone affects both in vitro and in vivo inflammatory responses in cells via binding proinflammatory mediators during cellular transduction (93). They also found that treatment with penetratin decreased expression of nuclear factor-kappaβ (NF-kappaβ) and tumor necrosis factor (TNF) in L929 fibroblasts and RAW 264.7 macrophages (93). Pouniotis et al. recently determined mice challenged with ovalbumin-expressing tumor cells were protected via T-cell activation following treatment with penetratin linked to the cytotoxic T lymphocyte peptide of ovalbumin (94).

VP22 Protein Transduction Domain

The VP22 structural protein contains a protein transduction domain of 40 amino acids in its C-terminal region and is responsible for the protein's novel cell trafficking properties (92). VP22 fusion proteins are secreted from cells, and the proteins target the nuclei of adjacent cells in the surrounding area (92). VP22 fusion proteins have been utilized to facilitate cell death with gangcyclovir (95), induce apoptosis in osteosarcoma cells when complexed with p53 (96), and induce apoptosis in HeLa cells when fused with neuroamidase (97) among other applications extensively reviewed in Dietz et al. (2004) (92). Although commercial kits are now available for the HSV-VP22 protein production system (Voyager™ Protein Production Kit, Invitrogen™ Life Technologies, Carlsbad, CA), it is not the protein transduction method of choice for a variety of reasons. The VP22 system requires that producer cells first be transfected with the cargo protein's cDNA. Consequently, it requires variable amounts of time for the cells to produce and secrete the fusion protein (92). In addition, the protein primarily targets cell nuclei;

therefore, it is not the method of choice when the biological activity of the fusion protein must be elicited in the cytoplasm.

Transportan Protein Transduction Domain

Transportan is a 27-amino acid cell permeable peptide produced by fusing the N-terminal fragment of the neuropeptide galanin with the wasp venom peptide mastoparan plus an additional lysine residue between the two peptides (92, 98). Like other PTDs, transportan transduces cells at 4ºC independently of endocytosis inhibitors. The transduction kinetics of transportan are reportedly faster than the Antennapedia-derived Antp peptide and Tat mediated transduction; in addition, it can also transduce epithelial cells more efficiently than the Antp peptide (92, 99). Transportan was utilized by Kaushik et al. (2002) in anti-HIV therapy to carry a TAR-targeted PNA to decrease replication of HIV virions by inhibiting Tat-mediated transactivation of HIV-1 LTR (92, 100). In addition, transportan was fused to a phosphotyrosine phosphatase antisense PNA to treat impaired glucose-induced insulin release in type 2 diabetes mellitus resulting in increased glucose-induced insulin secretion in mutant rat pancreatic islet cells in vitro (101). However, despite the biological potential of the transportan protein, it can elicit biological effects in cells independently of its cargo protein. Transportan has been reported to inhibit GTPase activity (102) and elicit an effect on the galanin receptors that are widely distributed in the nervous system (103). Therefore, although transportan effectively and rapidly transduces cells, it is not the most desirable cell permeable peptide because it has documented biological effects on cells independent of the cargo protein being carried into cells.

HIV-1 Tat Protein Transduction Domain

Groups investigating Tat-mediated protein transduction primarily focus on the 11-amino acid PTD from HIV-1 (amino acids 47-57) (84, 85). Tat proteins possess the unique ability to enter cells and translocate to the nucleus and nucleolus which is a property attributed to the basic region of the Tat protein (104-106). Dowdy and his associates have widely published Tat purification procedures and successfully produced multiple Tat fusion proteins such as Tat-$p27^{Kip1}$, Tat-Cdc42 and Tat-p16 (87, 107-111). Vocero-Akbani et al. (1999) engineered a transducable caspase-3 protein, Tat-Casp3, which entered 100% of target cells and induced apoptosis in HIV-infected cells (107).

Tat-conjugated proteins have been heavily investigated in cancer research due to their potential applications in cancer therapy. A Tat-p53 fusion protein was produced and induced apoptotic cell death in both p53 positive and negative human tumor cell lines (112). Guelen et al. (2004) produced a Tat-apoptin protein that transduced greater than 90% of its target cells and resulted in apoptotic activity in Saos-2 and HSC-3 cancer cells within 24 hours (113). Tat-apoptin was efficiently delivered to the nucleus of transformed cells and induced tumor cell apoptosis; however, in normal cells, the protein remained in the cytoplasmic compartment and did not elicit cell death (113). In 2006, Yan et al. reported transduction of a cell-permeable Survivin antagonist, Tat-Surv-T34A, in vivo into melanoma xenografts (114). Their results demonstrated 40-50% decreases in tumor mass and growth in melanoma xenografts (114). Their work supports systemic treatment of xenograft tumors with intraperitoneal injection of Tat fusion proteins to induce cell death in tumor cells via Tat-mediated protein transduction.

In addition to delivering therapeutic proteins to cells, the Tat PTD has been employed to deliver DNA into mammalian cells. Hashida et al. (2004) synthesized polylysine peptides containing the Tat-PTD, and they reported the DNA was transferred into most cancer cell lines as compared to the control.

Thus, substantial evidence is present in the literature to support Tat-mediated transduction of proteins into a variety of cell types.

Mechanism of Tat-Mediated Protein Transduction

The mode of protein transduction employed by Tat fusion proteins has been widely debated since the first publications regarding the use of the HIV-1 protein transduction domain. In 1998, Nagahara et al. (87) introduced an in-frame Tat bacterial expression vector with a hemagglutinin tag referred to as pTatHA. The vector contains an N-terminal 6-histidine tag which permits purification of the recombinant protein using affinity chromatography. The vector also contains a hemagglutinin tag required to detect transduction of recombinant proteins via immunocytochemistry. This vector was obtained from Steven Dowdy of the Howard Hughes Medical Institute in St. Louis, Missouri. The pTatHA vector was used to produce the recombinant proteins utilized in our research.

The characterization of the transduction properties of Tat fusion proteins has been widely investigated. The route of cellular internalization and intracellular trafficking of TFPs is still misunderstood and often appears to be a protein-specific phenomenon. Multiple pathways have been suggested to describe the transduction of TFPs including cellular membrane destabilization (115), membrane absorption via non-specific

endocytosis (116), lipid raft macropinocytosis (117) and caveolar endocytosis (118). Eguchi et al. reported that Tat transduction occurred independent of the classical endocytotic pathways by observing that the Tat-phage directly penetrated cell membranes and transduced into the cytoplasm compartment of cells via destabilizing the caveolar membrane (115). This membrane destabilization did not appear to be cytotoxic at high doses of Tat-phage, and the efficiency of transduction was not enhanced by endosomotropic agents (115). In 2003, Vives et al. claimed Tat analogues transduced into cells in a nonspecific endocytotic process that occurred actively, appeared energy dependent and exhibited rapid internalization of Tat proteins (116). Wadia et al. described a model of Tat transduction in which the PTDs are dependent on lipid rafts and Tat proteins preferentially bound cholesterol membrane constituents and triggered macropinocytosis (117). The macropinosomes are described as inherently leaky vesicles that do not fuse to lysosomes, but the exact mechanism of macropinosome release of Tat proteins remains unknown (117). Caveolar-mediated endocytosis of Tat fusion proteins has been reported to occur in a temperature dependent manner, and the endocytotic vesicles originate from lipid rafts (118-120). Tat proteins appear to co-localize in vesicles with markers of caveolar endocytosis, and the transduction of Tat proteins is impaired by drugs that disrupt lipid rafts (118-120). It has also been reported that Tat-mediated transduction is dependent on Tat binding cell surface heparan sulfate (HS) proteoglycans (121). Tyagi et al. found that cells defective in synthesis of HS proteoglycans exhibited impaired internalization of Tat proteins (121). Tat uptake was also decreased by agents degrading HS suggesting that HS proteoglycans are required for efficient intracellular delivery of Tat proteins (121). Additional methods to enhance Tat

mediated transduction of proteins have also been published. Upon cellular uptake of Tat proteins, the addition of chloroquine in vitro and in vivo has been suggested to enhance the endocytotic delivery of proteins via endosome disruption to promote nuclear targeting of Tat fusion proteins (122-124). Caron et al. reported that lysosomotropic agents such as chloroquine enhance the nuclear delivery of Tat fusion proteins up to 23-fold (122).

We intend to assess whether or not the addition of chloroquine enhances transduction efficiencies of our proposed E2F-1 Tat fusion proteins.

OVERVIEW OF PROPOSED RESEARCH

As reviewed above, the complex interaction of E2F-1 with the cell cycle can result in cell proliferation, cell cycle arrest or apoptosis. The effects of E2F-1 on cell growth and development are key actions directly relevant to cancer therapeutics. We propose producing E2F-1 Tat fusion proteins to impact gene expression in breast carcinoma cell lines. The majority of breast tumors overexpress the catalytic subunit of telomerase, hTERT; therefore, telomerase inhibition targeting hTERT is the first objective with our proposed E2F-1 Tat fusion protein therapy. We anticipate that introduction of E2F-1 Tat fusion proteins will repress expression of telomerase, thus forcing cancer cells into a senescent state or inducing cell death. In addition, 40-50% of carcinomas, including breast cancer, have mutations in the p53 tumor suppressor gene. In these cases, the p53 homologue protein, p73, is expected to trigger the same pathways as p53. We postulate that overexpression of E2F-1 will induce expression of p73 and result in cell cycle arrest via transactivation of the $p21^{WAF1/CIP1}$ protein. Significant

apoptotic activity is also expected to result following p73 overexpression and subsequent activation of BAX, PUMA and Noxa proteins leading to mitochondrial dysfunction and activation of effector caspases.

As reviewed, numerous studies report inducible apoptosis in tumor cells following viral gene therapy overexpressing E2F-1. We present an alternate form of a protein-based cancer therapy via attaching the E2F-1 gene to the Tat transduction domain of HIV-1. This Tat transduction domain has been proven to transduce proteins up to 120 kDa across cell membranes and the blood brain barrier (108, 110). Proteins attached to the HIV-1 Tat transduction domain have also been found to transduce 100% of target cells, and once Tat fusion proteins enter cells, they have the opportunity to elicit their biological effect followed by degradation (108, 110). Importantly, production of E2F-1 Tat fusion proteins would permit both optimal cellular transduction and a reversible overexpression of the E2F-1 protein in cells. Therefore, the production of biologically active E2F-1 Tat fusion proteins is a novel method of introducing the E2F-1 transcription factor into tumor cells to investigate its subsequent effects on gene expression. Collectively, we anticipate the findings of this research will lead to a greater understanding of E2F-1 and the development of an alternative therapy to augment conventional cancer treatments.

Table 1.1- Staging of Breast Cancer

Primary Tumor (T)	
T0	No evidence of primary tumor
TIS	Carcinoma in situ
T1	Tumor ≤2 cm
T1a	≤0.5 cm in greatest dimension
T1b	>0.5 but ≤1 cm in greatest dimension
T1c	>1 cm but ≤2 cm in greatest dimension
T2	Tumor >2 cm but ≤5 cm
T3	Tumor >5 cm
T4	Extension to chest wall, inflammation, satellite lesions, ulcerations
T4a	Extension to chest wall
T4b	Edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
T4c	Both (T4a and T4b)
T4d	Inflammatory carcinoma

Regional Lymph Nodes (N)	
N0	No regional lymph nodes
N1	Metastasis to movable ipsilateral nodes
N2	Metastasis to matted or fixed ipsilateral nodes
N3	Metastasis to ipsilateral internal mammary nodes

Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis (includes spread to ipsilateral supraclavicular lymph nodes)

Stage Grouping	**Tumor (T)**	**Lymph Node (N)**	**Metastasis (M)**
Stage 0	TIS	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 2.1 was modified from: Kasper DL, Fauci AS, Longo DL, Braunwald E, Hauser SL, Jameson JL, eds. Harrison's Principles of Internal Medicine, 16th edition. New York, NY: McGraw-Hill, 2005.

Table 1.2. Comparison of Breast Cancer Cell Lines

Cell Line	**HCC1599**	**HCC1937**	**MCF-7**	**MDA-MB-231**	**MDA-MB-435S**
ATCC Number	CRL-2331	CRL-2336	HTB-22	HTB-26	HTB-129
Patient Profile (age, sex, race)	44, Female, Caucasian	23, Female, Caucasian	69, Female, Caucasian	51, Female, Caucasian	31, Female, Caucasian
Tissue	mammary gland, primary ductal carcinoma	mammary gland, breast duct, primary ductal carcinoma	mammary gland; breast; epithelial; metastatic site- pleural effusion adenocarcinoma	mammary gland; breast; epithelial; metastatic site- pleural effusion adenocarcinoma	mammary gland; breast; duct; ductal carcinoma; metastatic site- pleural effusion
Morpho-logy	epithelial	epithelial	epithelial	epithelial	epithelial
Growth Properties	Cell line grows as multicellular aggregates in a suspension.	Cell line grows as large epithelial cells, adherent to flasks, float at high cell densities.	Cells are adherent.	Cells are adherent.	Cells are adherent.
Tumor Grade	TNM stage IIIA, grade 3	TNM stage IIB, grade 3	TMN stage IV	TMN stage IV	TMN stage IV
Receptors	ER/PR -	ER/PR -	ER +, PR -	EGF +, TGF alpha +	ER/PR-, EGF+, TGF alpha +
Oncogene	Her2/neu - p53- (missense mutation)	BRCA 1 (homozygous for 5382C mutation), Her2/neu - p53 - (nonsense mutation)	wnt7h + Tx-4 Her2/neu - p16 - (deletion) wildtype p53	p53- (missense mutation), p16 - (homozygous deletion)	p53- (missense mutation) Her2/neu -
Reference	(14)	(14, 17)	(10, 16)	(9)	(11-13, 15)

Table 1.3. Familial Cancer Syndromes Associated with the Development of Breast Cancer

Familial Cancer Syndrome	Characteristics	Gene Mutation	Locations	Functions/ Classification	Ref
Li-Fraumeni Li-Fraumeni-like syndrome (Hereditary Breast and Colorectal Cancer)	Autosomal dominant inheritance; early age of onset of breast cancer in addition to soft-tissue sarcomas, leukemia, lymphoma, brain cancer, lung cancer and adrenal cortical carcinoma	p53 hCHK2	17p13 22q12.1	Transcription factor, cell cycle regulation, apoptosis Checkpoint kinase, DNA damage response	(22, 23)
Hereditary Breast-Ovarian Cancer Syndrome	Autosomal dominant mode of inheritance; individuals at risk of developing both breast and ovarian cancer; BRCA2 mutations associated with breast cancer in men and prostate cancer; possible association with melanoma	BRCA1 BRCA2	17q21 13q12.3	DNA repair DNA repair	(20, 21, 23)
Peutz-Jeghers syndrome	Autosomal dominant inheritance; breast cancer, gastrointestinal tract carcinoma, testicular cancer, gynecological malignanies	STK11/ LKB	19p13.3	Serine-threonine kinase	(20)
Cowden Syndrome	Breast cancer, thyroid carcinoma, endometrial cancer	PTEN	10q23.3	Protein tyrosine phosphatase	(20)
Ataxia-Telangiectasia	Autosomal recessive; older patients susceptible to developing breast cancer, melanoma and Hodgkins disease	ATM	11q22-23	DNA repair, maintains genomic stability	(20)

E2F-1 Signaling Pathways Involved in Cell Cycle Control and Apoptosis

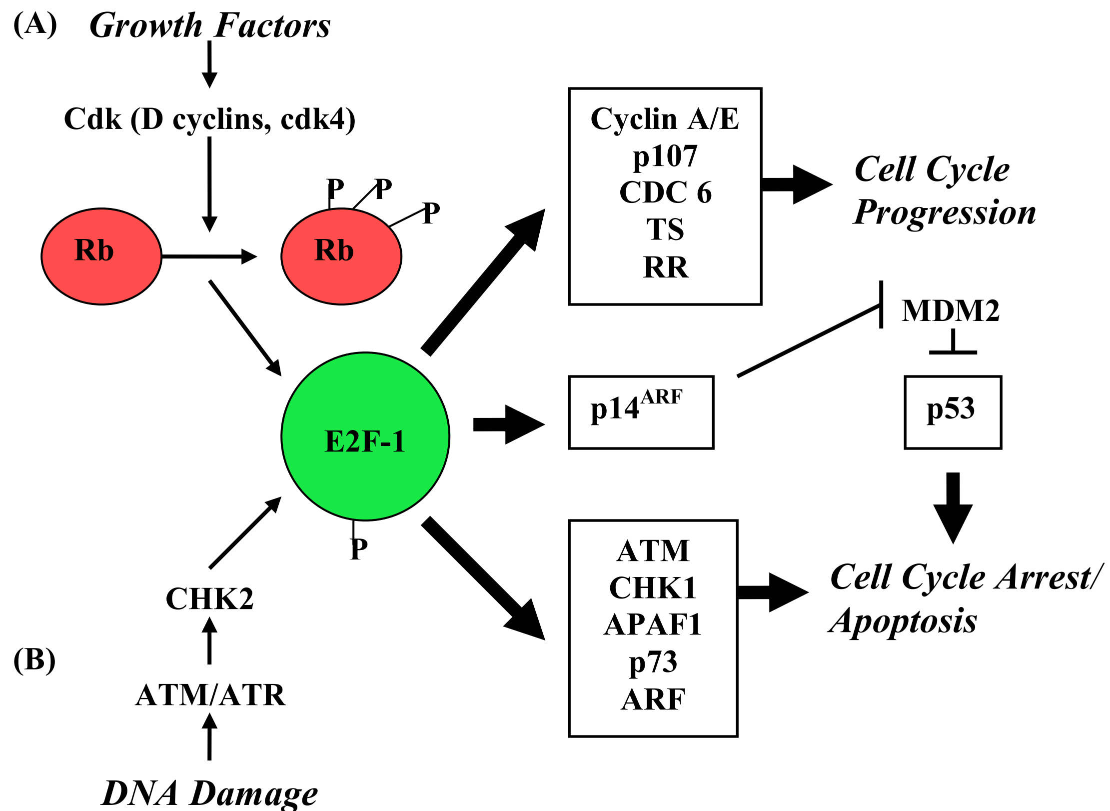

Figure 2.1 was modified from: Stevens, C. and La Thangue, N. B. The emerging role of E2F-1 in the DNA damage response and checkpoint control. DNA Repair (Amst), 3: 1071-1079, 2004.

Figure 2.1. Integral role of E2F-1 in control of cell cycle progression, cell cycle arrest and apoptosis. **(A)** Normally, E2F-1 functions to regulate expression of genes required for cell cycle progression such as thymidylate synthetase (TS), ribonucleotide reductase (RR) and p107 (35). E2F-1 is able to target these genes following stimulation via growth factors and subsequent activation resulting from phosphorylation of Rb. **(B)** In the presence of DNA damage, E2F-1 is phosphorylated by ATM/ATR and CHK2 kinases which stabilize the E2F-1 proteins. The high levels of E2F-1 protein resulting from DNA damage stimulates transcription of target genes. Activation of $p14^{ARF}$ functions to inhibit a negative regulator of p53, the MDM2 protein. Following inhibition of MDM2, p53 is transactivated resulting in cell cycle arrest and/or apoptosis (35). E2F-1 also binds the promoters of and transactivates expression of ATM, apoptosis protease-activating factor 1 (APAF1), alternative reading frame (ARF), CHK1 and p73 which also results in cell cycle arrest/apoptosis (35).

Effects of Telomerase Inhibition on Telomere Length over Time

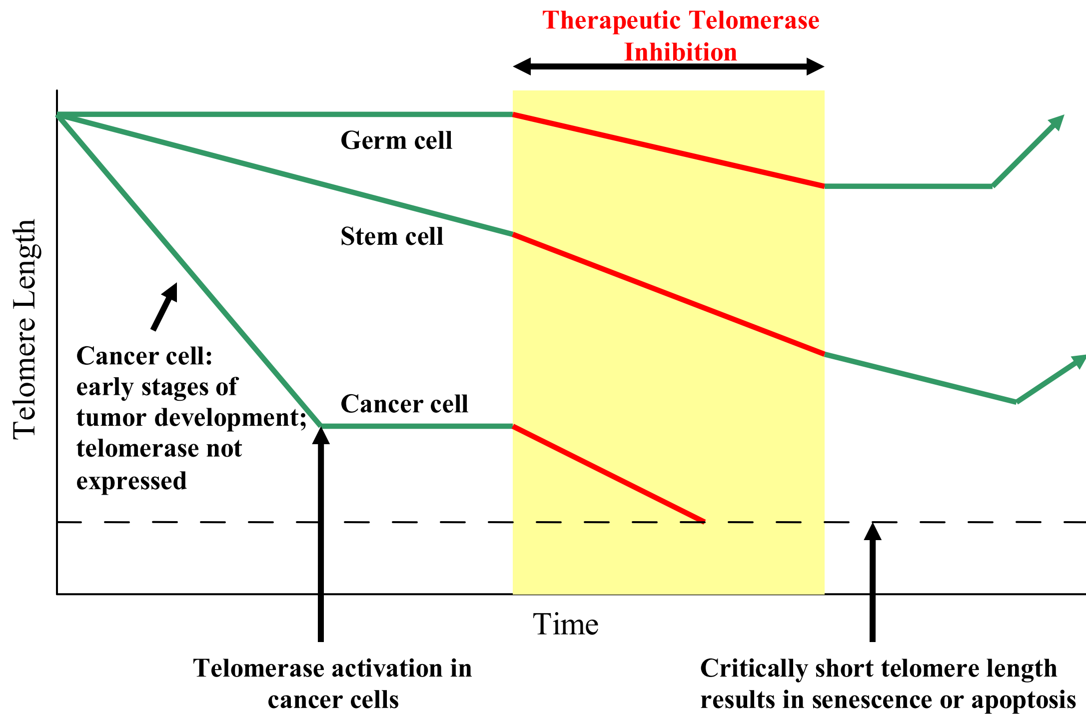

Figure 2.2 was modified from Figure 3 of: Keith, W. N., Bilsland, A., Evans, T. R., and Glasspool, R. M. Telomerase-directed molecular therapeutics. Expert Rev Mol Med, 2002: 1-25, 2002.

Figure 2.2. This figure illustrates the potential effects of telomerase inhibition on telomere length of different cell types over a period of time. In theory, telomerase inhibition should only impact cells actively expressing telomerase such as germ cells, stem cells and cancer cells. Following a period of telomerase inhibition, germ cells and stem cells would experience transient telomerase inhibition, but their recovery would be expected due to ample telomeric DNA. Cancer cells do not express telomerase in the early stages of tumor development; therefore, prior to application of a telomerase inhibitor, the telomeric DNA is significantly shortened as cancer cells proliferate (52). Telomerase is eventually reactivated which preserves the remaining telomeric DNA. However, upon application of a telomerase inhibitor, telomeric DNA is expected to shorten to a point where inherent apoptotic or senescent pathways are triggered within tumor cells (52). Overall, during application of a telomerase inhibitor, the primary systemic effects would be transient telomerase inhibition in germ cells and stem cells while tumor cells would be expected to undergo cell senescence/apoptosis.

Model of p53/p73 Pathways and Induction of Cell Cycle Arrest and/or Apoptosis

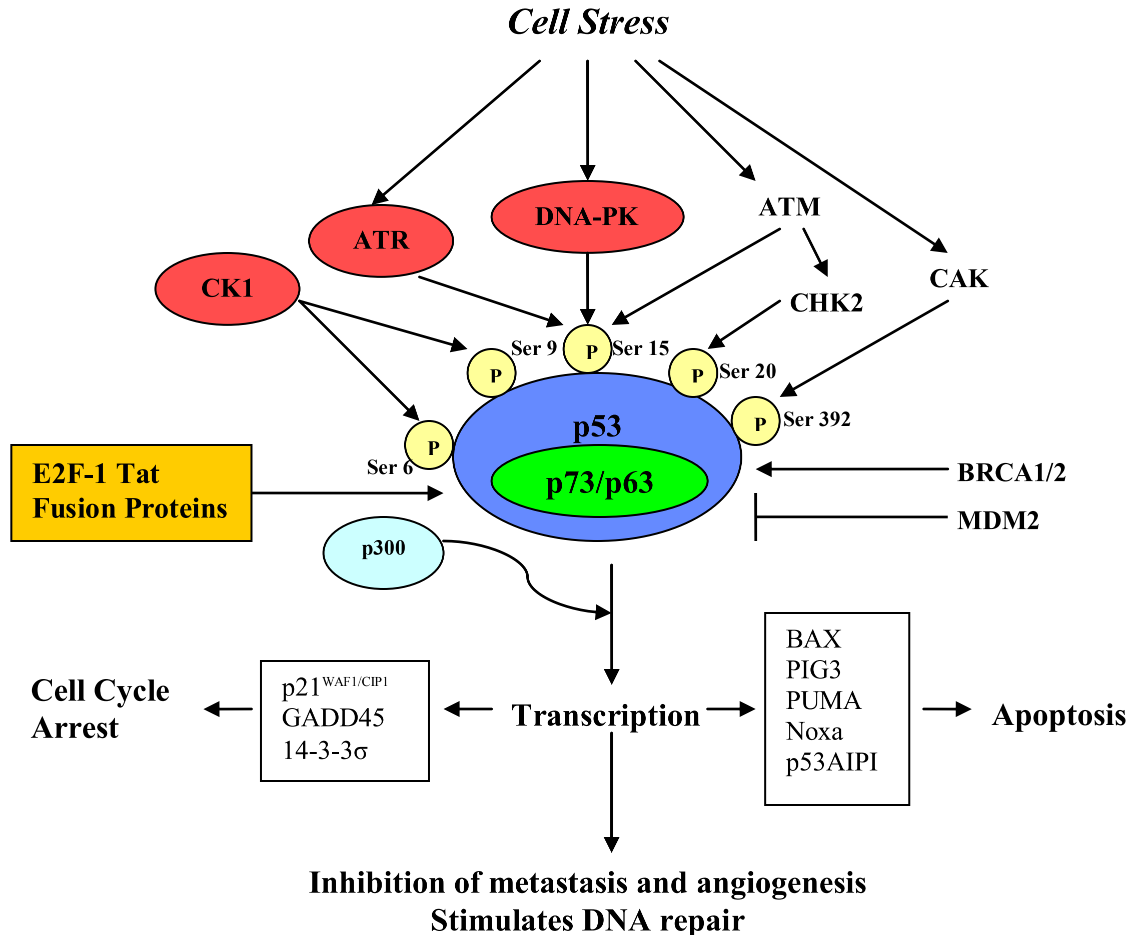

Figure 2.3 was modified from Figure 1 of: Gasco, M., Shami, S., and Crook, T. The p53 pathway in breast cancer. Breast Cancer Res, *4:* 70-76, 2002.

Figure 2.3. A simplified model of p53/p73 pathways and the induction of cell cycle arrest and/or apoptosis. Phosphorylation of the serine residues activates the p53/p73 protein for DNA binding and transcription of downstream effector genes (25). The p73 protein is activated directly in the absence of wildtype p53. E2F-1 Tat Fusion proteins (E2F-1/TatHA and E132/TatHA) have the ability to activate transcription of p53 and p73. Following acetylation of p53/p73 by p300, transcription of target genes is initiated resulting in transcriptional activation of genes leading to either cell cycle arrest or apoptosis (25). Importantly, p53/p73 transactivates expression of $p21^{WAF1/CIP1}$ which is imperative to cell cycle control. Alternatively, apoptosis can be triggered via activation of BAX, PIG3, Noxa and p53AIP1.

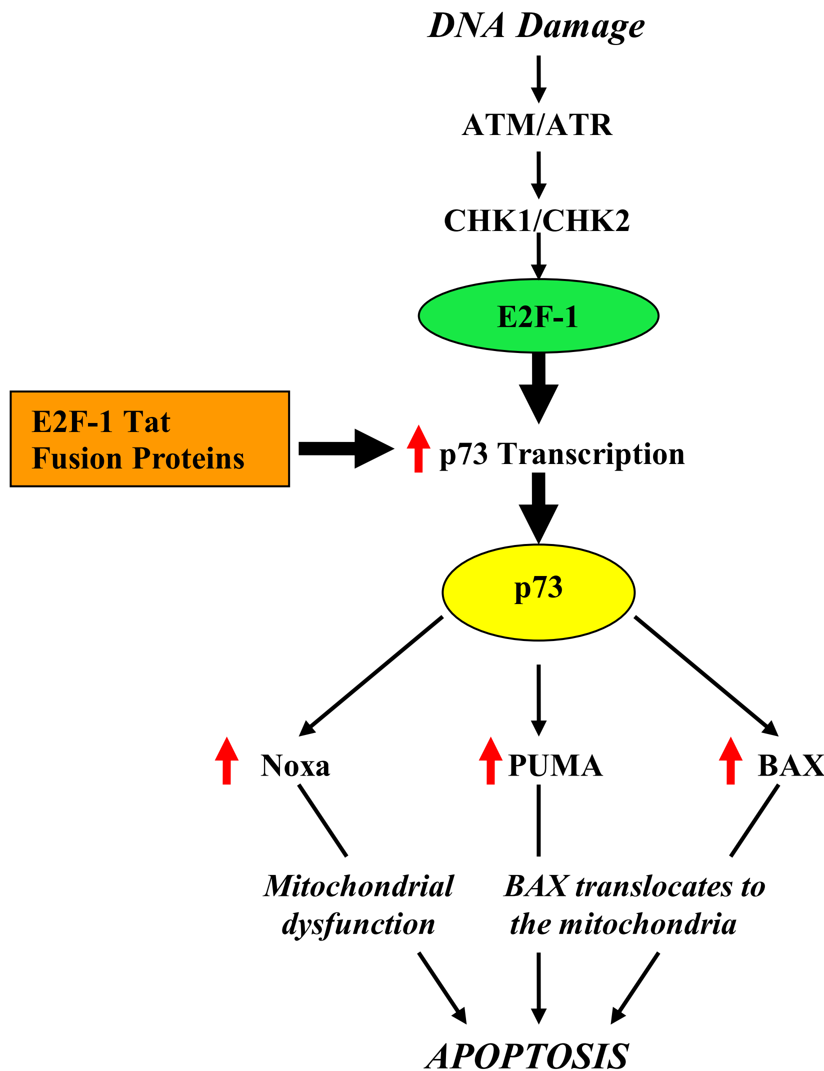

Figure 2.4 was modified from: *Roos, W. P. and Kaina, B. DNA damage-induced cell death by apoptosis. Trends Mol Med, 12: 440-450, 2006.*

Figure 2.4. Mechanism of p53-independent apoptosis. DNA damage activates ATM or ATR which subsequently activates CHK1 or CHK2 resulting in transactivation of native E2F-1. E2F-1 transcribes the p73 gene via binding E2F-1 sites in the proximal p73 promoter. The transactivation of p73 results in increased p73 protein levels in cells. The p73 protein activates transcription of the apoptosis-associated proteins Noxa, PUMA and BAX. Increased levels of Noxa results in mitochondrial dysfunction, releasing cytochrome c into the cytosol and triggering apoptosis via activation of effector caspases (78). Activation of PUMA and BAX results in translocation of BAX to the mitochondria subsequently releasing cytochrome c and activating caspases (78). A similar induction of apoptosis from transactivation of p73 is expected from artificial introduction of E2F-1 Tat fusion proteins into cells.

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CHAPTER III

PRODUCTION, PURIFICATION AND TRANSDUCTION OF E2F-1/TATHA AND E132/TATHA RECOMBINANT PROTEINS

ABSTRACT

Purification of Tat fusion proteins is highly dependent on the solubility and stability of the proteins under specific physiological parameters necessary for in vitro testing of cell lines. We have developed an effective methodology to generate constructs with the HIV-1 Tat transduction domain attached to E2F transcription factors in order to test their transduction potential and biological efficacy in carcinoma cell lines. Wildtype E2F-1 and mutant E2F-1 genes (E132; point mutation in DNA-binding domain) were cloned into the multiple cloning site (MCS) of the pTatHA expression vector to produce E2F-1/TatHA and E132/TatHA constructs. Proteins were produced in bacterial cell cultures and western blotting performed to verify the presence of the hemagglutinin (HA) tag. InVision™ staining was also utilized to identify presence of the 6-histidine tag prior to large scale production and purification of proteins. Using affinity chromatography and strong denaturants, the proteins were effectively isolated, partially refolded and purified via FPLC. Following dialysis into appropriate cell culture media, proteins were tested at

various concentrations to determine optimal transduction potentials of proteins. The addition of the lysosomotropic agent chloroquine was also tested to determine if transduction potentials and subsequent biological effects of Tat fusion proteins (TFPs) were enhanced in cell lines. We developed a successful technique to produce and purify recombinant E2F-1 Tat fusion proteins with greater than 95% transduction potentials. Additionally, the E2F-1 TFPs elicited their biological effect on breast cancer cell lines both with and without the addition of chloroquine. Overall, we developed a successful methodology to produce biologically active E2F-1 and E132 TFPs to impact gene expression in breast cancer cell lines.

INTRODUCTION

Protein therapeutics is a developing field oriented towards manipulating gene expression to impact cellular biology. However, effective protein therapy is highly dependent on several factors. Proteins must be able to effectively transduce through cellular membranes and target specific cell compartments. Appropriate folding of proteins must occur to preserve their biological activity, and proteins must have adequate half-lives to achieve their desired responses in cells. To produce proteins meeting the above criteria, multiple variables must be overcome during the production and purification process to preserve the proteins' capacity for transduction and biological efficacy.

Protein therapy is dependent on the direct delivery of proteins through the plasma membrane and into specific cell compartments. Small cationic peptides, commonly

referred to as protein transduction domains (PTDs), have the ability to deliver large proteins (up to 120 kDa) into mammalian cells both in vitro and in vivo (1, 2). One such cationic peptide is the 11-amino acid Tat-transduction domain derived from HIV-1 (amino acids 47-57) (1, 2). Tat proteins possess the unique ability to enter cells and translocate to the nucleus and nucleolus which is a property attributed to the basic region of the Tat protein (3-5). Dowdy and his associates have widely published Tat purification procedures and successfully produced multiple Tat fusion proteins such as Tat-Casp3, Tat-p27^{Kip1}, Tat-Cdc42 and Tat-p16 (6-11). Traditionally, Tat fusion proteins are produced as either soluble or insoluble proteins in bacterial expression systems, and the optimal biological activity of proteins is achieved via series of buffer exchanges, desalting and/or dialysis. However, the generation of biologically active proteins with PTDs is heavily dependent on the ability of proteins to fold correctly from an insoluble state during isolation and purification. Therefore, some groups are investigating mammalian expression systems to bypass the obstacles encountered with bacterial expression systems in order to generate biologically active TFPs for both in vitro and in vivo cell testing (12).

The characterization of the transduction properties of Tat fusion proteins has been widely investigated. The route of cellular internalization and intracellular trafficking of TFPs is still misunderstood and often appears to be a protein-specific phenomenon. Multiple pathways have been suggested to describe the transduction of Tat fusion proteins ranging from cellular membrane destabilization (13), membrane absorption via non-specific endocytosis (14), lipid raft macropinocytosis (15) and caveolar endocytosis (16). It has also been reported that Tat-mediated transduction is dependent on Tat

binding cell surface heparan sulfate proteoglycans for extracellular Tat internalization (17). Upon cellular uptake of Tat proteins, the addition of chloroquine in vitro and in vivo has been suggested to enhance the endocytotic delivery of proteins via endosome disruption to promote nuclear targeting of Tat fusion proteins (18-20).

In this study, we constructed E2F-1/TatHA and E132/TatHA expression vectors, produced them in a bacterial expression system and purified the recombinant proteins using affinity chromatography with FPLC. We also attempted to enhance transduction capabilities of proteins with the addition of chloroquine in anticipation of achieving a greater impact on target gene expression. Our lab modified existing purification protocols to produce an effective methodology produced biologically active E2F-1/TatHA and E132/TatHA proteins that successfully transduced breast cancer cells.

MATERIALS AND METHODS

Vector DNA

The vectors pCMV E2F-1 and pCMV E132 were obtained from Dr. Karen Vousden (Beatson Cancer Institute, UK) and the pTatHA vector was a kind gift from Dr. Steven Dowdy (University of Washington, St. Louis, MO). Vectors were received as plasmid DNA spotted on Whatman paper. DNA was extracted from paper via hydration in 50 µl Tris™-EDTA (TE) buffer overnight, and a 1 µl volume transformed into E. coli Top10F' cells (Invitrogen Life Technologies, Carlsbad, CA) using the transformation protocol described below. Large quantities of plasmid DNA were extracted from

bacterial DNA using a Qiagen Midiprep protocol (QIAGEN Inc., Valencia, CA) to yield sufficient DNA for cloning experiments.

E2F-1/TatHA Construct

Restriction Enzyme Digestion and Purification

The E2F-1 gene was directly cloned into the pTatHA vector via NcoI and EcoRI restriction enzyme sites. Both pCMV E2F-1 and pTatHA were digested with NcoI and EcoRI restriction enzymes overnight at 37ºC and samples run on 1% agarose gels containing ethidium bromide (0.5 µg/ml). Digested bands of DNA were visualized using low-intensity ultraviolet light, and DNA fragments (pTatHA= 3 kb, E2F-1=1.36 kb) excised with clean razor blades and placed in purification tubes.

Purification of DNA Fragments from Agarose Gel Using Siliconized Glass Wool

Purification tubes consisted of 0.6 ml microcentrifuge tubes that contained siliconized glass wool compacted in bottom one-third of tube in addition to a 22-gauge sized hole in tube base. Purification tubes were placed in 1.8 ml microcentrifuge tubes to collected liquefied agarose gel. Purification tubes containing excised DNA fragments were centrifuged at 10,000 g for 10 minutes at 4ºC. Liquefied fractions in 1.8 ml tubes were retained and volume adjusted with dH_2O to 200 µl. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) (~100 µl) was added to sample and tube vortexed for 20 seconds. Tubes were then centrifuged at 14,000 rpm for 5 minutes at 4ºC. Aqueous layers were carefully removed from phenol:chloroform and transferred to clean 1.8 ml microcentrifuge tubes. One-half volume (~100 µl) of 7.5 mM ammonium

acetate was added to assist with DNA precipitation. Samples were vortexed lightly and two volumes (~600 µl) of 100% ETOH added and samples incubated at -80ºC for 15-30 minutes to precipitate DNA. Samples were centrifuged at 14,000 rpm for 5 minutes at 4ºC and ETOH decanted without disrupting the DNA pellet. Next, 0.5 ml 70% ETOH added to tubes, tubes were inverted twice and samples again centrifuged as described above. Supernatants were removed by decanting, DNA pellets dried, and samples resuspended in 20 µl TE buffer (pH 8.0) for subsequent ligation of insert DNA into vector. Samples were quantified prior to ligation reactions.

Ligation of E2F-1 into MCS of pTatHA

Purified digested pCMV E2F-1 DNA was ligated into digested pTatHA using T4 DNA Ligase (Invitrogen Life Technologies, Carlsbad, CA) following the protocol provided by the manufacturer. In brief, 0.1 U T4 DNA Ligase was added to 5X Ligase Buffer, 0.1 µg pCMV E2F-1 DNA, 0.03 µg pTatHA DNA, and sufficient PCR H_2O to yield a 1X final Ligase Reaction Buffer concentration. Samples were incubated overnight at 16ºC, reactions diluted 5-fold with dH_2O and a 1 µl volume of the ligation reactions transformed into E. coli Top10F' competent cells (Invitrogen Life Technologies, Carlsbad, CA) following the protocol listed below.

Transformation Protocol for Top10F' Cells

Competent cells were thawed on ice and 3 µl of DMSO added to aliquots of cells. 50 µl samples of Top10F' cells were added to 1 µl volumes of diluted ligation reaction in 1.8 ml microcentrifuge tubes. Tubes were placed on ice for 30 minutes and gently mixed

2-3 times during this incubation period. Tubes were then heat shocked in a 42ºC water bath for one minute and immediately placed on ice for one minute. A 400 µl volume of 2YT broth was added to transformation reaction, tube placed in shaking water bath at 37ºC for 30 minutes, and a 30 µl volume of cell mixture spread on agar plates supplemented with 50 µg/ml ampicillin. Plates were incubated overnight at 37ºC, and the following day, single colonies from the transformation plate were screened for the E2F-1/TatHA construct via isolating plasmid DNA using traditional alkaline extraction (21). Plasmid DNA from select colonies were then DNA sequenced (Applied Biosystems, Model 373A Automated Sequencer, Oklahoma State University DNA/Protein Resource Facility, Stillwater, OK) to verify correct in-frame fusion of E2F-1 into pTatHA's multiple cloning site (MCS). The oligonucleotide primers used to sequence TatHA constructs are as follows: pTatHA forward primer 5'-CCCGCGAAATTAATACGAC-3' and pTatHA reverse primer 5'-GTCCCATTCGCCATTCAGG-3' (Sigma Genosys, Woodlands, TX). Illustration of the E2F-1/TatHA plasmid construction is represented by Figure 3.1.

E132/TatHA Construct

5' Site-Directed Mutagenesis of E132 via PCR

The E132 vector contained an artificial EcoRI site in the DNA binding domain and could not be directly inserted into pTatHA's MCS. Therefore, the E132 gene modified using 5' site-directed mutagenesis via PCR from plasmid pCMV-E132 using sense primer: 5'-gcgcgcaaccATGGCCTTGGCCGGG-3'and antisense primer: 5'-gcgcaggcatgcGGATCCAGCCCTGTC-3' (Sigma Genosys, Woodlands, TX) to generate

artificial NcoI and SphI sites (underlined in primer sequences). The PCR reaction used 2 Units Vent Taq polymerase (New England Biolabs, Beverly, MA), 1X Thermapol buffer (New England Biolabs, Beverly, MA), 500 nM of sense and antisense primers and 5% DMSO. The PCR product was amplified under the following conditions in a Perkin Elmer 9600 Thermocycler: initial denaturation: 94ºC- 3 minutes, 55ºC- 1 minute, 72ºC- 1 minute; 23 cycles: denaturation: 94ºC- 1 minute, annealing: 55ºC- 1 minute, extension: 72ºC- 1 minute, and final extension of 72ºC for 7 minutes. PCR products were then analyzed on 1% agarose gels and stained with ethidium bromide (0.5 μg/ml) to confirm generation of 1.36 kb amplified product (see Figure 3.3).

Restriction Enzyme Digestion and Klenow-Kinase-Ligase (KKL) Protocol

The pTatHA DNA was digested with restriction enzymes NcoI and SphI and purified using the protocol "Purification of DNA Fragments from Agarose Gel Using Siliconized Glass Wool" as described above. The E132 PCR product was digested with NcoI-SphI and subcloned into the NcoI-SphI sites of pTatHA to yield E132/TatHA using a modified Klenow-Kinase-Ligase (KKL) protocol (22). To summarize the KKL protocol, first, phenol:chloroform extracted PCR product was precipitated with ethanol and subsequently dissolved in distilled water (22). Next, 2 μl of 10X ligase buffer (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP) was added to the reaction in addition to the following reagents to yield a 25 μl final volume: 0.2 mM dNTPs, 5 U Klenow, 4 U T4 Polynucleotide Kinase, 2 U DNA Ligase (22). The reaction was incubated at 25ºC for 2 hours followed by heat inactivation at 70ºC for 10 minutes (22). The reaction was then diluted with distilled H_2O and restriction enzyme buffer

added to yield a 1X concentration. 40 U of restriction enzymes was then added to the reaction tubes followed by incubated of samples for 2 hours at 37ºC (22). Digested PCR product was then heat inactivated at 70ºC for 10 minutes followed by ligation to pTatHA vector via T4 DNA Ligase following protocol described above. Ligated constructs containing E132/TatHA were transformed into E. coli Top10F' competent cells (Invitrogen Life Technologies, Carlsbad, CA), screened with restriction enzyme digestions, and correct gene insertion verified by DNA sequencing of plasmid DNA as described above. Illustration of the E132/TatHA plasmid construction is represented by Figure 3.4.

Recombinant Protein Production

Western Blotting and InVision™ Staining

E2F-1/TatHA and E132/TatHA plasmids were transformed into BLR(DE3)pLysS competent cells (Novagen, Madison, WI) and recombinant proteins produced via inoculating 2 ml 2YT broth with single transformed colonies. After incubation in a shaking water bath (225 rpm) for 12-14 hours, 3 ml of fresh 2YT broth, 0.5 µg/ml ampicillin and 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) were added to cultures. Cultures were incubated an additional 6 hours to produce recombinant proteins via IPTG stimulation. Cultures were centrifuged at 6000 x g for 10 minutes and cell pellets resuspended in 100 µl of 20 mM Tris buffer (pH 8.0) followed by quantitation using a BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL) with BSA standards. 2X Laemmli Sample Buffer (21) was added to 50 µg of total cellular protein and samples were boiled for 3-5 minutes and immediately placed on ice. Samples were

then loaded onto 12% SDS-PAGE gels and ran on a Mini-PROTEAN® 3 (Bio-Rad Laboratories, Hercules, CA) protein electrophoresis unit using the Laemmli buffer system. Prestained 1 kb protein ladders (Fermentas Life Technologies, Hanover, MD) were also run on each gel to determine protein size.

Duplicate gels were run simultaneously- one gel for western blotting and a second gel for Coomassie blue staining. Gel designated for western blotting was transferred to nylon membranes using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions using 1X TBS-T transfer buffer.

Recombinant protein production was verified using primary mouse monoclonal HA antibodies (1:1,000 dilution) (CRP, Inc., Denver, PA) and secondary HRP-labeled goat anti-mouse antibodies (1:25,000 dilution) (KPL, Inc., Gaithersburg, MD). Membranes were blocked with TBS-T/5% milk solution for 1 hour, incubated with primary HA antibody for 1 hour, washed 3 times for 10 minutes each, and incubated with secondary HRP antibody for 1 hour. Following additional washings (3 x 10 minutes), western blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to manufacturer's instructions. Blots were exposed to X-ray film and developed with KODAK Developer (30 sec) and KODAK Fixer (30 sec). Immunoblots were also visualized using a KODAK Image Station 2000R (KODAK, Rochester, NY) according to manufacturer's instructions (see Figure 3.6).

Additional SDS-PAGE gels were also run to utilize an InVision™ His-Tag In-Gel Stain (Invitrogen Life Technologies, Carlsbad, CA) to verify the presence of 6-histidine

tag for purification purposes using HisTrap™ columns. The following protocol was slightly modified from the manufacturer's protocol. First, Tris-Glycine SDS-PAGE gels containing the recombinant protein and the positive control, BenchMark™ His-Tagged Protein Standard (Invitrogen Life Technologies, Carlsbad, CA), were fixed for 1 hour in a fixing solution containing the following: 50% ethanol, 20% acetic acid and 30% distilled water (v/v). Gels were then washed with distilled water (2 x 10 minutes) and incubated with 25 ml of the InVision™ His-Tag In-Gel Stain. Following incubation, gels were washed with 200 ml of 20 mM sodium phosphate wash buffer (pH 7.8) (3 x 10 minutes). The gel was then visualized using an UV transilluminator (302 nm) to verify presence of 6-histidine tag and an image captured with a CCD camera (see Figure 3.7).

Large-Scale Recombinant Protein Production

Recombinant E2F-1/TatHA and E132/TatHA proteins were purified under denaturing conditions with affinity chromatography using a modified procedure from Amersham Pharmacia (23). Single colonies from protein expressing cultures inoculated 200 ml of 2YT broth supplemented with ampicillin (50 µg/ml) and were incubated at 37ºC while shaking at 225 rpm for 16-18 hours. An additional 300 ml of broth was added along with freshly prepared ampicillin and IPTG (400 µM), and cultures incubated an additional 6 hours. Cultures were centrifuged for 10 minutes at 6000 x g, and the bacterial pellets frozen at -20ºC. Cell pellets were thawed for 20 minutes at 37ºC, and 25 ml of Buffer 1 (20 mM Tris-HCl, pH 8.0) was added to resuspend the cells with vigorous pipetting. Cells were sonicated on ice (3 x 15 seconds) and centrifuged (10 min x 6000 g). Cell pellets containing the inclusion bodies were then resuspended in 25 ml Buffer 2

(2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton™ X-100, pH 8.0). Samples were again sonicated on ice and centrifuged as described above. Pellets were resuspended in 25 ml Buffer 2 to wash the inclusion bodies and recentrifuged. A final wash of 25 ml of Buffer 1 was then performed, and following centrifugation, pellets were either used immediately or frozen at -20ºC for a maximum of two weeks prior to use.

Preparation of Inclusion Bodies for FPLC

Cell pellets were resuspended in 5-10 ml Buffer A1 (6 M guanidine-HCl, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) by stirring at room temperature for 60 minutes to solubilize recombinant proteins. Samples were centrifuged at 20,000 x g for 15 minutes to pellet residual cell debris. Protein supernatant containing denatured, soluble proteins was removed from residual cell debris. Supernatant was further clarified by passage through a 0.45 µM filter (Millipore, Bellerica, IL) to remove any remaining particles.

Isolation of Recombinant Proteins using Affinity Chromatography

Using an AKTA™ FPLC, nickel-charged HisTrap columns (Amersham Pharmacia, Piscataway, NJ) were equilibrated with 5 ml Buffer A1 and the proteins loaded at a flow rate of 0.5 ml/minute. Columns were washed with an additional 10 ml of Buffer A1 followed by a wash of 10 ml of Buffer A2 (6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A linear gradient from 6 M to 0 M urea was then performed to remove the denaturant and start refolding the proteins on the HisTrap™ column by gradually replacing Buffer A2 with Buffer B1 (0.5

M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A total volume of 40 ml of Buffer B1 was used to perform the gradient wash. The purified, refolded proteins were then eluted with Buffer B2 (0.5 M NaCl, 20 mM Tris-HCl, 500 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) using a gradient of 0 mM to 500 mM imidazole in the buffer. Fractions containing the purified protein were analyzed by SDS-PAGE gel electrophoresis and quantified with the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL).

Dialysis of Recombinant Proteins into Cell Culture Media

E2F-1/TatHA and E132/TatHA recombinant proteins were diluted with Buffer B2 to a concentration of 0.5 mg/ml to avoid protein precipitation cascades and injected into 12 ml Slide-A-Lyzer® Dialysis Cassettes (Pierce Biotechnology, Inc., Rockford, IL) using 21-gauge, 1-inch beveled hypodermic needles. Proteins were dialyzed in 1X PBS for 2 hours at room temperature. The cassettes were placed in fresh 1X PBS for an additional 2 hours at room temperature and then dialyzed overnight in appropriate cell culture media for in vitro experiments. Following dialysis, proteins were removed from cassettes and placed in sterile 50 ml conical centrifuge tubes and centrifuged at 9400 x g for 15 minutes to remove any protein precipitate. Protein supernatant was removed and the final concentrations of recombinant proteins determined using the Coomassie Plus™ Bradford Assay (Pierce Biotechnology, Inc., Rockford, IL) and SDS-PAGE gels using BSA protein standards. Contamination of proteins was minimized by passage through 0.2 μM filters (Millipore, Bellerica, IL) prior to testing proteins on carcinoma cell lines, and purified protein was supplemented with 10% heat-inactivated FBS (Hyclone, Logan,

UT) and 50 IU/ml penicillin/ 0.05 mg/ml Streptomycin. Proteins were tested at various concentrations to determine optimal transduction at different time points using immunocytochemistry to monitor cellular uptake of E2F-1/TatHA and E132/TatHA.

Synthesis of TatHA Control Protein

The TatHA control protein was artificially synthesized (amino acid sequence: YPYDVPDYAYGRKKRRQRRR) (24) on a Ranin Symphony/Multiplex Peptide Synthesizer (Center for Integrated BioSystems, Logan, UT). The TatHA peptide was reconstituted using Cellgro^{TM} RNase/DNase/Protease free dH_20 for cell culture (Mediatech Inc., Herndon, VA) and stored at -70ºC at a working concentration of 1 mg/ml (25).

Primary Carcinoma Cell Culture

Primary infiltrating ductal breast carcinoma cell lines HCC1937 (ATCC number: CRL-2336) and HCC1599 (ATCC number: CRL-2331) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI-1640 medium supplemented with 10% FBS, 50 IU/ml penicillin/ 0.05 mg/ml Streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were grown in a 37ºC humidified incubator with 5% CO_2 (Figure 3.13). Additional breast carcinoma cell lines were also utilized in our research: MDA-MB-231 (ATCC number: HTB-26), MDA-MB-435S (ATCC number: HTB-129), and MCF-7 cells (ATCC number: HTB-22). MDA-MB-231 and MDA-MB-435S cell lines were both grown in a 1:1 ratio of DMEM:Ham's F12 media supplemented with 10% FBS and 5 IU/ml penicillin/0.05mg/ml Streptomycin.

MCF-7 cells were grown in MEME cell culture media supplemented with 10% FBS, 50 IU/ml penicillin/ 0.05 mg/ml Streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate. Cell lines were grown in 25 cc^2 and 75 cc^2 tissue culture flasks and passaged every 2-4 days when 70-80% confluent. Cells were detached from flasks using 0.25% trypsin, 0.03% EDTA solution according to standard cell culture protocols (26).

Immunocytochemistry

HCC1937 and HCC1599 cells were exposed to 2 μM E2F-1/TatHA, E132/TATHA, and TatHA fusion peptides for various time points: 1, 6, 12 and 24 hours. Cells were washed 3 times with 1X PBS, fixed for 30 minutes in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in 1X PBS for 30 minutes. Cells were blocked for 30 minutes with 1% FBS and 0.1% Tween-20 in 1X PBS. Cells were incubated for 60 minutes with primary mouse HA antibodies (1:1000 dilution in 1X PBS), washed 3 times for 10 minutes each, and incubated for 60 minutes with a secondary FITC-labeled goat anti-mouse antibody (1:250 dilution in 1X PBS) (KPL, Inc., Gaithersburg, MD). For transduction experiments with counterstained cell nuclei, cells were washed, and incubated for 15 minutes with 1 μM TO-PRO-3 iodide (Molecular Probes, Eugene, OR). Cells from immunocytochemistry experiments were mounted on slides using the Prolong® Antifade Kit (Molecular Probes, Eugene, OR) and images captured with Bio-Rad MRC 1024 Laser Scanning Confocal Microscope.

Cell Culture Experiments with Recombinant Proteins

HCC1937 and HCC1599 cells were seeded at a density of 2 x 10^5 cells/well in CellStar® 6-well tissue culture plates. HCC1937 (adherent) cells were allowed 24 hours to adhere to plates before recombinant proteins were added. HCC1599 (suspension) cells were seeded directly into wells with TFPs. After specified treatment time of 6, 12, 24, 48 or 72 hours, RNA was isolated and analyzed via RT-qPCR to determine effects on gene transcription. In preliminary studies, 200 μM chloroquine was added to cell culture media with the TFPs to assess effects on transcription and gene expression. Chloroquine (Sigma-Aldrich, Inc., St. Louis, MO) stock solution (50 mg/ml) was prepared fresh, sterile filtered and only used for a maximum of two days for experiments.

RESULTS

We were able to successfully produce Tat fusion peptides utilizing both wildtype and mutant E2F-1 expression vectors to investigate their transcriptional effects on target genes. The vector pCMV E2F-1 contains the wildtype form of the human transcription factor E2F-1, and the pCMV E132 vector contains human E2F-1 with a mutation in its DNA-binding domain that produces an artificial EcoRI restriction enzyme site. The pTatHA vector utilized for our cloning experiments contains an N'-terminal 6-histidine tag, 11-amino acid Tat-transduction domain, a hemagglutinin (HA) tag and a polylinker referred to as the multiple cloning site (MCS) (10). Additionally, the Tat transduction domain is followed by multiple glycine residues for free bond rotation which promotes proper protein folding (10). In gene expression studies described in subsequent chapters,

E132/TatHA was designed to serve as a control protein along with an artificially produced TatHA alone control peptide.

The production of E2F-1/TatHA and E132/TatHA recombinant proteins is a multi-step process to ultimately produce biologically active TFPs with the ability to transduce all cell types. As diagramed in Figure 3.1, the E2F-1 gene from vector pCMV E2F-1 was directly cloned into the MCS of pTatHA via restriction enzyme digestion followed by ligation. Prior to DNA sequencing, restriction enzyme digestions with NcoI and EcoRI were performed re-excise the E2F-1 gene from the pTatHA vector to verify success of cloning experiments (see Figure 3.2), and DNA sequencing confirmed the in-frame insertion of wildtype E2F-1 into the MCS of pTatHA. The insertion of E132 into the MCS of pTatHA required additional effort due to the lack of two compatible restriction enzyme sites for cloning in order to leave the EcoRI DNA-binding domain mutation intact. To overcome this obstacle, 5' site-directed mutagenesis via PCR was used to generate an artificial SphI site on the 3' end of the E132 gene. The PCR reaction successfully generated the appropriately size fragment of DNA containing the E132 gene and the new 3' SphI site (see Figure 3.3). The pTatHA vector was then digested with NcoI and SphI to prepare the MCS for insertion of E132. Attempts to directly clone restriction enzyme digested, amplified E132 DNA into restriction enzyme digested pTatHA were unsuccessful. After using the Klenow-Kinase-Ligase protocol (22) described under Materials and Methods, the E132 gene was finally cloned into the MCS of pTatHA as outlined in Figure 3.4. Next, a series of restriction enzyme digestions were performed on E132/TatHA to verify presence of specific restriction enzyme sites and the

internal EcoRI mutation as demonstrated in Figure 3.5. DNA sequencing was also utilized to confirm the in-frame insertion of E132 into the MCS of pTatHA.

Following successful construction of E2F-1/TatHA and E132/TatHA expression vectors, plasmid DNA was transformed into BLR(DE3)pLysS competent cells. We also transformed BL21(DE3)LysS bacterial cells with our expression vectors, but the BLR(DE3)pLysS yielded greater levels of recombinant proteins. Additionally, our protocol used 2YT broth due to optimal protein production versus LB broth as suggested by Becker-Hapak et al. (8). Following optimization of small scale protein production as described under Materials and Methods, we verified the presence of both the HA tag and 6-histidine tag. The HA tag was confirmed via western blotting with primary monoclonal HA antibodies and secondary HRP-labeled antibodies. Figure 3.6 demonstrates confirmation of the HA tag in both E2F-1/TatHA and E132/TatHA expressing cultures. The 6-histidine tag was confirmed using the InVision™ His-Tag In-Gel Stain for SDS-PAGE gels. Presence of the 6-histidine tag was evident in both E2F-1/TatHA and E132/TatHA cultures and isolated proteins as demonstrated in Figure 3.7. The presence of both tags within our proteins was essential for protein isolation, purification (6-histidine tag was required to bind HisTrap™ columns) and to verify the transduction of our TFPs into carcinoma cell lines (via primary HA antibodies and secondary FITC-labeled antibodies).

Large scale protein production occurred either immediately before protein isolation via FPLC (for fresh pellets) or several weeks prior to isolation (pellets frozen at -20ºC). Large cell pellets were produced from the 500 ml cultures, and after initial trials, it was determined that E2F-1/TatHA and E132/TatHA proteins were produced as

insoluble inclusion bodies in the bacterial expression vectors. Complete sonication of our samples was an extremely important key to clean isolation of inclusion bodies, and multiple sonication steps were required throughout the purification process. Cell lytic agents and detergents were also tested to isolate the inclusion bodies, but sonication proved to be the most cost effective, optimal method to yield large quantities of inclusion bodies with minimal cell debris.

The FPLC procedure used by our lab employs one of the strongest denaturants, 6 M guanidine-HCl, in lieu of the 8 M urea denaturant reported by other labs. Initially, we utilized 8 M urea, but we determined that the proteins did not adequately bind the nickel-charged HisTrap™ columns used during the isolation process. Likewise, when we attempted to solubilize the inclusion bodies with 8 M urea, the combination was usually too viscous to filter or run through the HisTrap™ columns. The Buffer A1 solution containing 6 M guanidine-HCl effectively solubilized the inclusion bodies from both fresh and frozen pellets. Likewise, we were also able to filter the proteins to remove cell debris and prevent clogging of the HisTrap™ columns. Therefore, including 6 M guanidine-HCl in the purification protocol was a crucial step to denature the E2F-1/TatHA and E132/TatHA proteins to promote column binding.

FPLC was employed to isolated proteins for several reasons. We performed several buffer exchanges throughout the purification process, used large volumes of buffers for gradient washes and required a slow flow rate to promote His-tag proteins binding the column. Figure 3.8 outlines the FPLC purification protocol to isolate and purify the E2F-1/TatHA and E132/TatHA proteins. The gradual exchange of buffers and slow flow rate permitted the partial refolding of proteins on the column prior to their

elution. Optimal protein loading of the HisTrap™ columns occurred with a flow rate of 0.5 ml/min. The FPLC chromatograms in Figures 3.9 and 3.10 illustrate the isolation and refolding procedure for E2F-1/TatHA and E132/TatHA respectively. Proteins were solubilized in the buffer with 6 M guanidine-HCl, and following loading and washing of the proteins on the column, the buffer was changed to an 8 M urea-containing buffer to remove the guanidine and partially renature the proteins. Likewise, optimal biological activity of proteins was achieved with the transition from 6 M guanidine to 6 M urea followed by the removal of urea by washing with Buffer B1. A salt concentration of 0.5 M NaCl was used in all the buffers in addition to fresh β-mercaptoethanol to stabilize proteins. Figures 3.9 and 3.10 demonstrate the concentration of proteins (via UV absorbance at A_{280}) as they loaded onto the columns, were washed by buffers, and finally, eluted by a linear gradient of increasing concentrations of imidazole. The high absorbance peak during the loading phase illustrates that other contaminant proteins were unable to bind the nickel-charged column, thus they were washed out as the His-tagged proteins bound. The second large peak on the chromatograms demonstrates the elution of His-tagged E2F-1/TatHA and E132/TatHA proteins. On average, the elution peak represented 10-12 mg of recombinant protein eluted in a 1-3 ml volume of Buffer B2. Figures 3.11 and 3.12 represent the E2F-1/TatHA (Figure 3.11) and E132/TatHA (Figure 3.12) proteins throughout the production and purification process. Figures 3.11.A and 3.12.A are SDS-PAGE gels demonstrating the 60 kDa TFPs from the culture stage through FPLC isolation, and finally, following dialysis into cell culture media. Figures 3.11.B and 3.12.B show a more detailed view of the FPLC chromatogram elution peaks

and fractions. The SDS-PAGE gels demonstrate a sample of the purified protein from Fractions X and Y in lanes 4 and 5 of each gel respectively.

Following recombinant protein isolation via FPLC, the proteins were in a partially refolded state (23) and ready for dilution and dialysis. Proteins were diluted to a concentration of 0.5 mg/ml with elution buffer (Buffer A2) prior to dialysis to avoid protein precipitation cascades. Following dialysis and sterile filterization, proteins were added to several breast cancer cell lines to assess protein transduction. Figure 3.13 represents brightfield microscopy images of HCC1937 and HCC1599 cells to illustrate growth properties and morphology. The HCC1937 cells are adherent while the HCC1599 cells grow in suspension, thus, only immunocytochemistry images of HCC1937 are shown. The additional breast cancer cell lines studied in preliminary experiments, including MCF-7, MDA-MB-231 and MDA-MB-435S cells, all grow as adherent cells; therefore, immunocytochemistry experiments were easily performed.

Figures 3.14 and 3.15 demonstrate the transduction of E2F-1/TatHaA and E132/TatHA TFPs into the HCC1937 and MCF-7 cell lines. There were substantial cytoplasmic, perinuclear and nucleolar accumulations of the proteins. The recombinant TFPs appeared to successfully transduce all cell types, including the other abovementioned breast cancer cell lines. We added chloroquine to our cell culture media in several experiments in hopes of achieving even greater protein transduction and nuclear localization of the TFPs. However, chloroquine did not increase the transduction of TFPs as detected via fluorescent microscopy. Likewise, preliminary gene expression studies did not show increased activation or repression of target genes with the addition of chloroquine in concentrations ranging from 50 μM to 500 μM (data not shown).

Overall, it appeared the E2F-1/TatHA and E132/TatHA proteins were able to successfully transduce cell lines with and without the addition of chloroquine to cell culture media with no apparent enhancement of transcriptional activation or repression of target genes.

DISCUSSION

E2F-1/TatHA and E132/TatHA proteins were produced by our lab and confirmed to be biologically active in studies investigating their effects on gene expression. We modified our purification procedures accordingly to optimize their biological activity by avoiding protein precipitation cascade triggers. At any point in our protein experiments, if the E2F-1/TATHA and E132/TatHA proteins began to precipitate, they were discarded and fresh proteins produced and purified. Protein precipitation was observed as the formation of a cloudy white precipitate following protein dilution, dialysis or centrifugation. If proteins clogged the 0.2 μM filters used for filter sterilization, the proteins had initiated a protein precipitation cascade in most cases. Preliminary studies using proteins susceptible to precipitation, or proteins still in solution following removed of precipitate via centrifugation, commonly resulted in decreased protein transduction. Proteins with altered transduction capabilities unable to effectively transduce cells appeared as extracellular accumulations and clumps of proteins via immunocytochemistry. Additionally, a neglible effect on gene transcription was observed if these proteins were applied to cells (data not shown). Since biological activity of the proteins appeared to be affected by precipitation, we did not use any

proteins that experienced an initiation of a precipitation cascade or proteins suggestive of precipitation (i.e. clogged filters) in any of our formal experiments.

Prior to dialysis, proteins were always diluted to a 0.5 mg/ml concentration with elution buffer because concentrations greater than 0.5 mg/ml typically resulted in rapid protein precipitation. Only freshly dialyzed proteins were used in our experiments; however, proteins could be stored for a maximum of 5 days at 4ºC without evidence of precipitation in order to complete a series of experiments using the same batch of proteins. We did attempt to cryopreserve E2F-1/TatHA and E132/TatHA to preserve their biological activity by adding a 10-20% (v/v) final concentration of glycerol to the diluted proteins prior to flash-freezing and storage at -80ºC (8, 10). Unfortunately, the E2F-1/TatHA and E132/TatHA proteins were moderately susceptible to precipitation during the thawing process. Although precipitation was minimized with the addition of 20% glycerol, proteins would easily precipitate if we utilized dialysis to remove the high concentration of glycerol. We also performed a "glycerol shock study" to assess the toxic effects of glycerol on the breast cancer cell lines. Various glycerol concentrations [1%, 5%, 10%, 15% and 20% (v/v)] were added to HCC1937, HCC1599 and MCF-7 cells in their respective cell culture media and incubated for 24 hours with microscopic assessment for cell stress every 6 hours. Cells were sensitive to glycerol concentrations from 5-20% following 6 hours of incubation. At the 24 hour time point, cells were extremely stressed in the 20% glycerol concentration, and the majority of cells had detached (HCC1937 and MCF-7) and lysed (data not shown). Compelling evidence of cell detachment was also present in cells exposed to 5% glycerol at the 24 hour time point. In general, the breast cancer cell lines utilized in our studies were extremely

sensitive to glycerol concentrations greater than 1% following an incubation of 24 hours. Therefore, we omitted glycerol from our formal experiments and opted to only use freshly dialyzed proteins for in vitro testing.

The E2F-1/TatHA and E132/TatHA proteins produced in our lab possess the capability to transduce all cell types. The exact mechanism of E2F-1 TFP transduction has not been definitively determined but appeared to parallel the pattern of transduction observed in the study by Yang et al. (2002) where significant nuclear and nucleolar accumulation of proteins was observed (27). Likewise, it is plausible that internalization of the E2F-1/TatHA and E132/TatHA fusion proteins occurs in the rapid, lipid raft-dependent and receptor independent form of endocytosis not associated with caveolar- and clathrin-mediated endocytosis described by Wadia et al. (2004) (15, 18, 28). In all breast cancer cell lines tested, significant cytoplasmic, perinuclear and nucleolar accumulation of proteins were evident after all measured time points (1, 6, 12 and 24 hours). Due to the rapid, substantial perinuclear accumulation of the proteins, their half-lives also seemed to be greatly extended versus the native forms of the proteins. The wildtype E2F-1 protein and the mutant E2F-1 protein, E132, reportedly have biological half-lives of approximately 70 minutes (28). Caron et al. (2004) reported that perinuclear accumulation of proteins was suggestive of sequestration of the recombinant proteins within the Golgi apparatus and endoplasmic reticulum (ER) resulting in a sustained release of TFPs to the nuclear target and thus appearing to extend the protein half-lives (18). The extended half-lives of the E2F-1/TatHA and E132/TatHA recombinant proteins observed in our studies suggests that the transduction and probable Golgi and ER

accumulation in cells results in sustained biological activity of the proteins following 24 hours of treatment.

We attempted to enhance the transduction and biological effect of the E2F-1/TatHA and E132/TatHA proteins by adding various amounts of the lysosomotropic agent chloroquine to the cell culture media. No differences in transduction via immunocytochemistry were evident in chloroquine versus non-chloroquine treated cells. Similarly, no increased effect on gene expression was observed in chloroquine treated cells versus non-chloroquine treated cells after results were compared in larger preliminary test groups. Other research groups investigating the addition of chloroquine to enhance endosomal disruption have observed cytotoxicity with cell exposure to chloroquine concentrations greater or equal to 100 μM (15). The addition of chloroquine to our cell culture media also appeared to have a mildly toxic effect on our breast cancer cell lines. Following a minimum of 12 hours exposure to 200 μM chloroquine, adherent cell lines HCC1937 and MCF-7 began to detach in control groups exposed to cell culture media with chloroquine. A similar detachment and membrane blebbing was also observed in cells treated with TFPs in addition to chloroquine. Due to the possible toxicity of the chloroquine on our cell lines and negligible effects on gene transcription, chloroquine was not used in our formal experiments to assess the transcriptional effects of the TFPs in our breast carcinoma cell lines.

Our lab was successful at optimizing protocols to produce and purify soluble, biologically active E2F-1/TatHA and E132/TatHA recombinant proteins with the ability to transduce greater than 95% of target cells. However, there were specific limitations to the testing parameters of our Tat fusion proteins. First, the maximum testable

concentration of TFPs was 2 μM due to the susceptibility of the proteins to precipitate at higher concentrations. Second, we were unable to use cryopreserved TFPs on our cell lines because the cells were sensitive to the high concentration of glycerol. Our experiments with cells were also limited to less than a week per batch of purified proteins since protein storage was restricted to a maximum of 5 days to preserve biological activity. The instability of our proteins and their diminished biological activity in the presence of protein precipitation often delayed cell testing until the dilution and dialysis portion of our procedure was optimized. Therefore, future studies will be performed using mammalian expression systems to produce the E2F-1/TatHA and E132/TatHA proteins. We speculate that mammalian expression vectors may promote production of larger quantities of biologically active proteins possibly without the same protein precipitation issues encountered from our bacterial expression system.

Plasmid Map Illustrating Construction of E2F-1/TatHA Vector

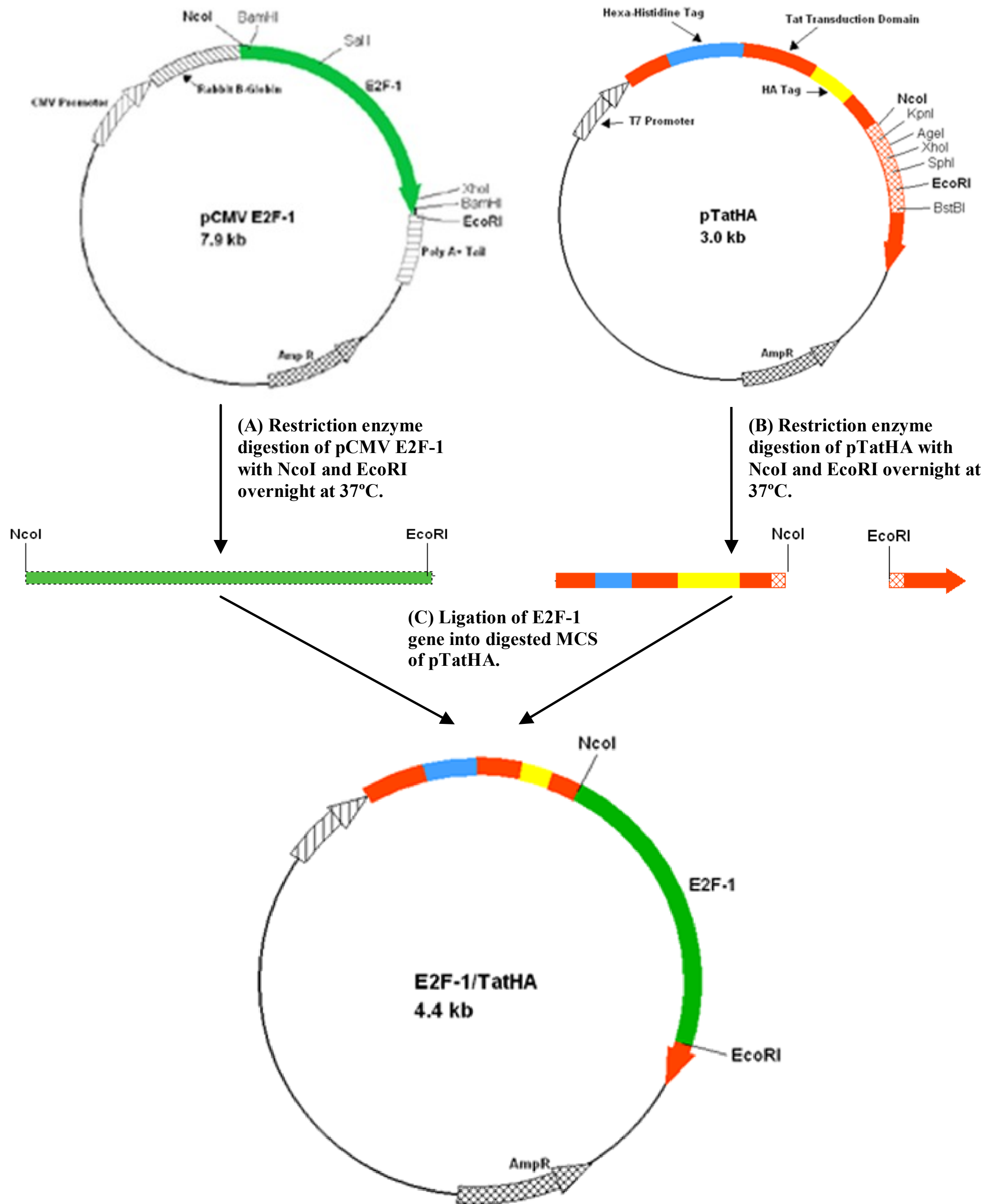


Figure 3.1. Plasmid map illustrating construction of E2F-1/TatHA vector. **(A)** Restriction enzyme digestion to excise E2F-1 gene from pCMV E2F-1. **(B)** Restriction enzyme digestion of pTatHA to prepare MCS for insertion of E2F-1 gene. **(C)** Ligation of purified digested E2F-1 DNA into digested pTatHA via NcoI and EcoRI restriction enzyme sites using T4 DNA Ligase protocol.

Restriction Enzyme Digestion of E2F-1/TatHA DNA

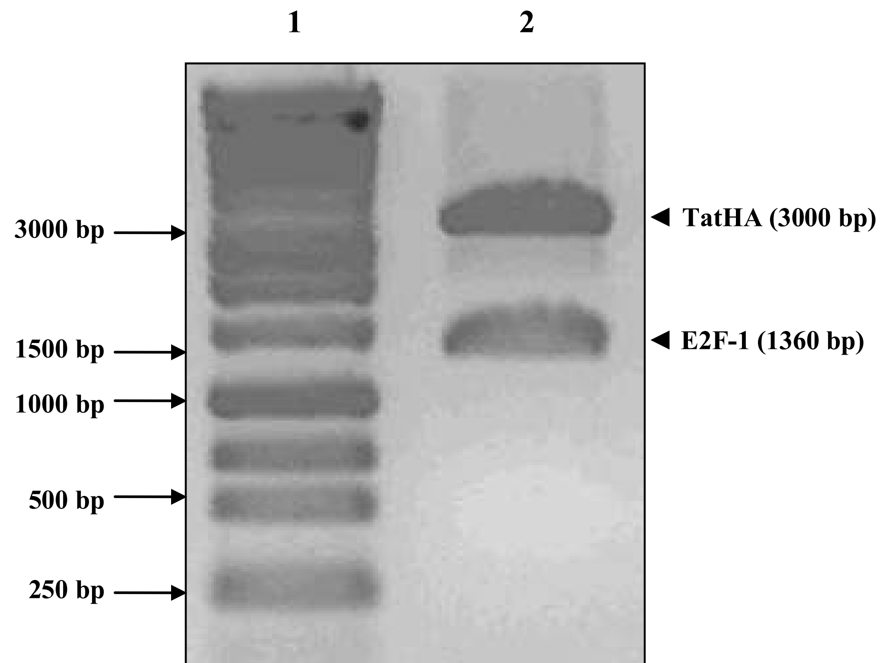


Figure 3.2. Agarose gel (1%) stained with ethidium bromide containing samples of E2F-1/TatHA DNA digested with restriction enzymes (RE) to verify presence of specific RE sites prior to DNA sequencing. *Lane 1*, DNA molecular weight marker with size indicated on left hand side of gel. *Lane 2*, E2F-1/TatHA digested with NcoI and EcoRI restriction enzymes to excise E2F-1 gene (1360 bp) from TatHA vector (3000 bp).

5' Site-Directed Mutagenesis of pCMV E132 via PCR Amplification

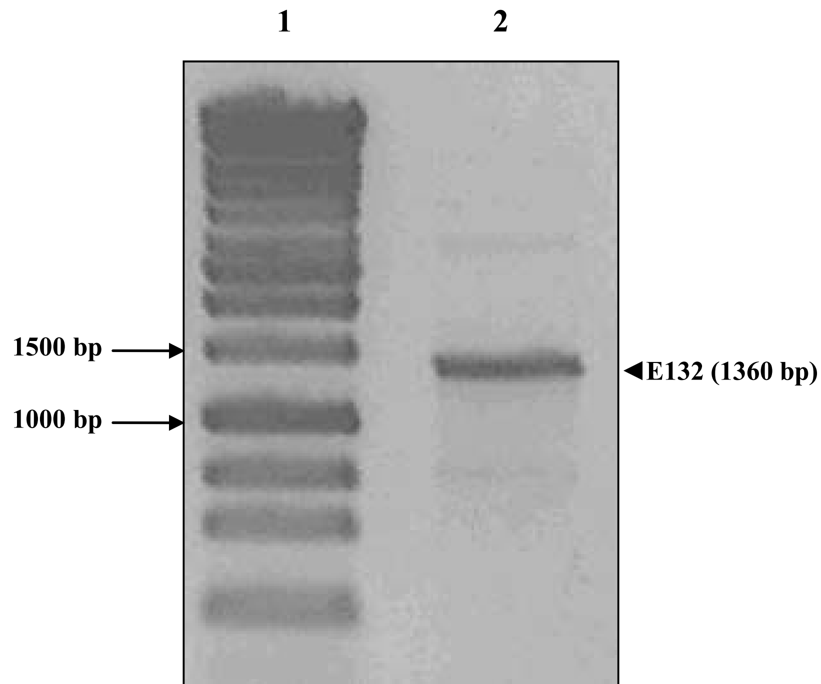


Figure 3.3. Agarose gel (1%) stained with ethidium bromide to confirm correct size of PCR product generated by 5' site-directed mutagenesis of the E132 gene. *Lane 1*, DNA molecular weight marker with specific basepair (bp) sizes illustrated on the left. *Lane 2*, E132 PCR product with the artificial restriction enzyme sites NcoI and SphI. E132 gene PCR product (1360 bp) indicated by arrow on right side. See Materials and Methods for detailed PCR protocol and primer sequences.

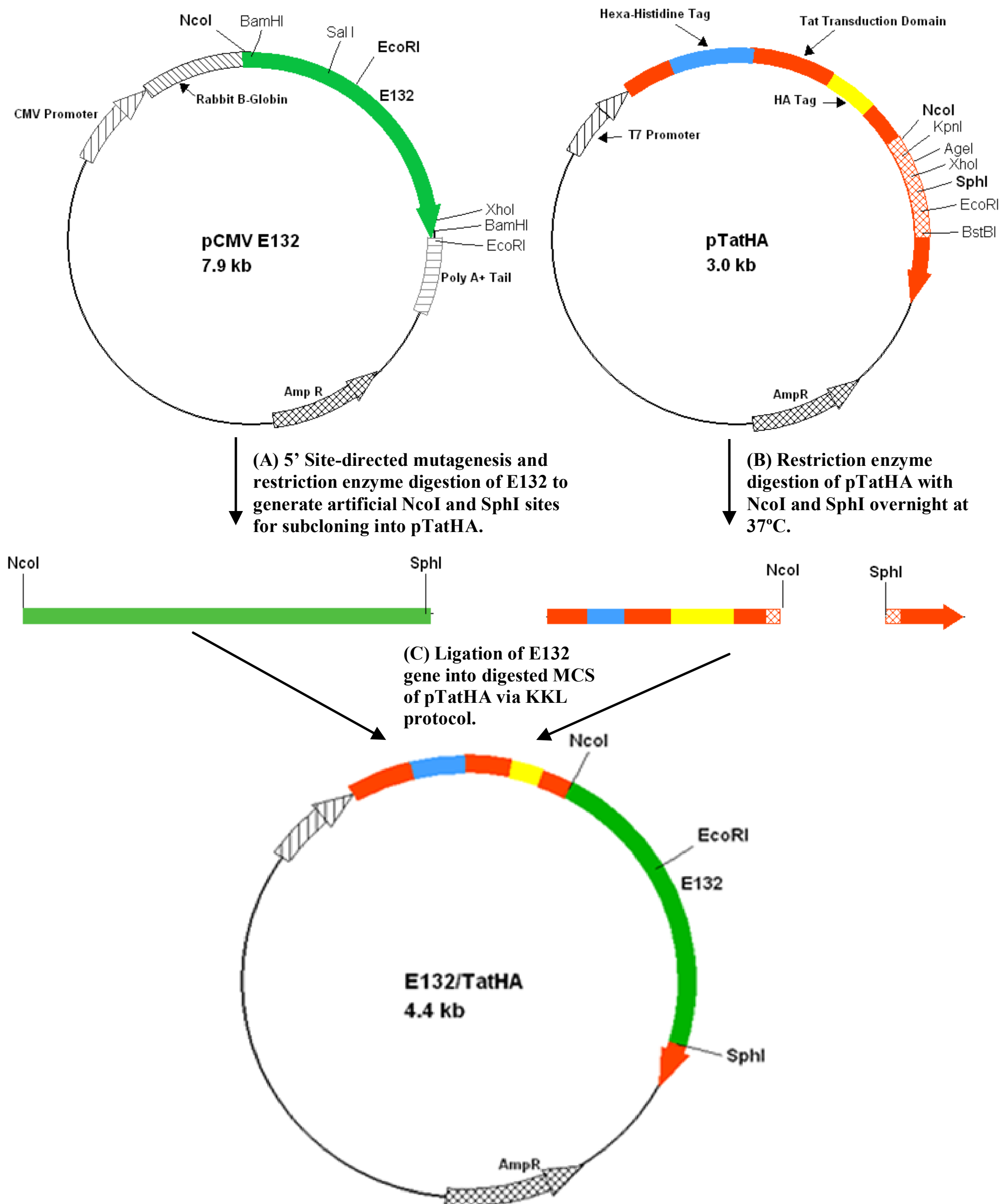


Figure 3.4. Plasmid map illustrating construction of E132/TatHA vector. **(A)** 5' Site-directed mutagenesis of E132 to generate NcoI and SphI restriction enzyme sites followed by restriction enzyme digestion with NcoI and SphI. **(B)** Restriction enzyme digestion of pTatHA to prepare MCS for insertion of E132 gene. **(C)** Ligation of purified digested E132 DNA into digested pTatHA via NcoI and SphI restriction enzyme sites using KKL and T4 DNA Ligase protocols.

Restriction Enzyme Digestion of E132/TatHA DNA

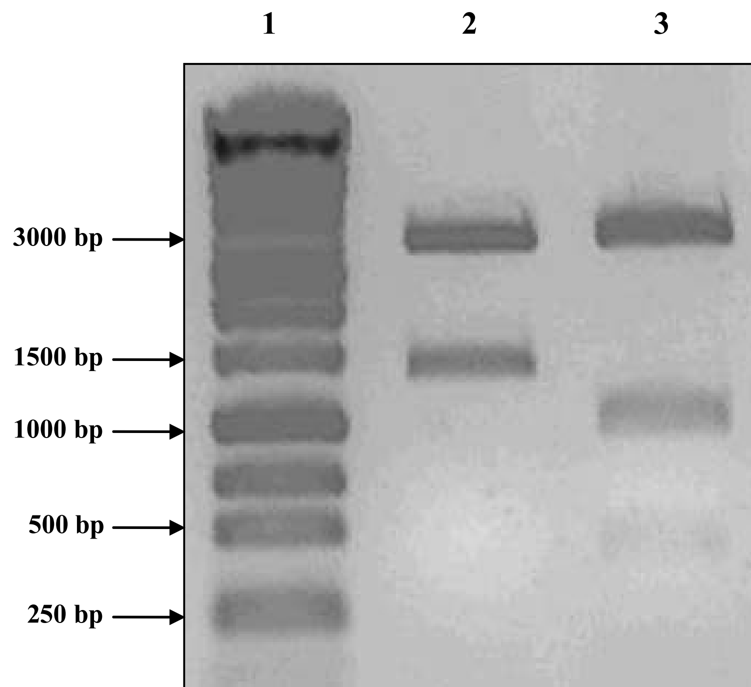


Figure 3.5. Agarose gel (1%) stained with ethidium bromide containing samples of E132/TatHA DNA digested with restriction enzymes (RE) to verify presence of specific RE sites prior to DNA sequencing. *Lane 1*, DNA molecular weight marker with size indicated on left hand side of gel. *Lane 2*, E132/TatHA digested with NcoI and SphI restriction enzymes to excise E132 gene (1360 bp) from TatHA vector (3000 bp). *Lane 3*, E132/TatHA digested with NcoI and EcoRI to cut E132 mutation site in DNA-binding domain to yield appropriately sized DNA fragments to verify intact mutation site.

Western Blot Verifying Production of E2F-1/TatHA and E132/TatHA Recombinant Proteins

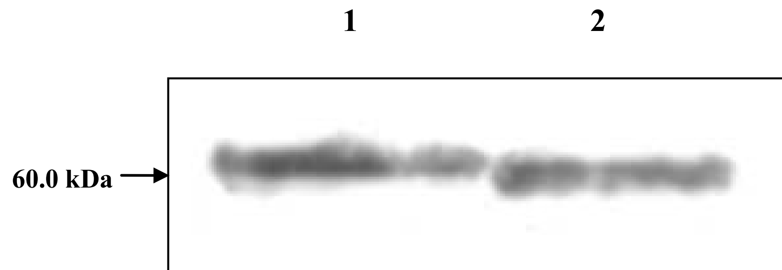


Figure 3.6. Western Blot analysis of lysates from bacterial cultures producing recombinant Tat fusion proteins. Protein (50 µg) was subjected to SDS-PAGE analysis and immunoblotting performed using primary mouse monoclonal HA antibodies (1:1000 dilution) and secondary HRP-labeled goat anti-mouse antibodies (1:25000 dilution). *Lane 1*, E2F-1/TatHA. *Lane 2*, E132/TatHA.

InVision^{TM} His-Tag Stained SDS-PAGE Gels to Verify Expression of 6-Histidine Tag in E2F-1/TatHA and E132/TatHA Proteins

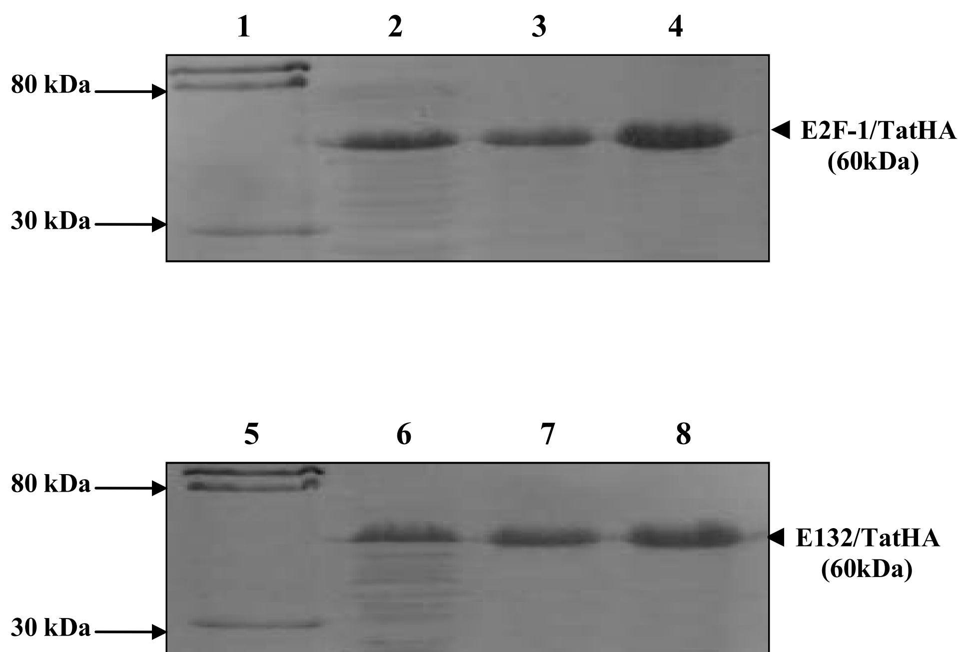


Figure 3.7. SDS-PAGE gels containing cell lysates from E2F-1/TatHA and E132/TatHA expressing cultures, isolated inclusion bodies, and purified recombinant proteins. Gels were stained with InVision^{TM} His-tag In-gel Stain to rapidly detect His-tagged fusion proteins. *Lane 1*, BenchMark^{TM} His-tagged Standard protein marker. *Lane 2,* sample of bacterial culture producing E2F-1/TatHA. *Lane 3,* sample of isolated inclusion bodies from E2F-1/TatHA culture. *Lane 4,* FPLC purified E2F-1/TatHA proteins. *Lane 5,* BenchMark^{TM} His-tagged Standard protein marker. *Lane 6,* sample of bacterial culture producing E132/TatHA. *Lane 7,* sample of isolated inclusion bodies from E132/TatHA culture. *Lane 8,* FPLC purified E132/TatHA proteins.

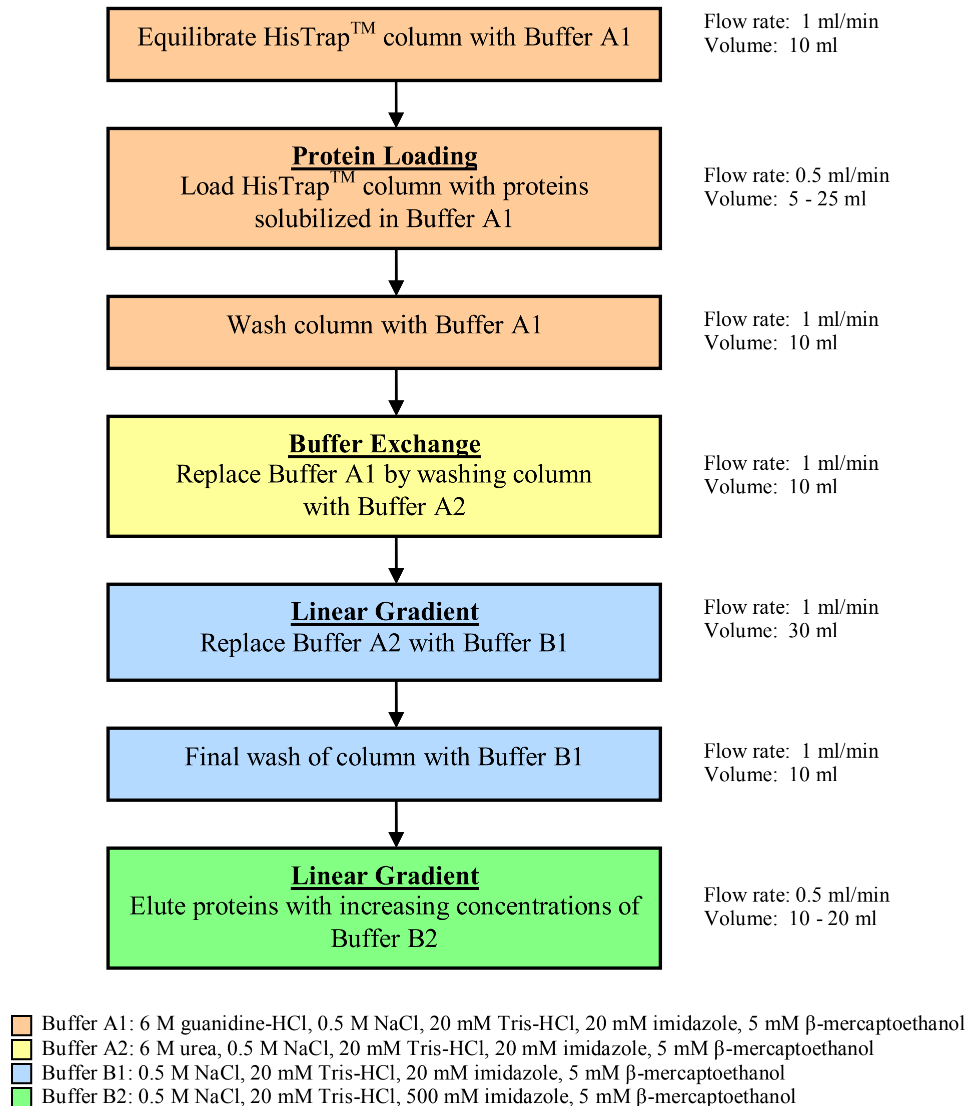


Figure 3.8. Flowchart illustrating FPLC purification protocol for E2F-1/TatHA and E132/TatHA recombinant proteins. A series of linear gradients and buffer exchanges were performed during the purification process to replace strong denaturants, slowly refold the proteins while they were bound to the column and thoroughly wash the proteins. Flow rates and volumes of buffers utilized are indicated to the right of the flowchart. Eluted proteins were analyzed via SDS-PAGE gels and further diluted and dialyzed to be used in cell culture experiments.

FPLC Chromatogram Representing Protein Purification of E2F-1/TatHA

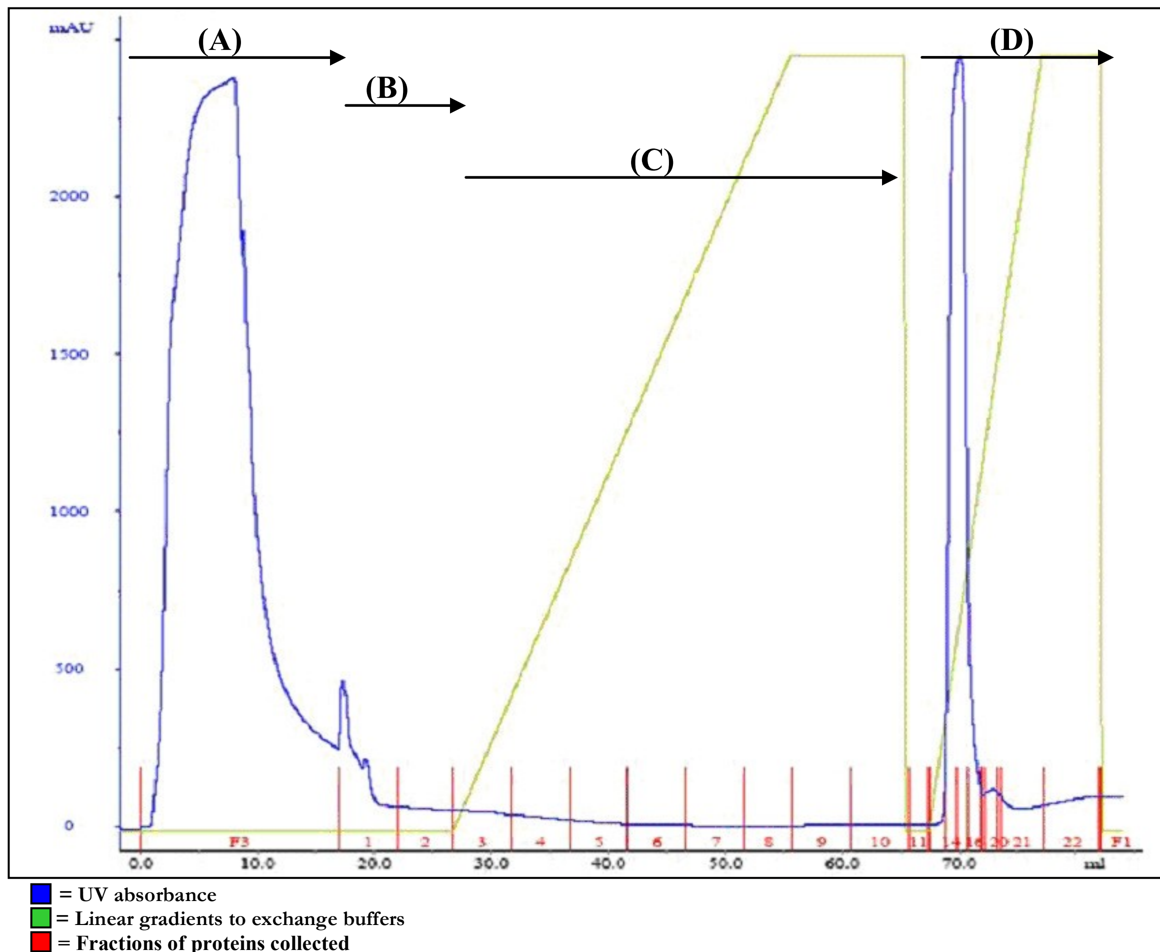


Figure 3.9. This figure represents the FPLC chromatogram for purification of E2F-1/TatHA recombinant proteins. On this graph, the ordinate represents UV absorbance at A_{280} to determine peak protein concentration. The abscissa represents the volume in ml of buffer flowing through HisTrap columns to load, wash and elute the E2F-1/TatHA proteins. **(A)** Buffer A1 wash to equilibrate HisTrap^{TM} column, load the recombinant protein and wash with an additional 10 ml of buffer. **(B)** Buffer A2 to replace Buffer A1, wash bound proteins and partially renature recombinant proteins. **(C)** A 30 ml linear gradient ranging from 6 M down to 0 M urea to exchange Buffer A2 with Buffer B1 to gradually refold and renature proteins bound to HisTrap^{TM} column. **(D)** A linear gradient ranging from 20 mM up to 500 mM imidazole performed to elute recombinant proteins by exchanging Buffer B1 with Buffer B2. Fractions of collected, purified proteins were subsequently analyzed on SDS-PAGE gels. Refer to Materials and Methods for specific details.

FPLC Chromatogram Representing Protein Purification of E132/TatHA

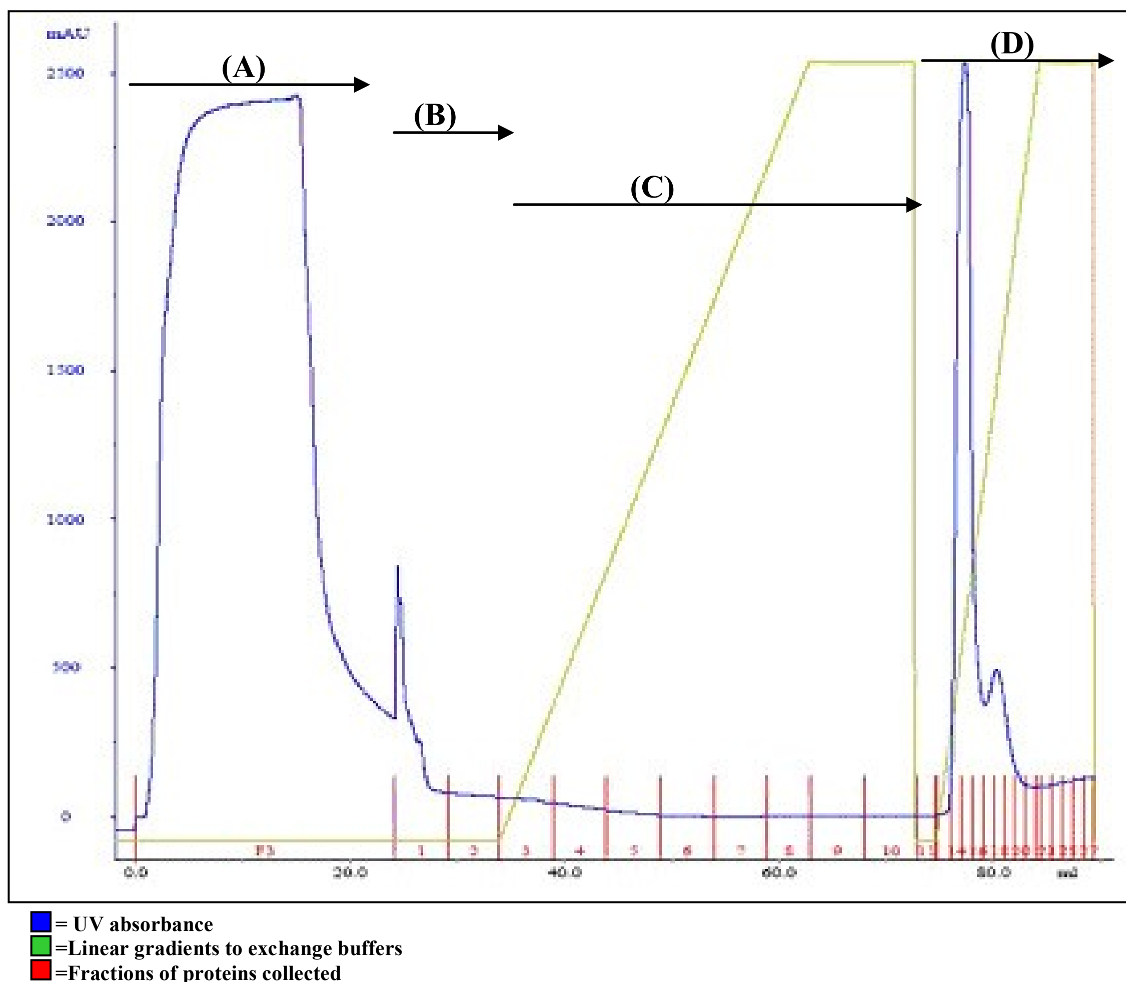


Figure 3.10. FPLC chromatogram illustrating the purification of E132/TatHA recombinant proteins. On this graph, the ordinate represents UV absorbance at A_{280} to determine peak protein concentration. The abscissa represents the volume in ml of buffer flowing through HisTrap^{TM} columns to load, wash and elute the E132/TatHA proteins. **(A)** Buffer A1 equilibration, loading and washing of proteins. **(B)** Buffer A2 wash. **(C)** Linear gradient to exchange Buffer A2 with Buffer B1. **(D)** Linear gradient to exchange Buffer B1 with Buffer B2. Fractions of collected, purified proteins were subsequently analyzed on SDS-PAGE gels. Refer to Materials and Methods for specific details.

FPLC Purification of E2F-1/TatHA Recombinant Proteins and Analysis with SDS-PAGE Gel Electrophoresis

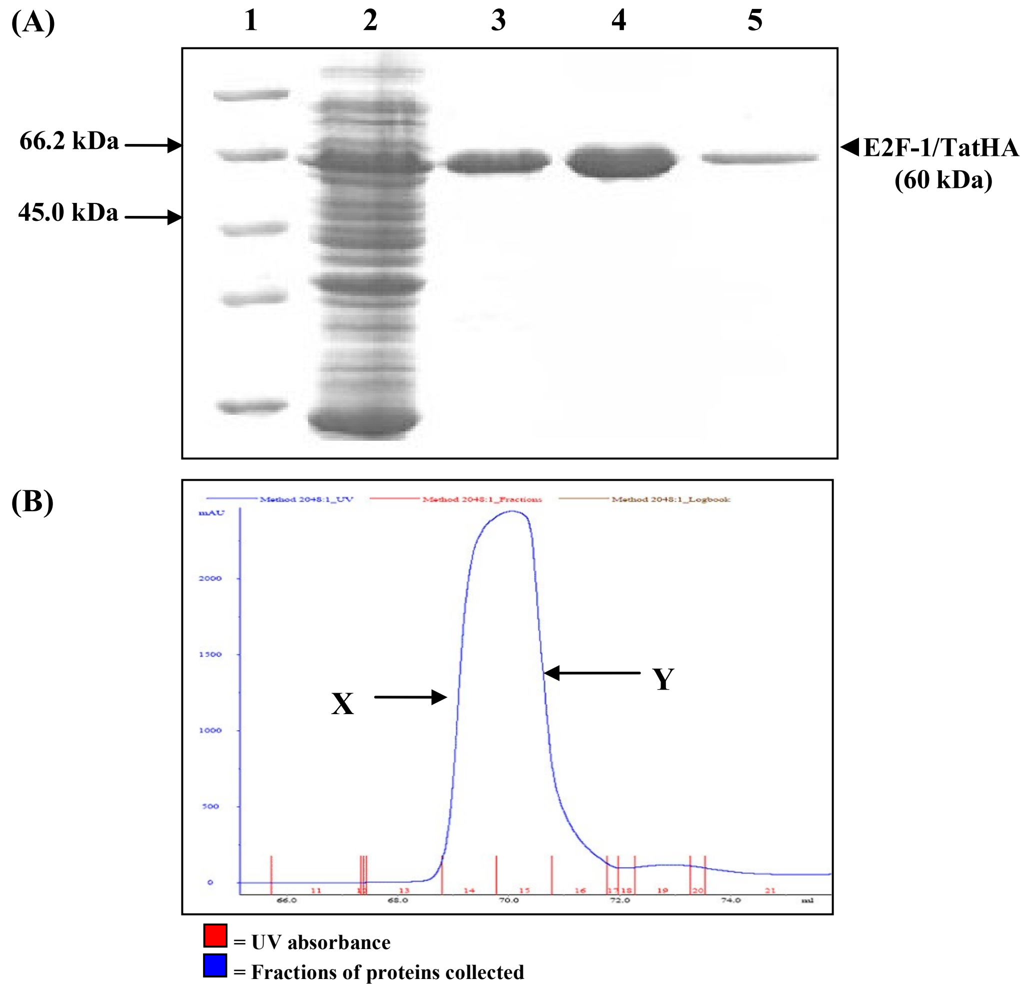


Figure 3.11. FPLC purification of E2F-1/TatHA recombinant proteins and analysis with SDS-PAGE gel electrophoresis. **(A)** SDS-PAGE gel of E2F-1/TatHA recombinant protein produced in culture and isolated via FPLC. *Lane 1*, protein molecular weight marker. *Lane 2*, sample of bacterial culture producing E2F-1/TatHA proteins (60 kDa). *Lane 3*, purified E2F-1/TatHA protein represented by FPLC fraction **X** (see Figure B) on chromatogram. *Lane 4*, purified E2F-1/TatHA protein represented by FPLC fraction **Y** (see Figure B). *Lane 5*, sample of E2F-1/TatHA diluted and dialyzed into cell culture media. **(B)** FPLC chromatogram illustrating eluted fractions of purified E2F-1/TatHA proteins. Ordinate is absorbance with peak protein concentration determined by absorbance at A_{280}, and abscissa represents eluted fractions of purified protein in Buffer B2. Fraction #14 is designated by **X** and Fraction #15 is represented by **Y**.

FPLC Purification of E132/TatHA Recombinant Proteins and Analysis with SDS-PAGE Gel Electrophoresis

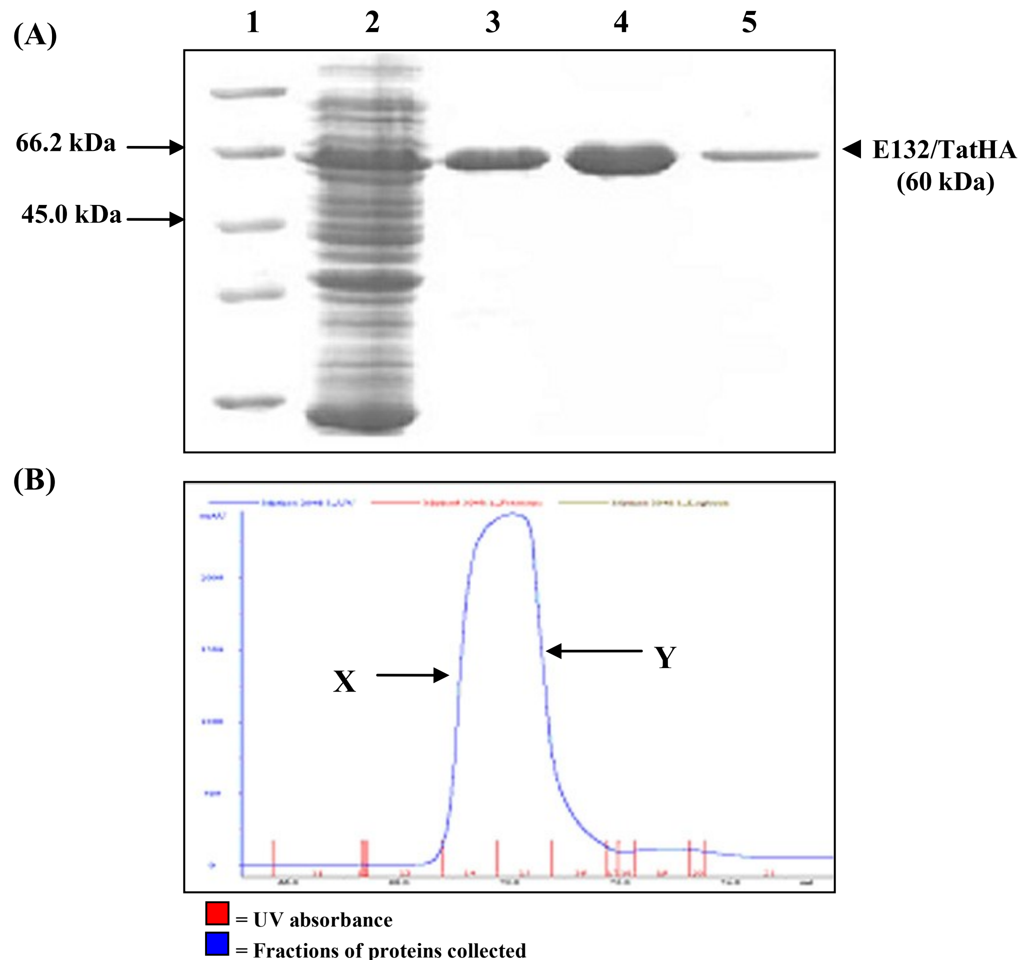
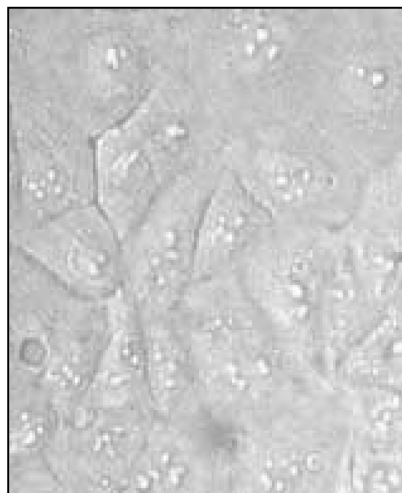


Figure 3.12. FPLC purification of E132/TatHA recombinant proteins and analysis with SDS-PAGE gel electrophoresis. **(A)** SDS-PAGE gel of E132/TatHA recombinant protein produced in culture and isolated via FPLC. *Lane 1,* protein molecular weight marker. *Lane 2,* sample of bacterial culture producing E132/TatHA proteins (60.0 kDa**.** *Lane 3*, purified E132/TatHA protein represented by FPLC fraction **X** (see Figure B) on chromatogram. *Lane 4*, purified E132/TatHA protein represented by FPLC fraction **Y** (see Figure B). *Lane 5*, sample of E132/TatHA diluted and dialyzed into cell culture media. **(B)** FPLC chromatogram illustrating eluted fractions of purified E132/TatHA proteins. Ordinate is absorbance with peak protein concentration determined by absorbance at A_{280}, and abscissa represents eluted fractions of purified protein in Buffer B2. Fraction #14 is designated by **X** and Fraction #15 represented by **Y**.

Brightfield Images of Infiltrating Ductal Breast Cancer Cell Lines

HCC1937



HCC1599

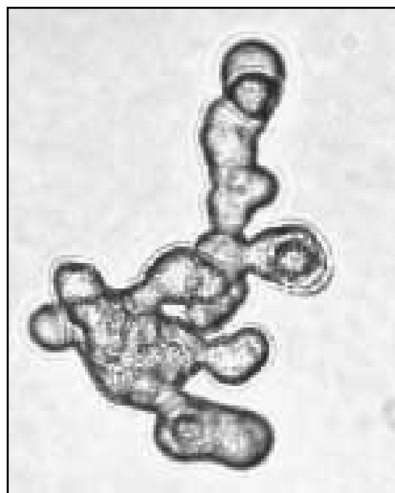


Figure 3.13. Brightfield microscopic images of breast cancer cell lines HCC1937 and HCC1599. The HCC1937 cell line grows as large, adherent epithelial cells. HCC1937 was isolated from a primary ductal carcinoma classified as a TNM Stage IIB, grade 3. HCC1937 is homozygous for BRCA1 5382C mutation, homozygous deletion of the PTEN gene and cells are negative for the expression of the following genes: Her2/*neu*, estrogen receptor, progesterone receptor and for expression of p53 (29, 30). The HCC1599 cell line grows as multicellular aggregates in suspension. HCC1599 was established from an invasive ductal carcinoma classified as TNM stage IIIA, grade 3. The HCC1599 cells are negative for the expression of p53, estrogen receptor, progesterone receptor, and Her2/*neu* (29, 30).

Transduction of E2F-1/TatHA and E132/TatHA Proteins into HCC1937 Cells

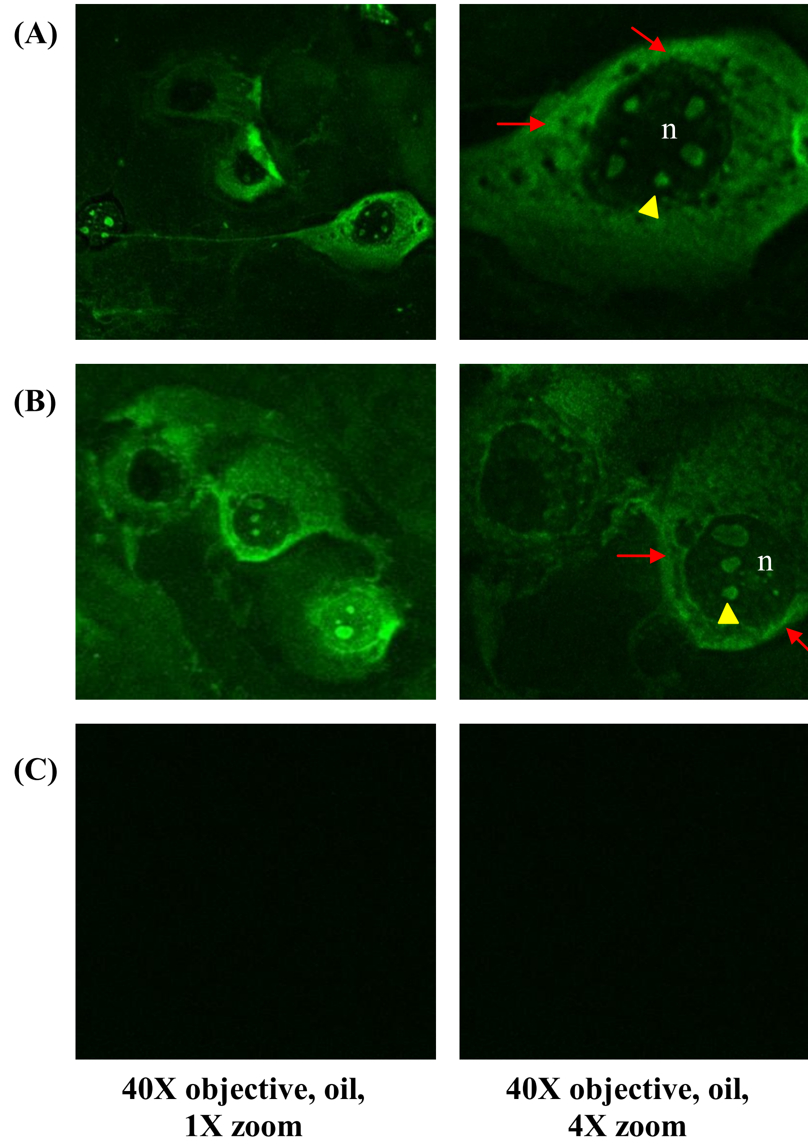


Figure 3.14. Transduction of E2F-1/TatHA and E132/TatHA proteins into HCC1937 cells. Immunocytochemistry was performed with primary HA antibodies and secondary FITC-labeled antibodies as described under Materials and Methods. **(A)** Cells treated with 2 μM E2F-1/TatHA. **(B)** Cells treated with 2 μM E132/TatHA. **(C)** Cells untreated with Tat fusion peptides. Legend: n, nucleus of cell; arrow, perinuclear accumulation of proteins; arrowhead, nucleolar localization of TFPs. Images were captured using the 40X objective on the Bio-Rad MRC 1024 Laser Scanning Confocal Microscope with oil immersion and either 1X or 4X zoom.

Transduction of E2F-1/TatHA and E132/TatHA Proteins into MCF-7 Cells

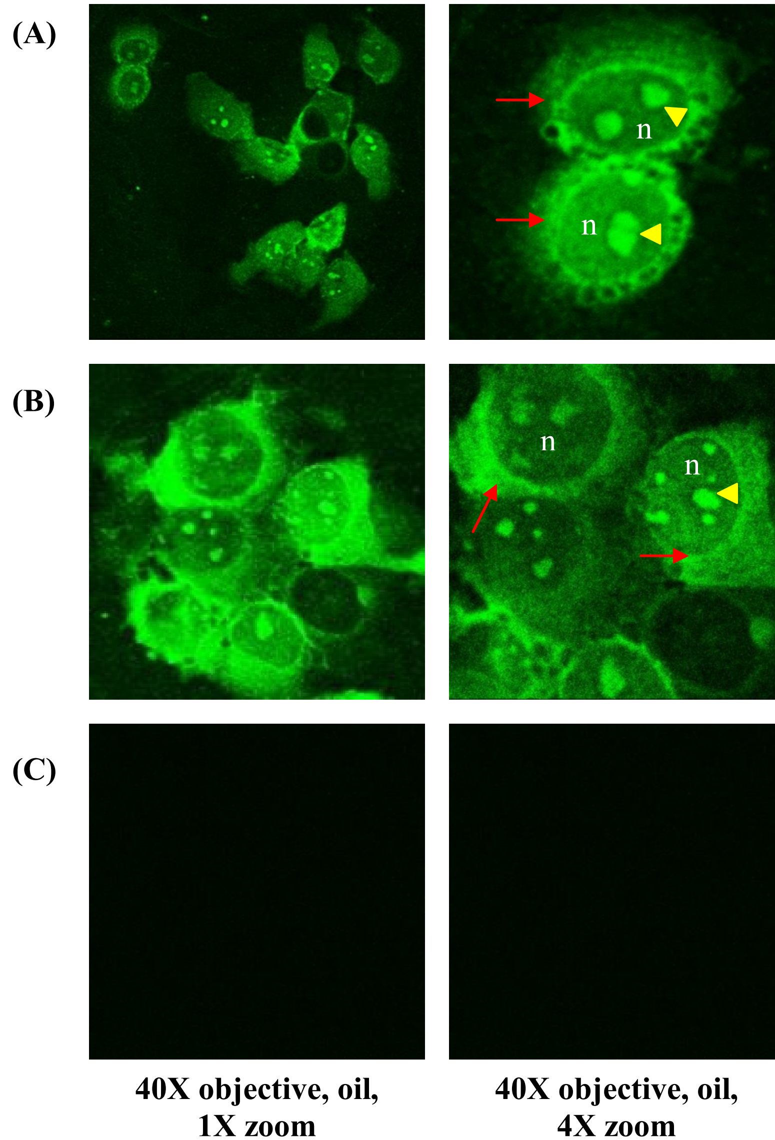


Figure 3.15. Transduction of E2F-1/TatHA and E132/TatHA proteins into MCF-7 cells. Immunocytochemistry was performed with primary HA-antibodies and secondary FITC-labeled antibodies as described under Materials and Methods. **(A)** Cells treated with 2 μM E2F-1/TatHA. **(B)** Cells treated with 2 μM E132/TatHA. **(C)** Cells untreated with Tat fusion peptides. Legend: n, nucleus of cell; arrow, perinuclear accumulation of proteins; arrowhead, nucleolar localization of TFPs. Images were captured using the 40X objective on the Bio-Rad MRC 1024 Laser Scanning Confocal Microscope with oil immersion and either 1X or 4X zoom.

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CHAPTER IV

TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS REPRESSES EXPRESSION OF HTERT IN PRIMARY DUCTAL BREAST CARCINOMA CELL LINES

ABSTRACT

The objective of this study was to investigate repression of telomerase activity in primary ductal breast cancer cells through transcriptional regulation of the catalytic subunit hTERT. We hypothesized that inhibition of telomerase expression could be achieved via Tat mediated protein transduction of the repressor protein E2F-1. Protein purification techniques were refined to yield biologically active Tat fusion proteins (TFPs) capable of transducing the breast cancer cell lines HCC1937 and HCC1599. Cell lines were treated with wildtype E2F-1 (E2F-1/TatHA), mutant E2F-1 (E132/TatHA) and a control Tat peptide (TatHA) for 24 hours. Real-time RT-qPCR results revealed significant repression of the catalytic subunit of telomerase (hTERT) in both HCC1937 and HCC1599 cells. In HCC1937 cells, hTERT was repressed 3.5-fold by E2F-1/TatHA in comparison to E132/TatHA ($p<0.0012$) and the TatHA peptide controls ($p<0.0024$). In HCC1599 cells, hTERT was also repressed with E2F-1/TatHA treatment by 4.0-fold

when compared to the E132/TatHA control ($p<0.0001$). A slightly lower hTERT repression of 3.3-fold was observed with E2F-1/TatHA in the HCC1599 cells when compared to the TatHA control ($p<0.0001$). These results suggest that transduction of E2F-1/TatHA fusion proteins in vitro is an effective repressor of hTERT expression in the primary ductal breast cancer cell lines HCC1937 and HCC1599.

INTRODUCTION

Telomerase activity is detectable in 80-90% of malignancies and is absent in most normal somatic cells (1). Breast cancer has been identified as an important target for developing telomerase inhibitors. Importantly, fine-needle aspirations of malignant breast tumors revealed that 81% were positive for telomerase (2), and Hiyama et al. (2000) also reported that 95% of advanced stage breast cancers express telomerase (3). Due to the majority of tumor cells expressing telomerase, this protein is being evaluated as a tumor marker for breast cancer and other solid malignancies (4).

Telomerase, a ribonucleoprotein, synthesizes tandem repeats of the DNA sequence TTAGGG at the terminal ends of chromosomes permitting continuous genomic replication and cell division. Telomerase includes an RNA component (hTR) that serves as the template for telomeric DNA and a protein catalytic subunit (hTERT) with reverse transcriptase activity (5). The activity of telomerase is regulated by the transcription of hTERT, and the cloning and characterization of the hTERT-promoter has permitted examination of elements controlling transcriptional activation and repression of hTERT (6).

To impact expression of hTERT in breast cancer cells, our research will utilize the E2F-1 transcription factor. The E2F family of transcription factors mediates cell cycle progression, and they are released upon phosphorylation of Retinoblastoma (Rb) family proteins (7-9). While most of these factors are associated with inducing gene expression, E2F-1 has been identified as a transcriptional repressor of the hTERT gene, specifically binding to two separate sites (-174 bp and -98 bp) of the proximal hTERT promoter (7). In addition, a study that examined the apoptotic function of E2F-1 found that cell death generated by ectopic expression of E2F-1 was independent of the p53 regulatory pathway (8). Therefore, even in cells with intact p53 suppressor gene function, overexpression of E2F-1 should inhibit transcription of the hTERT gene and potentially induce apoptosis. Previous studies utilizing adenoviral-mediated transfection of E2F-1 genes induced apoptosis of melanoma cells (10), and an additional study revealed that E2F-1 suppressed cell growth while decreasing telomerase activity in the Tu-167 SCCHN cell line (11). Adenoviral-mediated overexpression of E2F-1 also induced apoptosis in human breast and ovarian carcinoma cell lines independently of p53 (12). We anticipate that telomerase repression via E2F-1 protein therapy could induce apoptotic activity and/or cell senescence in cancer cells more effectively than the viral-based studies described above.

Tat-mediated protein transduction was utilized in our research to effectively target cancer cells. Transduction occurs in a receptor- and transporter-independent fashion, targets the lipid bilayer, and also crosses the blood-brain barrier (13). Tat has been reported to transduce 100% of mammalian cells, and studies also suggest the transcriptional effects of Tat-mediated protein transduction are reversible and have no

detrimental effects because clearance of transduced proteins is dependent on the half-life of the protein (13-15). Becker-Hapak et al. (14) also reported that Tat-mediated transduction is concentration dependent, reaches maximum intracellular concentration in less than 5 minutes, and nearly equal intracellular concentrations of the fusion proteins have been detected between all transduced cells.

Our research investigated primary infiltrating ductal carcinoma cells, both with and without a BRCA1 gene mutation. In 2001, published results by Ho et al. (16) determined that E2F-1 and E2F-4 were decreased in primary breast carcinomas, and 70% of the tumors revealed decreased expression of E2F-1. Additionally, the metastatic nodal tissue examined indicated that 100% of the tissue samples had significantly low levels of E2F-1 as compared to normal breast tissue. Ho et al. also suggested that E2Fs act as tumor suppressors in breast cancer and their down-regulation may be important in the development of metastases (16). It is important to determine the repressive effects of E2F-1 on hTERT transcription in tumor cells with and without the normal BRCA1 protein to determine any variance between BRCA1 status and inhibition of telomerase. Recently, Wang et al. (17) determined that the BRCA1 promoter was transactivated in a dose-dependent manner by E2F-1 and served as a target for E2F-dependent transcriptional regulation. Other studies have also demonstrated that BRCA1 is a potent inducer of apoptosis by binding directly to p53 and stimulating transcriptional activation of pro-apoptotic genes such as Bax (18, 19). Therefore, telomerase inhibition in the primary infiltrating ductal carcinoma cell line with a BRCA1 gene mutation, HCC1937, will be compared to a breast cancer cell line without detectible BRCA1 gene mutations,

HCC1599. Aside from variance in BRCA1 status, both HCC1937 and HCC1599 cell lines carry similar mutations in p53, Her2/neu and ER/PR receptors (20).

Here we report the first results of the repressive effects of E2F-1 on telomerase activity using Tat-mediated transduction. These studies validated our proposed hypothesis that telomerase activity can be repressed in infiltrating ductal carcinoma cells via transcriptional regulation of hTERT utilizing protein transduction techniques with E2F-1 Tat fusion proteins.

MATERIALS AND METHODS

Vector DNA

The vectors pCMV E2F-1 and pCMV E132 were obtained from Dr. Karen Vousden (Beatson Cancer Institute, UK) and the pTatHA vector was a kind gift from Dr. Steven Dowdy (University of Washington, St. Louis, MO).

E2F-1/TatHA and E132/TatHA Constructs

The wildtype E2F-1 gene was directly cloned into the pTatHA vector via NcoI and EcoRI restriction enzyme sites. The E132 vector (mutant E2F-1) contained an artificial EcoRI site in the DNA binding domain and could not be directly inserted into pTatHA's multiple cloning site (MCS). Therefore, the E132 gene was modified using 5' site-directed mutagenesis via PCR from plasmid pCMV-E132 using sense primer: 5'-gcgcgcaaccATGGCCTTGGCCGGG-3'and antisense primer: 5'-gcgcagcatgcGGATCCAGCCCTGTC-3' to generate artificial NcoI and SphI sites

(underlined in primer sequences). The PCR reaction used 2 Units Vent Taq polymerase (New England Biolabs, Beverly, MA), 1X Thermapol buffer (New England Biolabs, Beverly, MA), 500 nM of sense and antisense primers and 5% DMSO. The PCR product was amplified under the following conditions: initial cycle 94ºC- 3 minutes, 55ºC- 1 minute, 72ºC- 1 minute followed by 23 cycles of 94ºC- 1 minute, annealing, 55ºC- 1 minute, 72ºC- 1 minute and a final extension of 72ºC for 7 minutes. The pTatHA DNA was digested with restriction enzymes NcoI and SphI and purified using phenol/chloroform extraction. The E132 PCR product was digested with NcoI-SphI and subcloned into the NcoI-SphI sites of pTatHA to yield E132/TatHA using a Klenow-Kinase-Ligase (KKL) protocol (21).

Recombinant Protein Production

E2F-1/TatHA and E132/TatHA plasmids were transformed into BLR(DE3)pLysS competent cells (Novagen, Madison, WI) and recombinant proteins produced via inoculating 2 ml 2YT broth with single transformed colonies. After incubation in shaking water bath (225 rpm) for 12-14 hours, 3 ml of fresh 2YT broth, 0.5 µg/ml ampicillin, and 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) were added to cultures. Cultures incubated an additional 6 hours to produce recombinant proteins via IPTG stimulation. Cultures were centrifuged at 6000 x g for 10 minutes and cell pellets resuspended in 100 µl of 20 mM Tris buffer (pH 8.0) followed by quantitation using a BCA™ Protein Assay Kit (Pierce Biotechnologies, Inc., Rockford, IL) with BSA standards. 2X Laemmli Sample Buffer was added to 50 µg of total cellular protein and samples were boiled for 3-5 minutes and immediately placed on ice (22). Samples were

then loaded onto 12% SDS-PAGE gels and run on a Mini-PROTEAN® 3 (Bio-Rad Laboratories, Hercules, CA) protein electrophoresis unit using the Laemmli buffer system. Prestained 1 kb protein ladders (Fermentas Life Technologies, Hanover, MD) were also run on each gel to determine protein size (refer to Figure 4.1).

Western Blotting

For Western blotting experiments, duplicate gels were run simultaneously- one gel for western blotting and a second gel for Coomassie blue staining. The gel designated for western blotting was transferred to nylon membranes using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions using 1X TBS-T transfer buffer. Recombinant protein production was verified using primary mouse monoclonal anti-HA antibodies (1:1,000 dilution) (CRP, Inc., Denver, PA) and secondary HRP-labeled goat anti-mouse antibodies (1:25,000 dilution) (KPL, Inc., Gaithersburg, MD). Membranes were blocked with TBS-T/5% milk solution for 1 hour, incubated with primary anti-HA antibody for 1 hour, washed 3 times for 10 minutes each, and incubated with secondary HRP antibody for 1 hour. Following additional washings (3 x 10 minutes), western blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to manufacturer's instructions. Blots were exposed to X-ray film and developed.

Large-Scale Recombinant Protein Production

Recombinant E2F-1/TatHA and E132/TatHA proteins were purified under denaturing conditions with affinity chromatography using a modified procedure from Amersham Pharmacia (23). Single colonies from protein expressing cultures inoculated 200 ml of 2YT broth supplemented with ampicillin (50 μg/ml) and were incubated at 37ºC while shaking at 225 rpm for 16-18 hours. An additional 300 ml of broth was added along with fresh ampicillin and IPTG (400 μM), and cultures incubated an additional 6 hours. Cultures were centrifuged for 10 minutes at 6000 x g, and the pellets frozen at -20ºC. Cell pellets were thawed for 20 minutes at 37ºC, and 25 ml of Buffer 1 (20 mM Tris^{TM}-HCl, pH 8.0) added to resuspend the cells with vigorous pipetting. Cells were sonicated on ice (3 x 15 sec) and centrifuged (10 min x 6000 g). Cell pellets containing the inclusion bodies were then resuspended in 25 ml Buffer 2 (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton^{TM} X-100, pH 8.0). Samples were again sonicated on ice and centrifuged as described above. Pellets were resuspended in 25 ml Buffer 2 to wash the inclusion bodies and recentrifuged. A final wash of 25 ml of Buffer 1 was then performed, and following centrifugation, pellets were either used immediately or frozen at -20ºC for a maximum of two weeks prior to use.

Preparation of Inclusion Bodies for FPLC

Cell pellets were resuspended in 5-10 ml Buffer A1 (6 M guanidine-HCl, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) by stirring at room temperature for 60 minutes to solubilize recombinant proteins. Samples were centrifuged at 20,000 x g for 15 minutes to pellet residual cell debris. Protein supernatant

containing denatured, soluble proteins was removed from residual cell debris. Supernatant was further clarified by passage through a 0.45 μM filter (Millipore, Bellerica, IL) to further remove any particles.

Isolation of Recombinant Proteins using Affinity Chromatography

HisTrap™ Ni^{++} charged columns (Amersham Pharmacia, Piscataway, NJ) were equilibrated with 5 ml Buffer A1 and the proteins loaded at a flow rate of 0.5 ml/minute. Columns were washed with an additional 10 ml of Buffer A1 followed by a wash of 10 ml of Buffer A2 (6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A linear gradient from 6 M to 0 M urea was then performed to remove the denaturant and start refolding the proteins on the HisTrap™ column by gradually replacing Buffer A2 with Buffer B1 (0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A total volume of 40 ml of Buffer B1 was used to perform the gradient wash. The purified, refolded proteins were then eluted with Buffer B2 (0.5 M NaCl, 20 mM Tris-HCl, 500 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) using a gradient of 0 mM to 500 mM imidazole in the buffer. Fractions containing the purified protein were analyzed by SDS-PAGE gel electrophoresis and quantified with the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL).

Dialysis of Recombinant Proteins into Cell Culture Media

E2F-1/TatHA and E132/TatHA recombinant proteins were diluted with Buffer B2 to a concentration of 0.5 mg/ml to avoid protein precipitation cascades and injected into 12 ml Slide-A-Lyzer® Dialysis Cassettes (Pierce Biotechnology, Inc., Rockford, IL)

using 21-gauge, 1-inch beveled hypodermic needles. Proteins were dialyzed in 1X PBS for 2 hours at room temperature. The cassettes were placed in fresh 1X PBS for an additional 2 hours at room temperature and then dialyzed overnight in appropriate cell culture media for designed cell line experiments. Following dialysis, proteins were removed from cassettes and placed in sterile 50 ml conical centrifuge tubes and centrifuged at 9400 x g for 15 minutes to remove any protein precipitate. Protein supernatant was removed and the final concentrations of recombinant proteins determined using the Coomassie Plus™ Bradford Assay (Pierce Biotechnology, Inc., Rockford, IL) and SDS-PAGE gels using BSA protein standards. Contamination of proteins was minimized by passage through 0.2 µM filters (Millipore, Bellerica, IL). Purified protein was supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and 50 IU/ml penicillin/0.05 mg/ml streptomycin. Prior to testing on carcinoma cell lines, various µM concentrations were tested to determine optimal transduction at different time points using immunocytochemistry to monitor cellular uptake of E2F-1/TatHA and E132/TatHA.

Synthesis of TatHA Control Protein

The TatHA control protein was artificially synthesized (amino acid sequence: YPYDVPDYAYGRKKRRQRRR) (24) on a Ranin Symphony/Multiplex Peptide Synthesizer (Center for Integrated BioSystems, Logan, UT). The TatHA peptide was reconstituted using Cellgro™ RNase free/DNase/Protease free dH_20 for cell culture (Mediatech Inc., Herndon, VA) and stored at -70ºC at a working concentration of 1 mg/ml (25).

Primary Carcinoma Cell Culture

Primary infiltrating ductal breast carcinoma cell lines HCC1937 (ATCC number: CRL-2336) and HCC1599 (ATCC number: CRL-2331) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI-1640 medium supplemented with 10% FBS, 50 IU/ml penicillin/ 0.05 mg/ml streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were grown in a 37ºC humidified incubator with 5% CO_2. Cell lines were grown in 25 cc^2 and 75 cc^2 tissue culture flasks and passaged every 2-4 days when 70-80% confluent. Cells were detached from flasks using 0.25% trypsin, 0.03% EDTA solution according to standard cell culture protocols (26).

Immunocytochemisty

HCC1937 and HCC1599 cells were exposed to 2 μM E2F-1/TatHA, E132/TATHA, and TatHA fusion peptides for various time points: 1, 6, 12 and 24 hours. Cells were washed 3 times with 1X PBS, fixed for 30 minutes in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in 1X PBS for 30 minutes. Cells were blocked for 30 minutes with 1% FBS and 0.1% Tween-20 in 1X PBS. Cells were incubated for 60 minutes with primary mouse HA antibodies (1:1000 dilution in 1X PBS), washed 3 times for 10 minutes each, and incubated for 60 minutes with a secondary FITC-labeled goat anti-mouse antibody (1:250 dilution in 1X PBS) (KPL, Inc., Gaithersburg, MD). Cells were then washed, and incubated for 15 minutes with 1 μM TO-PRO-3 iodide (Molecular Probes, Eugene, OR) to counterstain the cell nuclei. Cells were then mounted

on slides using Prolong® Antifade (Molecular Probes, Eugene, OR) and images captured with Bio-Rad MRC 1024 Laser Scanning Confocal Microscope (see Figure 4.2).

Cell Culture Experiments with Recombinant Proteins

HCC1937 and HCC1599 cells were seeded at a density of 2 x 10^5 cells/well in CellStar® 6-well tissue culture plates. HCC1937 (adherent) cells were allowed 24 hours to adhere to plates before recombinant proteins were added. HCC1599 (suspension) cells were seeded directly into wells with TFPs. After 24 hours of co-incubation, RNA was isolated and analyzed via RT-qPCR as described below.

RNA Isolation, Reverse Transcription and Quantitative PCR

Total RNA was isolated from cells using the Mini RNA Isolation II™ kit (Zymo Research, Orange, CA) using the following protocol. After treatment with TFPs, adherent cell lines were washed at least three times with 1X PBS and detached from tissue culture plates using 0.5 ml HyQ®tase™ (Hyclone, Logan, UT). Cells were added to 1.8 ml microcentrifuge tubes and pelleted by centrifugation at 14,000 rpm for 1 minute. Supernatant was decanted and excess fluid removed via micropipetting. A 600 µl volume of ZR RNA Buffer was added to cells, tubes vortexed 30 seconds, and sample transferred to a Zymo-Spin III column placed in a 2 ml collection tube. Zymo-Spin III column was centrifuged at 14,000 rpm for 1 minute. A 350 µl volume of RNA Wash Buffer was added to the Zymo-Spin III column and centrifuged as described above to wash column. A second wash was performed with 350 µl RNA Wash Buffer to further clean RNA. The spin column was then transferred to a sterile 1.8-ml microcentrifuge

tube. A 50 µl volume of RNase-free water was added directly to the membrane of the Zymo-Spin III Column and column centrifuged for 15 seconds to elute RNA. The eluted RNA was quantified using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

A two-step reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) was performed for all the experiments described in this study. A 200 ng quantity of RNA was treated with DNase (1 U/µg) (Fisher Scientific, Pittsburgh, PA) in a 10 µl digestion reaction volume including a final 1X concentration of RQ1 RNase-Free DNase Reaction Buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) (Fisher Scientific, Pittsburgh, PA). Samples were incubated at 37ºC for 30 minutes followed by the addition of 1 µl of RQ1 DNase Stop Solution (20 mM EGTA, pH 8.0) (Fisher Scientific, Pittsburgh, PA) and samples incubated at 65ºC for 10 minutes to inactivate the DNase. The 11 µl volume of DNase-digested RNA was used for the RT-PCR reaction.

Production of cDNA from Total RNA

The 11 µl volume containing the RNA was combined with 2 µl random hexadeoxynucleotides (0.5 µg/ul) (Fisher Scientific, Pittsburgh, PA) and a 4 µl volume of dNTP mix (10 mM per dNTP) (New England Biolabs, Beverly, MA). Tubes were heated for 5 minutes at 70ºC to anneal primers to RNA, samples then placed on ice, and the following components added to the reaction tube to yield a 20 µl volume: 2 µl 10X RT Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, pH 8.3) (New England Biolabs, Beverly, MA), 10 U of RNase Inhibitor (Fisher Scientific, Pittsburgh, PA), and 25 U M-MuLV Reverse Transcriptase (New England Biolabs, Beverly, MA).

Reaction tubes were incubated at 42ºC for 1 hour to generate cDNA, and the RT enzyme heat inactivated at 95ºC for 5 minutes. A 0.5 µl volume containing 2.5 U of RNase H (New England Biolabs, Beverly, MA) was added to tubes and samples incubated at 37ºC for 20 minutes to degrade RNA. RNase H was then heat inactivated at 95ºC for 5 minutes. Each cDNA pool was diluted to 50 µl and stored at -20ºC until analyzed via real-time qPCR reaction.

Real-time Quantitative PCR

Real-time qPCR was performed using the MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). A 2 µl sample of each cDNA pool served as template for the qPCR reaction using IQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) and 300 nM of each primer: hTERT sense & antisense primers (5'-GGAGCAAGTTGCAAAGCATTG-3' & 5'-CCCACGACGTAGTCCATGTT-3') (27), B-actin sense & antisense primers (5'-CACTCTTCCAGCCTTCCTTCC-3'& 5'-CTGTGTTGGCGTACAGGTCT-3') (28). All qPCR reactions were performed in at least triplicate for each sample using 96-well plates. The following cycling program was used for all primer sets. Initial denaturation step at 95ºC for 3 minutes followed by 45 cycles at 94ºC- 30 sec., 59.5 ºC- 30 sec., 72ºC- 30 sec., and an 80ºC- 15 sec. data acquisition step. A melt curve was performed for every reaction plate. Fold changes in gene expression were determined using the $2^{-\Delta\Delta C_T}$ method (29). The C_T data were imported into Microsoft Excel. C_T values for hTERT and B-actin were averaged for each sample, and ΔC_T calculated ($\Delta C_T = C_{T,\text{ avg. hTERT}} - C_{T,\text{ avg. B-actin}}$). $\Delta\Delta C_T$ was calculated in the following manner: $\Delta\Delta C_T$ = Avg. $\Delta C_{T,\text{ hTERT from E2F-1 or E132 TFP treatment}}$ - $\Delta C_{T,\text{ hTERT from}}$

$_{\text{TatHA control}}$. Mean fold change in gene expression was determined by the following calculation: $2^{\wedge-\Delta\Delta CT}$.

Statistical Analysis

Statistical significance of mean fold changes in gene expression was determined by Student's paired t-test using Microsoft Excel and Analyze-it™ (Analyze-it Software, Ltd., vsn 1.71). Results were considered significant if two-tailed P values were <0.05.

RESULTS

We were able to successfully produce E2F-1/Tat fusion peptides utilizing both wildtype and mutant E2F-1 expression vectors to investigate their transcriptional effects on hTERT. Techniques were modified to produce both E2F-1/TatHA and E132/TatHA recombinant proteins (see Figure 4.1) and recombinant protein production was verified via western blotting. Protein transduction was determined in each cell line via immunocytochemistry, and the results supported effective transduction of E2F-1/TatHA, E132/TatHA and TatHA into all cell types. The TFPs were detected throughout the cell cytoplasm with perinuclear localization and detectable nuclear accumulation (Figure 4.2). The series of immunocytochemistry experiments ranging from 1 hour to 24 hours incubation with TFPs all revealed successful transduction of recombinant proteins into greater than 95% of carcinoma cells.

Our experiments were only performed with freshly dialyzed proteins to avoid protein precipitation and to ensure biological activity of proteins. The proteins were

added to cells for 24 hours to assess differences in hTERT expression between the wildtype E2F1/TatHA protein, mutant E2F-1 (E132/TatHA) and the TatHA control protein. Following RNA isolation and real-time RT-qPCR, results revealed significant repression of hTERT in both HCC1937 and HCC1599 breast cancer cells. In HCC1937 cells, hTERT was repressed 3.5-fold by E2F-1/TatHA in comparison to the TatHA control peptide ($p < 0.0024$) (Figure 4.3). The cells treated with E132/TatHA did not reveal effective repression of hTERT in comparison to TatHA ($p < 0.6484$). When comparing the wildtype E2F-1/TatHA protein to its mutated counterpart, E132/TatHA, statistically significant 3.5-fold repression of hTERT gene expression occurred in HCC1937 cells with E2F-1/TatHA protein treatment versus the E132/TatHA control protein ($p < 0.0012$) (Figure 4.4). Overall, there was no discernable effect on hTERT expression between the control E132/TatHA and control TatHA alone fusion proteins. In HCC1937 cells, E2F-1/TatHA greatly repressed hTERT in comparison to both E132/TatHA and TatHA control proteins.

In HCC1599 cells, a significant 3.3-fold repression of hTERT gene expression was observed with E2F-1/TatHA protein treatment versus the TatHA control protein ($p < 0.0001$) following 24 hours of treatment (Figure 4.3). Interestingly, a 4.0-fold repression of hTERT expression was observed with E2F-1/TatHA protein treatment of HCC1599 cells versus the E132/TatHA control protein ($p < 0.0001$) (Figure 4.4). A slightly increased expression of hTERT (1.15-fold increase) was observed in TatHA treated HCC1599 cells versus the E132/TatHA protein, but this difference was only marginally statistically significant ($p < 0.0102$) (Figure 4.3). Further testing will be

performed to assess differences between E132/TatHA and TatHA control proteins in larger in vitro test groups.

Minimal differences in hTERT expression were detected in HCC1937 and HCC1599 cells based on the BRCA1 status. As previously mentioned, the HCC1937 cell line has a homozygous BRCA1 mutation (5382C) in addition to mutations in p53, Her2/neu and ER/PR receptors. In contrast, the HCC1599 cell line expresses the wildtype BRCA1 tumor suppressor gene but also contains mutations in p53, Her2/neu and ER/PR receptors. A slightly higher repression of hTERT was observed in HCC1599 cells (4.0-fold) versus HCC1937 (3.5-fold) when E2F-1/TatHA was compared to the E132/TatHA control, but this slight difference in hTERT repression is most likely not attributed to the difference in BRCA1 status.

DISCUSSION

The results from this study suggest that E2F-1/TatHA is a moderately effective repressor of hTERT. In comparison to E132/TatHA and TatHA controls, hTERT was repressed by 3.3 to 4.0-fold in HCC1937 and HCC1599 breast carcinoma cells. The efficient transduction of the E2F-1/TatHA peptides into breast cancer cells combined with their transcriptional effects on hTERT expression suggests an alternate route to inhibit telomerase expression in breast cancer cells. The extent of hTERT repression in our study is comparable to the results observed by Crowe et al.'s study (2001) which suggests that our alternative route of introducing the E2F-1 transcription factor into cancer cells does not result in greater repression of hTERT than liposome-mediated

transfection of E2F-1(7). However, the introduction of biologically active E2F-1/TatHA proteins has potential benefits. Treatment with our wildtype and mutant TFPs results in reversible repression of hTERT which could serve as a modality to augment the effects of other chemotherapeutic or apoptotic agents. This particular study did not examine the direct effects on apoptosis, but continued treatment with E2F-1/TatHA proteins could serve to effectively repress hTERT leading to long-term telomerase repression.

To interpret the results of our hTERT repression studies, it is important to review the expected effects of E2F-1/TatHA treatment of cells versus E132/TatHA and control peptide TatHA. Crowe et al. determined the normal E2F-1 expression vector binds to two sites of the hTERT promoter and represses its transcription by approximately 4-fold in their study (7). The negative control, mutant E2F-1, referred to as E132/TatHA in our research, has a point mutation in the DNA-binding domain and cannot bind to the E2F-1 binding sites on the hTERT promoter and exhibited no repressive effects on hTERT (7). We expected to observe similar results in our study, and since Tat-mediated protein transduction is an effective method of delivering biologically active proteins into cells, we hoped to achieve greater than 4.0-fold repression of hTERT in our study. However, as demonstrated by our data following 24 hours of treatment with E2F-1/TatHA, only a maximum of 4-fold hTERT repression was observed in HCC1599 cells.

The characterization of E2F-1 as a transcriptional repressor is a controversial role for this factor because it has traditionally been denoted as a transactivator of various genes. Hsieh et al. (30) reported that transcriptional repression rather than the transactivation function of E2F-1 is involved in the induction of apoptosis. Zhang et al. (19) provided the first direct evidence that telomerase is required for maintaining the

vitality of both human tumor and immortal cells. Zhang et al. also reported that cell death in immortal cells was telomere-length dependent, and upon inhibiting telomerase in cells with short telomeres, the resulting damage to the chromosomes triggered apoptotic cell death (19). Interestingly, Yamasaki et al., (31) determined that E2F-1 null mutant mice developed a variety of malignant tumors, and Field et al. (32) indicated that E2F-1 functions in mice to promote apoptosis and suppress cell proliferation. Tumor formation in the absence of E2F-1 was also suggested by Crowe et al. (7). Their study utilized E2F-1 expression vectors and liposomal transfection into SCC25 cells resulting in transcriptional repression of hTERT via consensus binding in the hTERT- promoter at putative E2F-1 sites (7). Importantly, Crowe et al. reported that transfection of the E2F-1 constructs reduced endogenous hTERT mRNA levels by 4-fold, and the reduced hTERT mRNA levels were accompanied by decreasing telomerase activity (7). The study by Crowe et al. was one of the first to correlate the novel transcriptional repressive effects of E2F-1 on the hTERT gene promoter while monitoring subsequent telomerase activity (7). However, the repressive effects of E2F-1 on hTERT must be further examined in future studies. The liposome-mediated transfection of E2F-1 via the Lipofectamine™ kit (Invitrogen Life Technologies, Carlsbad, CA) as described in Crowe et al. does not guarantee transfection of 100% of the cancer cells (7), nor do any of the adenoviral-mediated studies previously mentioned (10-12). Therefore, to determine if E2F-1 can completely repress hTERT transcription, the E2F-1 protein must be effectively transduced into 100% of the cancer cells. Our research suggested greater than 95% transduction rates with E2F-1 TFPs, but maximum hTERT repression was only 4-fold with our alternative mode of transduction which is comparable to the results published by

Crowe et al (7). Complete repression of hTERT was not observed in our study using TFPs suggesting that a combination of transcriptional repressors may be required using Tat-mediated transduction to completely inhibit expression of hTERT.

The breast cancer cells lines utilized in our study represent non-metastatic primary ductal carcinoma cell lines. HCC1937 cells were derived from a TNM stage IIB, grade 3 breast tumor, and the HCC1599 cell line was started from a TNM stage IIIA, grade 3 primary ductal carcinoma (20). Our research suggests that E2F-1/TatHA is an effective repressor of hTERT in primary ductal carcinoma cells that are actively expressing telomerase. Although we have not fully investigated metastatic breast carcinoma cell lines, preliminary studies (results not shown) with MDA-MB-231 and MDA-MB-435S cells, both of which are derived from pleural effusions from metastatic breast cancer (TNM stage IV) (17), did not reveal statistically significant repression of hTERT. Therefore, the E2F-1/TatHA proteins investigated in this study would be potential adjuvants to therapy in nonmetastatic, primary breast tumors.

Collectively, these studies were undertaken to develop an effective technique to achieve complete transduction of cancer cells with fusion peptides. The hypothesized repression of telomerase activity in infiltrating ductal carcinoma cells via E2F-1 repressor proteins is supported by significant hTERT repression in both HCC1937 and HCC1599 cells. However, the ability to achieve complete repression of hTERT via the E2F-1 transcription factor still remains elusive. Future experiments will further investigate the transcriptional repressive effects of E2F-1 and its effects on cell-cycle progression and telomerase repression.

SDS-PAGE Gels of Recombinant E2F-1/TatHA and E132/TatHA Proteins

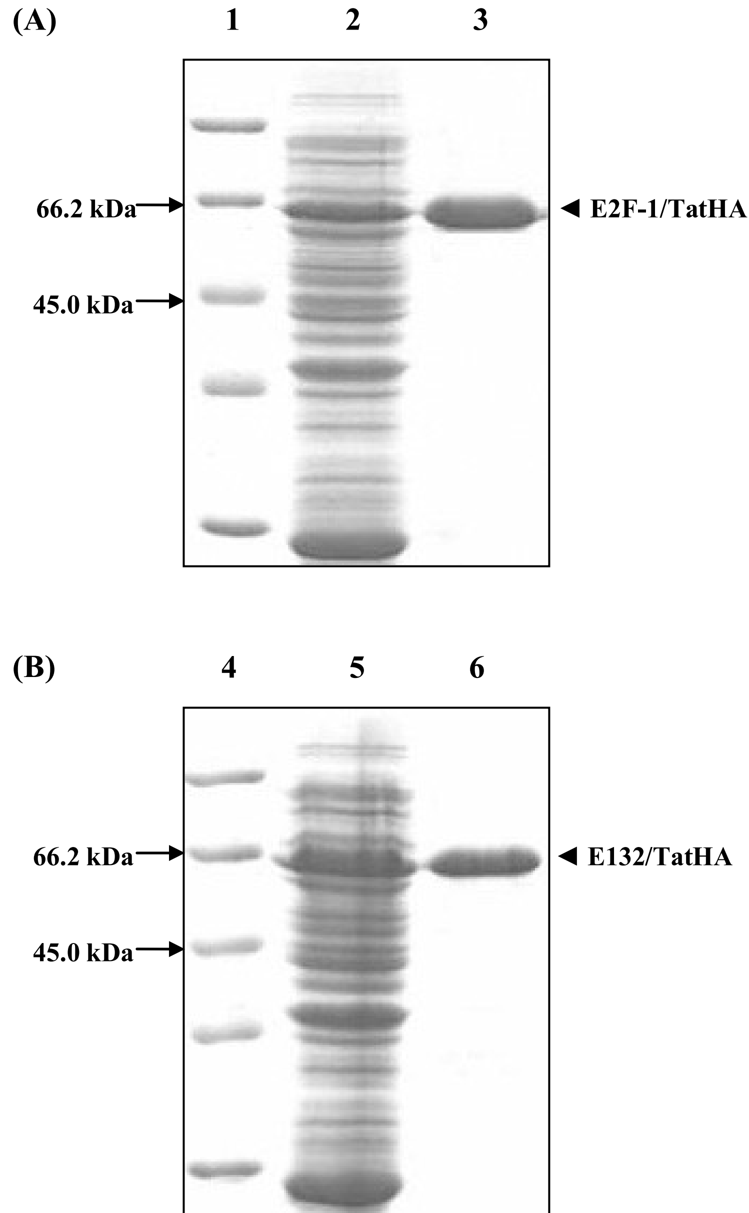

Figure 4.1. SDS-PAGE gels of recombinant E2F-1/TatHA and E132/TatHA proteins. (A) SDS-PAGE gel of E2F-1/TatHA purified protein. *Lane 1*, Protein molecular weight marker. *Lane 2*, E2F-1/TatHA expressing culture. *Lane 3*, FPLC purified E2F-1/TatHA protein (60 kDa). (B) SDS-PAGE gel of E132/TatHA purified protein. *Lane 4*, Protein molecular weight marker. *Lane 5*, E132/TatHA expressing culture. *Lane 6*, FPLC purified E132/TatHA protein (60 kDa).

Immunocytochemistry Images of HCC1937 Cells

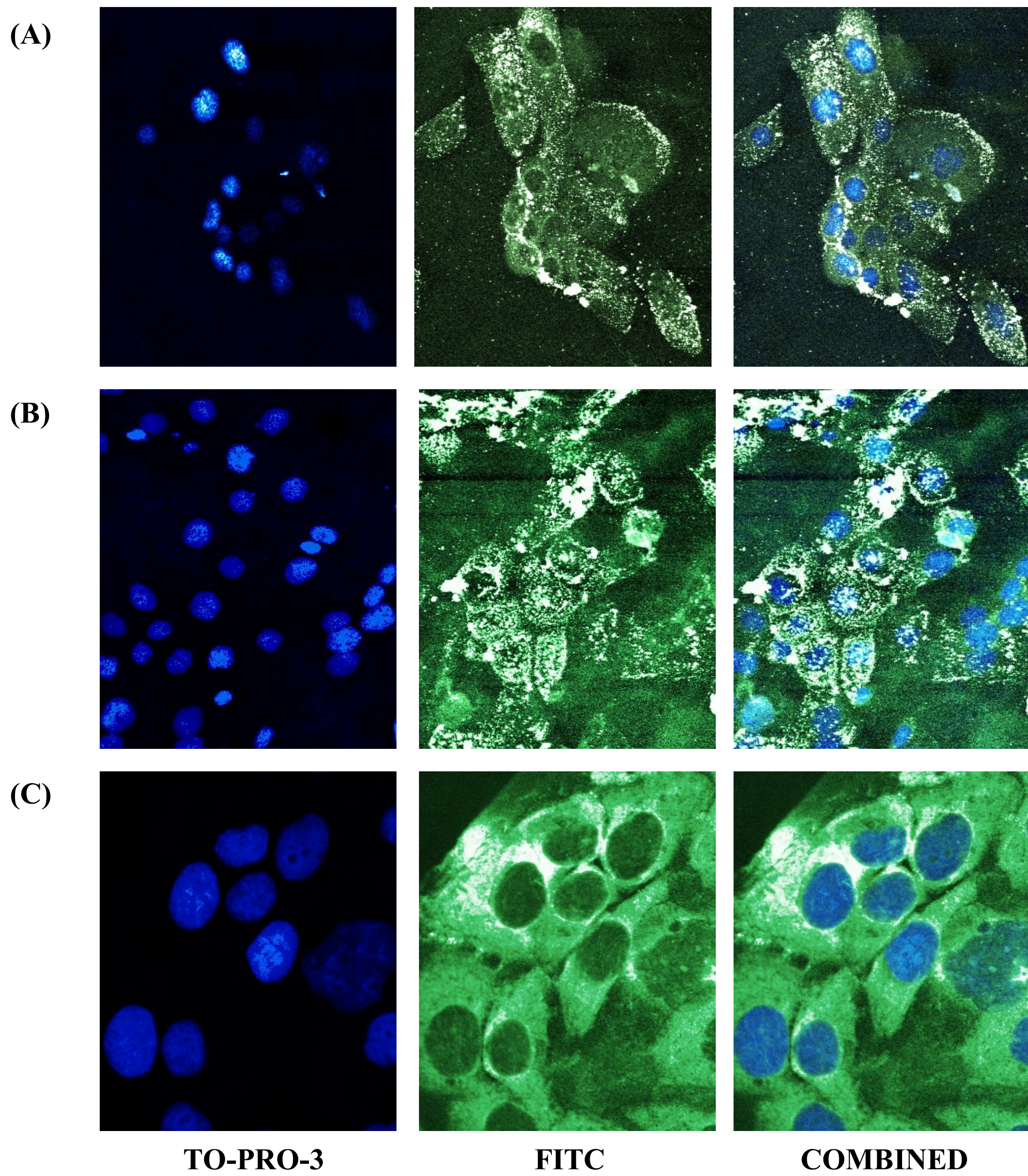

Figure 4.2. Immunocytochemistry images of HCC1937 cells transduced with Tat fusion proteins for 24 hours. (A) Cells treated with 2 μM E2F-1/TatHA. (B) Cells treated with 2 μM E132/TatHA. (C) Cells treated with 2 μM TatHA control peptide. Images were captured using the 40X objective on the Bio-Rad MRC 1024 Laser Scanning Confocal Microscope.

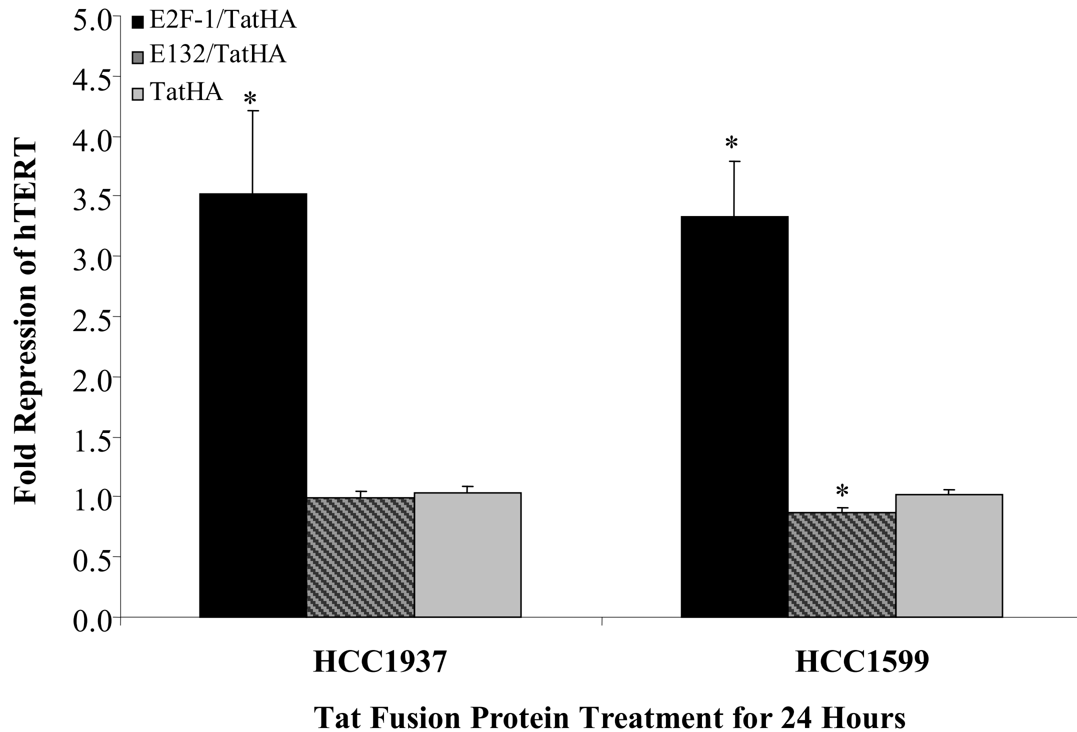

Figure 4.3. Fold changes in hTERT gene expression compared to TatHA control in breast cancer cell lines. HCC1937 and HCC1599 cells were treated for 24 hours with 2 μM Tat fusion proteins. RNA was isolated and RT-qPCR performed as described under Materials and Methods. The asterisks (*) indicate statistically significant fold changes in gene expression. A significant 3.5-fold repression of hTERT gene expression was observed in HCC1937 cells treated with E2F-1/TatHA proteins in comparison to treatment with TatHA control proteins ($p<0.0024$). Repression of hTERT in HCC1937 cells treated with E132/TatHA proteins versus the TatHA control proteins was not statistically significant ($p<0.6484$). A 3.3-fold repression of hTERT was observed in HCC1599 cells treated with E2F-1/TatHA proteins versus the TatHA control protein ($p<0.0001$). hTERT gene expression was slightly increased (1.15-fold increase) in HCC1599 cells treated with E132/TatHA proteins versus the TatHA control proteins ($p<0.0102$). The results are means +/- SEM of three separate experiments (HCC1937, n=17; HCC1599, n=18).

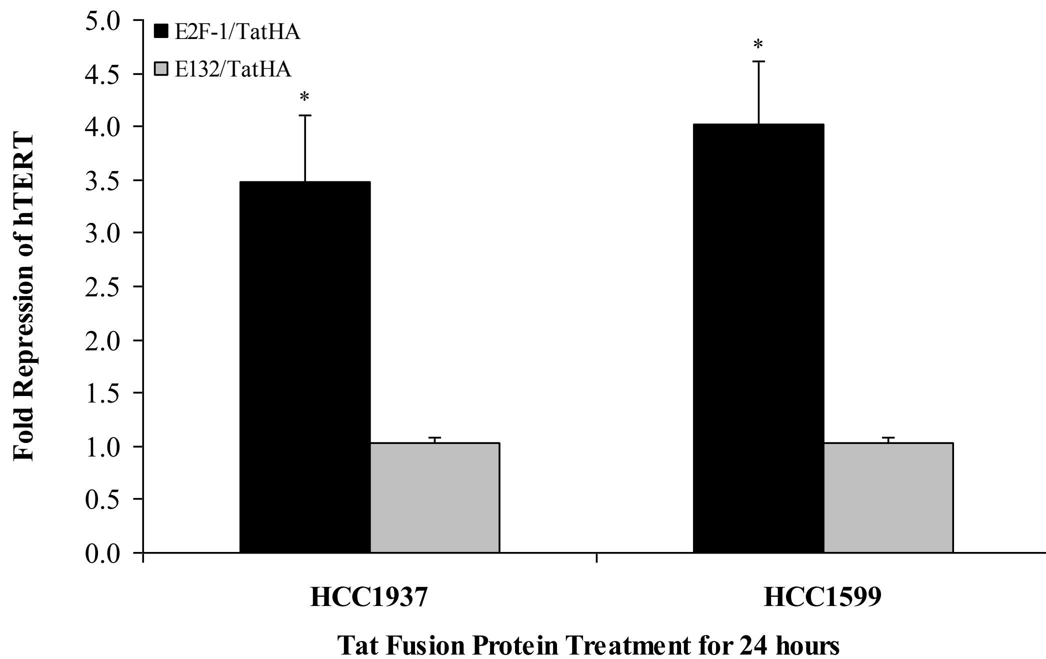

Figure 4.4. Fold changes in hTERT gene expression compared to E132/TatHA control in breast cancer cell lines. HCC1937 and HCC1599 cells were treated for 24 hours with 2 μM Tat fusion proteins. RNA was isolated and RT-qPCR performed as described under Materials and Methods. The asterisks (*) indicate statistically significant fold changes in gene expression. In HCC1599 cells, hTERT gene expression was repressed 4.0-fold by treatment with E2F-1/TatHA proteins in comparison to the E132/TatHA control proteins ($p<0.0001$). In HCC1937 cells, hTERT gene expression was repressed 3.5-fold by treatment with E2F-1/TatHA proteins versus the E132/TatHA control proteins ($p<0.0012$). The results are means +/- SEM of three separate experiments (HCC1937, n=17; HCC1599, n=18).

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CHAPTER V

TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS ACTIVATES THE p73 AND p21$^{WAF1/CIP1}$ TUMOR SUPPRESSOR PATHWAYS IN p53-MUTATED BREAST CARCINOMA CELLS

ABSTRACT

Mutations in the p53 tumor suppressor gene are present in 40-50% of breast carcinomas (1, 2). In cancer cells harboring a p53 mutation, overexpression of a p53 homologue (the p73 tumor suppressor) induces apoptosis (3, 4). Upregulation of p73 and subsequent cell death have been achieved via transfection of the E2F-1 transcription factor gene (5, 6). Here we report an alternative, reversible methodology to induce p73 expression in breast carcinoma cells utilizing protein transduction with E2F-1 Tat fusion proteins (TFPs) to target and kill breast cancer cells. We produced recombinant E2F-1/TatHA fusion proteins which transduced greater than 95% of treated cells. Biological activity of the E2F-1 TFP was determined by monitoring induction of p73 expression using real-time RT-qPCR, and apoptosis was assessed by microscopic examination and via TUNEL assay. Two p53 deficient breast cancer cell lines, HCC1937 and HCC1599, were exposed to 2 μM E2F-1 TFP or control TatHA protein for 24 hours. Greater than 2-fold induction of p73 was observed in E2F-1 TFP treated cells as compared to TatHA

treated controls. A significant increase in the p53-responsive gene, p21$^{WAF1/CIP1}$, was also observed in cell lines via real-time qPCR. Following E2F-1 TFP treatment for 24 hours, apoptotic activity was evident in the breast cancer cells. In conclusion, the E2F-1/TatHA protein effectively transduced HCC1599 and HCC1937 cells, activated transcription of p73 and induced apoptosis. This study supports the hypothesis that transduced E2F-1/TatHA fusion protein activates the p73 and p21$^{WAF1/CIP1}$ tumor suppressor pathways in p53-mutated breast carcinoma cell lines.

INTRODUCTION

The majority of neoplastic cells exhibit aberrations in cell cycle control which can lead to increased cell-cycle progression. Common mutations that lead to diminished cell-cycle control include alterations in the p53 tumor suppressor gene. Approximately 50% of cancers, including breast cancer, harbor mutations of the p53 tumor suppressor gene (1); however, a p53-homologue, p73, is rarely mutated in carcinoma cells (7). The transcriptionally active form of p73 (commonly referred to as TA-p73) was investigated by our research group. Various isoforms of the transcriptionally active p73 tumor suppressor exist and have the ability to activate p53-responsive genes. Isoforms such as p73α and p73β contain N-terminal transactivation domains responsible for activation of various p53 target genes associated with apoptosis and cell cycle control (8, 9).

An important regulator of p73 expression, the E2F-1 transcription factor, has been found to activate p73 expression in select studies. Activation of the p73 tumor suppressor pathway has been observed in adenoviral and retroviral studies transfecting

the E2F-1 gene into carcinoma cells (5, 6). Additionally, upregulation of p53-responsive genes has been observed in cells upon activation of p73 (10, 11). Specifically, stimulation of the p73 tumor suppressor gene has induced p21$^{WAF1/CIP1}$ expression leading to cell growth arrest and apoptosis (11, 12).

Our research used an alternative method of introducing the E2F-1 transcription factor into breast carcinoma cells by employing Tat-mediated protein transduction. Proteins attached to the HIV-1 Tat transduction domain have been found to transduce 100% of target cells (13-17). Once Tat fusion proteins enter cells, they have the opportunity to elicit their biological effect followed by degradation, resulting in the reversible therapeutic application of the E2F-1 transcription factor. Our production of E2F-1/TatHA fusion peptides has permitted an alternative route of assessing p73 activation and apoptosis in p53-mutated primary ductal breast cancer cells. Upon verification that E2F-1 TFPs transduced HCC1937 and HCC1599 cells, we determined fold changes in gene expression of p73 via real-time RT-qPCR.

Two different E2F-1 TFPs were utilized in this study: E2F-1/TatHA (wildtype E2F-1) and E132/TatHA (E2F-1 with a DNA binding domain mutation) (see Figure 5.1). Previous studies revealed that only one of the six domains within the E2F-1 protein is critical to bind the promoter of p73 and activate transcription (5). Hallstrom and Nevins (2003) reported that the "marked-box domain" was crucial to increasing expression of p73 (5); therefore, we determined if the E132/TatHA protein (intact marked-box domain; mutant DNA binding domain) elicited the same apoptotic effect as wildtype E2F-1/TatHA. In addition, we determined if treatment with E2F-1/TatHA and E132/TatHA proteins resulted in activation of the p73-target gene p21$^{WAF1/CIP1}$ in comparison to a

control TatHA protein. We also monitored carcinoma cell lines for increases in $p21^{WAF1/CIP1}$ expression in response to TFP treatment to assess if p53-responsive genes were upregulated via overexpression of p73.

The $p21^{WAF1/CIP1}$ gene functions as an inhibitor of cyclin-dependent kinases and is directly induced by the p53 tumor suppressor protein and its homologues. The $p21^{WAF1/CIP1}$ gene is an uncommon target for mutational inactivation in breast cancer (18); however, in the absence of p53, its innate role in controlling the cell cycle may be compromised. Additionally, the level of $p21^{WAF1/CIP1}$ gene expression in cells is reportedly affected by the BRCA1 tumor suppressor protein (19). Overexpression of BRCA1 in ovarian and breast cancer cell lines has been found to reduce cellular proliferation (20). BRCA1 may also function to regulate the $p21^{WAF1/CIP1}$ promoter and serve as an essential factor required for adequate $p21^{WAF1/CIP1}$ activation. BRCA1 has a direct role in cell cycle checkpoints and inhibits cell cycle progression into S-phase which was observed in studies that overexpressed the BRCA1 protein revealing subsequent activation of $p21^{WAF1/CIP1}$ in a p53-independent manner (19). Additionally, in cell lines lacking $p21^{WAF1/CIP1}$, overexpression of BRCA1 did not lead to G1 arrest, thus supporting the concept that the arrest of the cell cycle in G1 phase is dependent on the expression of $p21^{WAF1/CIP1}$ (19). The induction of $p21^{WAF1/CIP1}$ via p73 overexpression will be monitored by real-time RT-qPCR in our experiments to distinguish possible differences in BRCA1 status on $p21^{WAF1/CIP1}$ expression and subsequent apoptotic activity.

Our research examines the repressive effects of E2F-1/TatHA, E132/TatHA and TatHA treatment on p53-mutated primary infiltrating ductal carcinoma cell lines

HCC1937 and HCC1599. The HCC1937 cell line contains a homozygous BRCA1 mutation (5382C) while the HCC1599 cell line expresses the wildtype BRCA1 gene (21, 22). We propose that decreased expression of BRCA1 in the HCC1937 cell line may impact the ability of p73 overexpression to activate $p21^{WAF1/CIP1}$. Overall, we expect to observe increased fold induction of p73 in all cell lines tested with possible variations in $p21^{WAF1/CIP1}$ expression based on BRCA1 status of tumor cells. Additionally, several other metastatic breast cancer cell lines were studied on a preliminary basis to determine if E2F-1/TatHA and E132/TatHA TFP treatments impact advanced stage breast cancers.

MATERIALS AND METHODS

Vector DNA

The vectors pCMV E2F-1 and pCMV E132 were obtained from Dr. Karen Vousden (Beatson Cancer Institute, UK) and the pTatHA vector was a kind gift from Dr. Steven Dowdy (University of Washington, St. Louis, MO).

E2F-1/TatHA and E132/TatHA Constructs

The E2F-1 gene was directly cloned into the pTatHA vector via NcoI and EcoRI restriction enzyme sites. Purified digested pCMV E2F-1 DNA was ligated into digested pTatHA using T4 DNA Ligase (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The E132 vector contained an artificial EcoRI site in the DNA binding domain and could not be directly inserted into pTatHA's multiple cloning site (MCS). Therefore, the E132 gene was modified using 5' site-directed mutagenesis

via PCR from plasmid pCMV-E132 using sense primer: 5'-gcgcgcaaccATGGCCTTGGCCGGG-3'and antisense primer: 5'-gcgcagcatgcGGATCCAGCCCTGTC-3' (Sigma Genosys, Woodlands, TX) to generate artificial NcoI and SphI sites (underlined in primer sequences). The PCR reaction included 2 Units Vent Taq polymerase (New England Biolabs, Beverly, MA), 1X Thermapol buffer (New England Biolabs, Beverly, MA), 500 nM of sense and antisense primers and 5% DMSO. The PCR product was amplified under the following conditions in a Perkin Elmer 9600 Thermocycler: initial denaturation: 94ºC- 3 minutes, 55ºC- 1 minute, 72ºC- 1 minute; 23 cycles: denaturation: 94ºC- 1 minute, annealing: 55ºC- 1 minute, extension: 72ºC- 1 minute, and final extension of 72ºC for 7 minutes. PCR products were analyzed on 1% agarose gels and stained with ethidium bromide (0.5 μg/ml) to confirm generation of 1.36 kb amplified product. The pTatHA DNA was digested with restriction enzymes NcoI and SphI and purified. E132 PCR product was digested with NcoI-SphI and subcloned into the NcoI-SphI sites of pTatHA to yield E132/TatHA using a Klenow-Kinase-Ligase (KKL) protocol (23). Ligated constructs containing E132/TatHA were transformed into E. coli Top10F' competent cells (Invitrogen Life Technologies, Carlsbad, CA) and correct gene insertion verified by DNA sequencing of plasmid DNA.

Small-Scale Recombinant Protein Production

E2F-1/TatHA and E132/TatHA plasmids were transformed into BLR(DE3)pLysS competent cells (Novagen, Madison, WI) and recombinant proteins produced via inoculating 2 ml 2YT broth with single transformed colonies. After incubation in

shaking water bath (225 rpm) for 12-14 hours, 3 ml of fresh 2YT broth, 0.5 μg/ml ampicillin, and 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) were added to cultures. Bacterial cultures were incubated an additional 6 hours to produce recombinant proteins. Cultures were centrifuged at 6000 x g for 10 minutes and cell pellets resuspended in 100 μl of 20 mM Tris buffer (pH 8.0) followed by quantitation using a BCA™ Protein Assay Kit (Pierce Biotechnologies, Inc., Rockford, IL) with BSA standards. 2X Laemmli Sample Buffer (24) was added to 50 μg of total cellular protein and samples were boiled for 3-5 minutes and immediately placed on ice. Samples were then loaded onto 12% SDS-PAGE gels and electrophoresis performed using a Mini-PROTEAN® 3 (Bio-Rad Laboratories, Hercules, CA) protein electrophoresis unit with the Laemmli buffer system. Prestained 1 kb protein ladders (Fermentas Life Technologies, Hanover, MD) were also ran on each gel to determine protein size.

Western Blotting

Duplicate gels were electrophoresed simultaneously- one gel for western blotting and a second gel for Coomassie blue staining. Gel designated for western blotting was transferred to nylon membranes using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions using 1X TBS-T transfer buffer. Recombinant protein production was verified using primary mouse monoclonal anti-HA antibodies (1:1,000 dilution) (CRP Inc., Denver, PA) and secondary HRP-labeled goat anti-mouse antibodies (1:25,000 dilution) (KPL Inc., Gaithersburg, MD). Membranes were blocked with TBS-T/5% milk solution for 1 hour, incubated with primary anti-HA antibody for 1 hour, washed 3 times

for 10 minutes each, and incubated with secondary HRP antibody for 1 hour. Following additional washings (3 x 10 minutes), western blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to manufacturer's instructions. Blots were exposed to X-ray film and developed with KODAK Developer (30 sec) and KODAK Fixer (30 sec). Immunoblots were also visualized using a KODAK Image Station 2000R (KODAK, Rochester, NY) according to manufacturer's instructions.

Large-Scale Recombinant Protein Production

Recombinant E2F-1/TatHA and E132/TatHA proteins were purified under denaturing conditions with affinity chromatography using a modified procedure from Amersham Pharmacia (25). Single colonies from protein expressing cultures inoculated 200 ml of 2YT broth supplemented with ampicillin (50 µg/ml) and were incubated at 37ºC while shaking at 225 rpm for 16-18 hours. An additional 300 ml of broth was added along with fresh ampicillin and IPTG (400 µM), and cultures incubated an additional 6 hours. Cultures were centrifuged for 10 minutes at 6000 x g, and the bacterial pellets frozen at -20ºC. Cell pellets were thawed for 20 minutes at 37ºC, and 25 ml of Buffer 1 (20 mM Tris^{TM}-HCl, pH 8.0) added to resuspend the cells with vigorous pipetting. Cells were sonicated on ice (3 x 15 sec) and centrifuged (10 min x 6000 g). Cell pellets containing the inclusion bodies were then resuspended in 25 ml Buffer 2 (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton^{TM} X-100, pH 8.0). Samples were again sonicated on ice and centrifuged as described above. Pellets were resuspended in 25 ml Buffer 2 to wash the inclusion bodies and recentrifuged. A final wash of 25 ml of

Buffer 1 was then performed, and following centrifugation, pellets were either used immediately or frozen at -20ºC for a maximum of two weeks prior to use.

Preparation of Inclusion Bodies for FPLC

Cell pellets were resuspended in 5-10 ml Buffer A1 (6 M guanidine-HCl, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) by stirring at room temperature for 60 minutes to solubilize recombinant proteins. Samples were centrifuged at 20,000 x g for 15 minutes to pellet residual cell debris. Protein supernatant containing denatured, soluble proteins was removed from debris and further clarified by passage through a 0.45 μM filter (Millipore, Bellerica, IL) to remove impurities.

Isolation of Recombinant Proteins Using Affinity Chromatography

Using an AKTA™ FPLC™, Ni^{++} charged HisTrap™ columns (Amersham Pharmacia, Piscataway, NJ) were equilibrated with 5 ml Buffer A1 and the proteins loaded at a flow rate of 0.5 ml/minute. Columns were washed with an additional 10 ml of Buffer A1 followed by a wash of 10 ml of Buffer A2 (6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A linear gradient from 6 M to 0 M urea was then performed to remove the denaturant and start refolding the proteins on the HisTrap™ column by gradually replacing Buffer A2 with Buffer B1 (0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0). A total volume of 40 ml of Buffer B1 was used to perform the gradient wash. The purified, refolded proteins were then eluted with Buffer B2 (0.5 M NaCl, 20 mM Tris-HCl, 500 mM imidazole, 5 mM β-mercaptoethanol, pH 8.0) using a gradient of 0 mM to 500 mM

imidazole in the buffer. Fractions containing the purified protein were analyzed by SDS-PAGE gel electrophoresis and quantified with the BCA™ Protein Assay Kit (Pierce Biotechnologies).

Dialysis of Recombinant Proteins into Cell Culture Media

E2F-1/TatHA and E132/TatHA recombinant proteins were diluted with FPLC elution buffer B2 to 0.5 mg/ml to avoid protein precipitation cascades. Proteins were injected into 12 ml Slide-A-Lyzer® Dialysis Cassettes (Pierce Biotechnology, Rockford, IL) for dialysis into PBS followed by overnight dialysis into cell culture media per manufacturer's protocol. Proteins were freshly dialyzed for each set of experiments and never frozen. Following dialysis, proteins were centrifuged, sterile filtered with 0.2 µM filters (Millipore, Bellerica, MA) and the final concentrations of recombinant proteins determined using the Coomassie Plus™ Bradford Assay (Pierce Biotechnology, Rockford, IL) and SDS-PAGE gels using BSA protein standards. Recombinant proteins in media were supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and 50 IU/ml penicillin/ 0.05 mg/ml streptomycin.

Synthesis of TatHA Control Protein

The TatHA control protein was artificially synthesized (amino acid sequence: YPYDVPDYAYGRKKRRQRRR) (26) on a Ranin Symphony/Multiplex Peptide Synthesizer (Center for Integrated BioSystems, Logan, UT). The TatHA peptide was reconstituted using Cellgro™ RNase/DNase/Protease free dH_20 for cell culture

(Mediatech Inc., Herndon, VA) and stored at -70ºC at a working concentration of 1 mg/ml (27).

Primary Carcinoma Cell Culture

Primary infiltrating ductal breast carcinoma cell lines HCC1937 (ATCC number: CRL-2336) and HCC1599 (ATCC number: CRL-2331) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI-1640 medium supplemented with 10% FBS, 50 IU/ml penicillin/ 0.05 mg/ml streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Cells were grown in a 37ºC humidified incubator with 5% CO_2. Additional breast carcinoma cell lines were also utilized in our research: MDA-MB-231 (ATCC number: HTB-26), MDA-MB-435S (ATCC number: HTB-129) and MCF-7 cells (ATCC number: HTB-22). MDA-MB-231 and MDA-MB-435S cell lines were both grown in a 1:1 ratio of DMEM:Ham's F12 media supplemented with 10% FBS and 5 IU/ml penicillin/ 0.05mg/ml streptomycin. MCF-7 cells were grown in MEME cell culture media supplemented with 10% FBS, 50 IU/ml penicillin/ 0.05 mg/ml streptomycin, 10 mM HEPES and 1 mM sodium pyruvate. Cell lines were grown in 25 cc^2 and 75 cc^2 tissue culture flasks and passaged every 2-4 days when 70-80% confluent. Cells were detached from flasks using 0.25% trypsin, 0.03% EDTA solution according to standard cell culture protocols (28).

Immunocytochemisty and TUNEL Assay

Proteins were tested at various µM concentrations to determine optimal transduction at different time points using immunocytochemistry to monitor cellular

uptake of E2F-1/TatHA, E132/TatHA and TatHA proteins. For final experiments, HCC1937 and HCC1599 cells were exposed to 2 μM E2F-1/TatHA, E132/TATHA and TatHA fusion peptides for the following time points: 1, 6, 12 and 24 hours. Cells were washed 3 times with 1X PBS, fixed for 30 minutes in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in 1X PBS for 30 minutes. Cells were blocked for 30 minutes with 1% FBS and 0.1% Tween-20 in 1X PBS. Cells were incubated for 60 minutes with primary mouse HA antibodies (1:1000 dilution in 1X PBS), washed 3 times for 10 minutes each, and incubated for 60 minutes with a secondary FITC-labeled goat anti-mouse antibody (1:250 dilution in 1X PBS) (KPL, Inc., Gaithersburg, MD). For immunocytochemistry experiments, cells were washed, and incubated for 15 minutes with 1 μM TO-PRO-3 iodide (Molecular Probes, Eugene, OR) to counterstain the cell nuclei. To perform the TUNEL assay, we used a TMR red In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Cells were fixed, permeabilized, and incubated with antibodies as described above to label recombinant proteins. Following the kit's instructions, samples were then incubated with TUNEL reagents to label terminal DNA strand breaks. Cells from immunocytochemistry and TUNEL experiments were mounted on slides using the Prolong® Antifade Kit (Molecular Probes, Eugene, OR) and images captured with Bio-Rad MRC 1024 Laser Scanning Confocal Microscope using the 40X objective.

Cell Culture Experiments with Recombinant Proteins

HCC1937 and HCC1599 cells were seeded at a density of 2×10^5 cells/well in CellStar® 6-well tissue culture plates. HCC1937 (adherent) cells were allowed 24 hours

to adhere to plates before recombinant proteins were added. HCC1599 (suspension) cells were seeded directly into wells with TFPs. For preliminary studies, MDA-MB-231, MDA-MB-435S and MCF-7 cells were allowed to adhere to plates for 24 hours prior to experiments with TFPs. Following the specific treatment times of 6, 12 or 24 hours, RNA was isolated and analyzed via RT-qPCR as described below.

RNA Isolation, Reverse Transcription, and qPCR

Total RNA was isolated from cells using the Mini RNA Isolation II™ kit (Zymo Research, Orange, CA). After treatment with TFPs, adherent cell lines were washed at least three times with 1X PBS and detached from tissue culture plates using 0.5 ml HyQ®tase™ (Hyclone, Logan, UT). RNA isolation was performed according to the manufacturer's protocol. The eluted RNA was quantified using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and stored at -80ºC. We used two-step reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) for all the experiments described in this study. A 200 ng quantity of RNA was treated with DNase (1 U/µg) (Fisher Scientific, Pittsburgh, PA) in a 10 µl digestion reaction volume including a final 1X concentration of RQ1 RNase-Free DNase Reaction Buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) (Fisher Scientific, Pittsburgh, PA). Samples were incubated at 37ºC for 30 minutes, 1 µl of RQ1 DNase Stop Solution (20 mM EGTA, pH 8.0) (Fisher Scientific, Pittsburgh, PA) was added to the reactions and samples incubated at 65ºC for 10 minutes to inactivate the DNase. The 11 µl volume of DNase-digested RNA was used for the RT-PCR reaction.

Production of cDNA from Total RNA via RT-PCR

The 11 µl volume containing the RNA was combined with 2 µl random hexadeoxynucleotides (0.5 µg/ul) (Fisher Scientific, Pittsburgh, PA) and a 4 µl volume of dNTP mix (10 mM per dNTP) (New England Biolabs, Beverly, MA). Tubes were heated for 5 minutes at 70ºC to anneal primers to RNA, samples then placed on ice, and the following components added to the reaction tube to yield a 20 µl volume: 2 µl 10X RT Buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, pH 8.3) (New England Biolabs, Beverly, MA), 10 U of RNase Inhibitor (Fisher Scientific, Pittsburgh, PA), and 25 U M-MuLV Reverse Transcriptase (New England Biolabs, Beverly, MA). Reaction tubes were incubated at 42ºC for 1 hour to generate cDNA, and the RT enzyme heat inactivated at 95ºC for 5 minutes. A 0.5 µl volume containing 2.5 U of RNase H (New England Biolabs, Beverly, MA) was added to tubes and samples incubated at 37ºC for 20 minutes to degrade RNA. RNase H was then heat inactivated at 95ºC for 5 minutes. Each cDNA pool was diluted to 50 µl and stored at -20ºC until analyzed via real-time qPCR reaction.

Real-time qPCR Using SYBR® Green

Real-time qPCR was performed using the MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). A 2 µl sample of each cDNA pool served as template for the qPCR reaction using IQ™ SYBR® Green Supermix (Bio-Rad Laboratories) and 300 nM of each primer. Primers were obtained from Fisher Scientific (Pittsburgh, PA) and sequences are as follows: p73 sense primer 5'-GGATTCCAGCATGGACGTCTT-3' (29), p73 antisense primer 5'-

GCGCGGCTGCTCATCT-3’ (29), p21 sense primer 5’-GATTAGCAGCGGAACAAGGA-3’, p21 antisense primer 5’-CAACTACTCCCAGCCCCATA-3’, β-actin sense 5’-CACTCTTCCAGCCTTCCTTCC-3’ (30) and β-actin antisense primer 5’-CTGTGTTGGCGTACAGGTCT-3’ (30). All qPCR reactions were performed in at least triplicate for each sample using a 96-well real-time plate. The following cycling program was used for all primer sets. Initial denaturation step at 95ºC for 3 minutes followed by 45 cycles using with following parameters: 94ºC- 30 seconds (denaturation), 59.5 ºC- 30 seconds (annealing step), 72ºC-30 seconds (extension), and 80ºC- 15 seconds (data acquisition step). A melt curve was performed for every reaction plate. Fold changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method (31). Following analysis with real-time quantitative PCR, the C_T data were imported into Microsoft Excel. C_T values for p73, p21, and β-actin were averaged for each sample, and ΔC_T calculated ($\Delta C_T = C_{T,\text{ avg. p73, p21}} - C_{T,\text{ avg. }\beta\text{-actin}}$). $\Delta\Delta C_T$ was calculated in the following manner: $\Delta\Delta C_T$ = Avg. $\Delta C_{T,\text{ p73/p21 from E2F-1 or E132 TFP treatment}}$ - $\Delta C_{T,\text{ p73/p21 from TatHA control}}$. Mean fold change in gene expression was determined by the following calculation: $2^{\wedge -\Delta\Delta CT}$.

Statistical Analysis

Statistical significance of mean fold changes in gene expression was determined by Student’s paired t-test using Microsoft Excel and Analyze-it™ (Analyze-it Software, Ltd., vsn 1.71). Results were considered significant if two-tailed P values were <0.05.

RESULTS

Our lab successfully produced and purified biologically active E2F-1 Tat fusion proteins with the ability to transduce all breast cancer cell lines that underwent testing (transduction rates >95%). Figure 5.2 represents a western blot verifying the hemagglutinin-tag in the 60 kDa recombinant E2F-1/TatHA and E132/TatHA proteins from bacterial culture isolates. Following purification of the recombinant proteins, transduction experiments using immunocytochemistry in TFP treated HCC1937 and HCC1599 cells revealed significant perinuclear and cytoplasmic accumulation of the recombinant proteins with trace to moderate nuclear and nucleolar accumulation of proteins (data not shown). HCC1599, MCF-7, MDA-MB-231 and MDA-MB-435S all exhibited effective transduction of the TFPs (data not shown).

Importantly, our experiments were only performed with freshly dialyzed recombinant E2F-1/TatHA and E132/TatHA proteins to avoid the addition of glycerol required for cryopreservation and to minimize protein precipitation. The E2F-1 Tat fusion proteins were highly sensitive to precipitation during purification, dialysis and following attempted storage at -20ºC and -80ºC. E2F-1/TatHA and E132/TatHA recombinant proteins were especially vulnerable to pH changes during FPLC and temperature changes during dialysis. If a precipitation cascade was initiated, the proteins were not utilized in experiments due to diminished biological activity (data not shown). Following the protein purification procedure describe in Materials and Methods, the TFPs that maintained biological activity throughout the purification and dialysis process were able to impact gene expression in the p53-mutated primary ductal breast cancer cell lines.

The effects of 2 μM E2F-1/TatHA, E132/TatHA and TatHA on p73 gene expression were examined in HCC1599 cells at several different time points: 6, 12 and 24 hours. Figure 5.3 demonstrates the gradual increase of p73 gene expression following both E2F-1/TatHA and E132/TatHA treatments as compared to the TatHA peptide control. After six hours of TFP treatment, p73 expression was increased by 1.3-fold in E2F-1/TatHA treated cells ($p<0.0001$, $n=16$) and 1.4-fold in E132/TatHA treated cells ($p<0.0001$, $n=16$) in comparison to the TatHA control peptide. Treatment with TFPs for 12 hours resulted in a 2.2-fold increase of p73 gene expression with E2F-1/TatHA treated cells ($p<0.0001$, $n=17$) and a 2.2-fold increase in E132/TatHA treated cells ($p<0.0001$, $n=17$) in comparison to the TatHA control peptide. Following TFP treatment, the greatest fold increase in p73 gene expression was observed following 24 hours of TFP exposure. Treatment with E2F-1/TatHA ($p<0.0001$, $n=18$) for 24 hours resulted in 2.7-fold increase in p73 expression and E132/TatHA treatment ($p<0.0001$, $n=18$) resulted in a similar 2.2-fold increase of p73. For all three time points, no significant difference in gene expression was detected after 6 and 12 hours of treatment with E2F-1/TatHA versus E132/TatHA (6 hours: $p<0.4212$, $n=16$; 12 hours: $p<0.9577$, $n=17$). However, a small statistically significant difference was observed after 24 hours of treatment with E2F-1/TatHA versus E132/TatHA ($p<0.0178$, $n=17$).

In HCC1937 cells, treatment with 2 μM TFPs for 24 hours resulted in a 2.0-fold increase in p73 gene expression with exposure to both E2F-1/TatHA ($p<0.0001$, $n=17$) and E132/TatHA ($p<0.0001$, $n=17$) (see Figure 5.4). Again, no significant differences were observed between E2F-1/TatHA and E132/TatHA treatment ($p<0.8520$, $n=17$). In preliminary experiments with MDA-MB-231, MDA-MB-435S and MCF-7 cells,

treatment with TFPs for 24 hours resulted in no significant increases in p73 gene expression (results not shown).

Following 24 hours of TFP treatment, total RNA from cells was analyzed for changes in $p21^{WAF1/CIP1}$ expression associated with alterations in p73 gene expression. In HCC1599 cells, $p21^{WAF1/CIP1}$ gene expression increased by 3.3-fold with E2F-1/TatHA treatment ($p<0.0001$, $n=18$) and by 3.4-fold with E132/TatHA treatment ($p<0.0001$, $n=17$) in comparison to the TatHA control peptide (refer to Figure 5.5). No significant difference was observed between E2F-1/TatHA treatment versus E132/TatHA treatment ($p<0.7829$, $n=17$). In the HCC1937 cell line, a lesser degree of increased $p21^{WAF1/CIP1}$ gene expression was observed (Figure 5.5). Following 24 hours of treatment with TFPs, a small 1.4-fold increase in $p21^{WAF1/CIP1}$ was detected in both E2F-1/TatHA ($p<0.0046$, $n=16$) and E132/TatHA treated cells ($p<0.0223$, $n=16$). An obvious difference in $p21^{WAF1/CIP1}$ gene expression was appreciated in the wildtype BRCA1 cell line HCC1599 versus the BRCA1 mutated cell line HCC1937. These results support the diminished ability of increased levels of p73 to activate $p21^{WAF1/CIP1}$ expression in breast cancers with BRCA1 mutations.

Both HCC1599 and HCC1937 cells were examined for apoptotic activity following 24 hours of treatment with TFPs. Initially, flow cytometric analysis was attempted with TUNEL assays to quantitate apoptotic activity; however, both cells lines were not compatible with FACS analysis due to severe cell clumping regardless of troubleshooting techniques and cell dissociation agent utilized. In the adherent cell line, HCC1937, cells were examined using fluorescent microscopy to quantitate apoptotic activity following TFP treatment (refer to Materials and Methods). Figure 5.6 illustrates

the apoptotic activity observed in HCC1937 cells following treatment with TFPs. Approximately 11.7% of cells were TUNEL-positive with E2F-1/TatHA treatment ($p<0.0001$, $n=9$) and 10.5% of cells TUNEL-positive with E132/TatHA treatment ($p<0.0001$, $n=9$). Treatment with the TatHA control peptide only revealed 0.46% of TUNEL-positive cells. Therefore, following treatment with TFPs, significant apoptotic activity was detected in both E2F-1/TatHA and E132/TatHA treated breast cancer cells. Greater statistically significant apoptotic activity was appreciated with E2F-1/TatHA versus E132/TatHA treatment ($p<0.0067$, $n=9$) which could be associated with the greater expression of p73 in cells treated with E2F-1/TatHA for 24 hours.

Figure 5.7 demonstrates apoptotic activity in HCC1937 and HCC1599 cells using immunocytochemistry combined with the TUNEL-assay. Transduction of the different TFPs is represented by the fluorescein-labeled proteins that exhibit cytoplasmic, perinuclear and nucleolar accumulation in both cell lines. Texas Red fluorescing nuclei representing apoptotic activity were visualized in both cells lines. Although the apoptotic activity in HCC1937 cells could be quantitated, in the HCC1599 cells, fluorescent microscopy was only used to qualitatively assess apoptotic activity (see Figure 5.7). Due to irreversible cell clumping, the number of cells undergoing apoptosis could not be accurately quantified microscopically. It is speculated that in comparison to the level of apoptosis observed in HCC1937 cells, a comparable number of HCC1599 cells underwent apoptosis suggesting about 10% of E2F-1/TatHA and E132/TatHA treated cells exhibited apoptotic activity following 24 hours of treatment.

DISCUSSION

Approximately 80% of breast cancers are primary infiltrating ductal carcinomas; therefore, it is important to examine the biological effects of E2F-1/TatHA on this stage of tumor development. Our research investigated an alternate route to modulate cancer cell growth in anticipation of augmenting the chemotherapeutic effects of conventional therapies in breast tumors that express mutant p53 tumor suppressor genes but maintain transcriptionally active p73. Utilizing p53-mutated primary ductal breast cancer cell lines, HCC1937 and HCC1599, we were able to effectively induce expression of p73 with both E2F-1/TatHA and E132/TatHA protein treatments versus the control peptide. Despite the mutation in the DNA-binding domain present in E132/TatHA, the intact marked-box domain enabled this protein to elicit its effect on p73. However, following 24 hours of treatment, the wildtype E2F-1/TatHA protein increased p73 by 2.7-fold versus the 2.2-fold observed from E132/TatHA treatment. This difference could be attributed to altered biological activity of the E132/TatHA proteins due to the DNA-binding domain mutation affecting protein folding and capacity to bind the p73 promoter. Further evaluation of these differences will be assessed in future experiments. In comparison to the study by Marabese et al. (2003), our results that suggest increased p73 expression resulting from E2F-1/TatHA and E132/TatHA treatment supports their reported findings of 2.5-fold induction of p73 in the neuroblastoma cell line SH-SY5Y following treatment with doxorubicin (29). Our results parallel the findings of Marabese et al. (29) and support the ability of E2F-1 and E132 Tat fusion peptide treatment to

induce changes in gene expression comparable to the effects elicited by chemotherapeutic agents.

It is widely accepted that p53 has a central role in the induction of apoptosis. Due to the high prevalence of p53 mutations in breast cancer cells, alternative pathways must be activated to impact the cell cycle. This study supports the effective activation of p73 and induction of p53-responsive genes in p53-mutated cell lines. In HCC1599 cells, E2F-1/TatHA and E132/TatHA treatment increased $p21^{WAF1/CIP1}$ expression by at least 3.3-fold. In contrast, the BRCA1 mutated cell line, HCC1937, demonstrated a significantly smaller 1.4-fold activation of $p21^{WAF1/CIP1}$. These results support the importance of wildtype BRCA1 tumor suppressor proteins in the activation of p53-target genes. Apoptotic activity was still present in both cell lines suggesting additional pathways and target genes may have been induced by TFP treatment. These results suggest that E2F-1/TatHA treatment of breast cancer cells would be potentially more beneficial in tumors expressing wildtype BRCA1. Future evaluation of gene and protein expression following TFP treatment using microarray technology may reveal additional upregulated and downregulated pathways resulting from E2F-1 TFP treatment to further distinguish the association between $p21^{WAF1/CIP1}$ and BRCA1.

The E2F-1/TatHA fusion proteins tested in our experiments affected p73 expression in non-metastatic primary ductal breast cancer cell lines. The metastatic breast cancer cell lines investigated in our preliminary experiments did not reveal significant fold increases in p73. However, both MDA-MB-231, MDA-MB-435S and MCF-7 already overexpress p73 (7, 32-34) suggesting that advanced, metastatic breast cancer would not be responsive to the E2F-1/TatHA or E132/TatHA treatments. No

morphological changes suggesting apoptosis were observed in the MDA-MB-231 or MDA-MB-435S cell lines with TFP treatment (data not shown). The MCF-7 cells treated with the TFPs did not experience any changes in p73 and $p21^{WAF1/CIP1}$ gene expression as detected by real-time RT-qPCR. However, the MCF-7 cells did exhibit morphological changes consistent with apoptosis such as cell detachment, cell shrinkage and membrane blebbing after 24 hours of treatment with TFPs. Further evaluation of apoptotic pathways using microarray analysis will be pursued to assess the affects of E2F-1 TFP treatment in MCF-7 cells.

In summation, we were able to successfully produce biologically active E2F-1 Tat fusion proteins that elicited a biological effect in primary, p53-mutated breast cancer cell lines HCC1937 and HCC1599. Real-time RT-qPCR revealed significant fold increases in both p73 expression and the p53-responsive gene $p21^{WAF1/CIP1}$. Evidence of apoptosis was observed in both cell lines after 24 hours of treatment suggesting that longer treatment times with TFPs could significantly augment therapies targeting primary breast cancer cells. Additionally, our research supports an alternative route of impacting gene expression in primary breast tumors by using E2F-1 Tat fusion proteins which could enhance the current modalities of cancer therapeutics.

Construction of Expression Vectors and Specific Domains within E2F-1 Tat Fusion Proteins

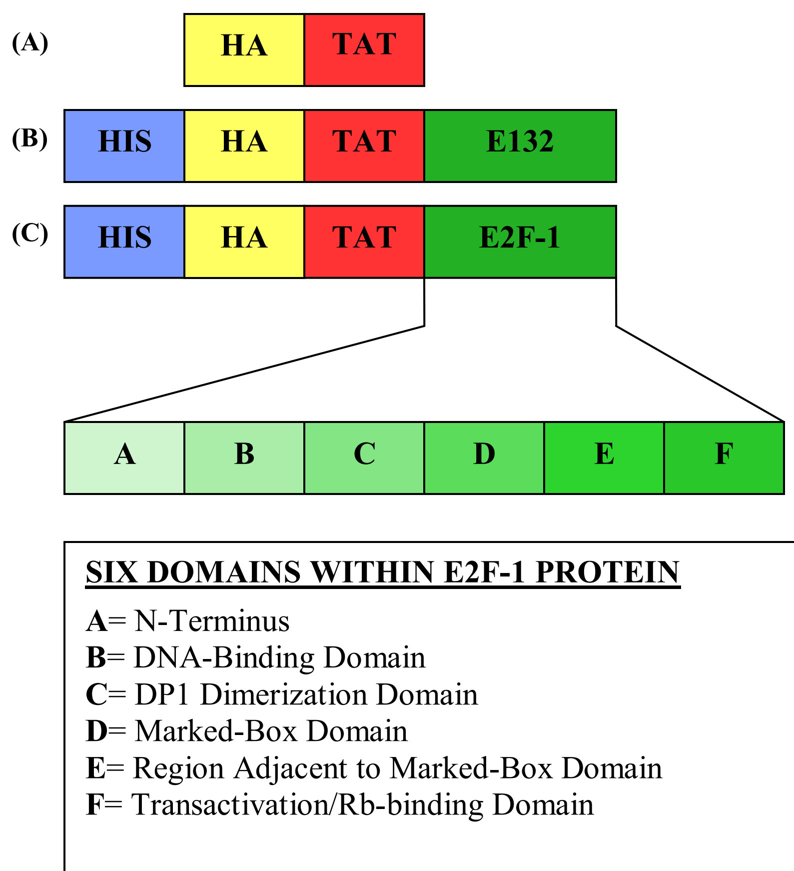

Figure 5.1. Construction of expression vectors and specific domains within E2F-1 Tat Fusion Proteins. Abbreviations: HIS= 6-Histidine tag used in protein purification, HA= Hemagglutinin tag, TAT= HIV-1 Tat transduction domain, E132= mutant variant of E2F-1 (mutation in DNA-binding domain), E2F-1= wildtype E2F-1 transcription factor. (A) TatHA control peptide, (B) E132/TatHA peptide, (C) E2F-1/TatHA peptide with specific E2F-1 protein domains illustrated.

Western Blot Analysis of E2F-1/TatHA and E132/TatHA Proteins

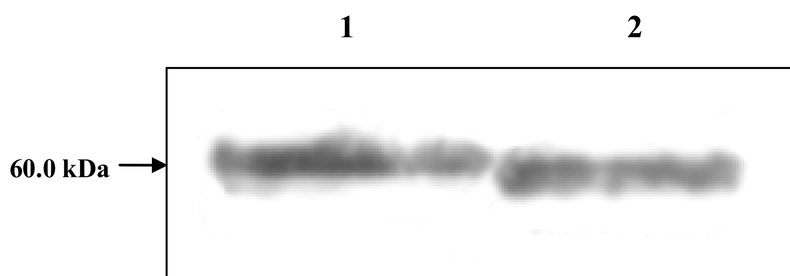

Figure 5.2. Western Blot analysis of lysates from bacterial cultures producing recombinant Tat fusion proteins. Protein (50 μg) was subjected to SDS-PAGE analysis and immunoblotting performed using primary mouse monoclonal HA antibodies (1:1000 dilution) and secondary HRP-labeled goat anti-mouse antibodies (1:25000 dilution). *Lane 1*, E2F-1/TatHA. *Lane 2*, E132/TatHA.

Fold Change in p73 Gene Expression in HCC1599 Cells

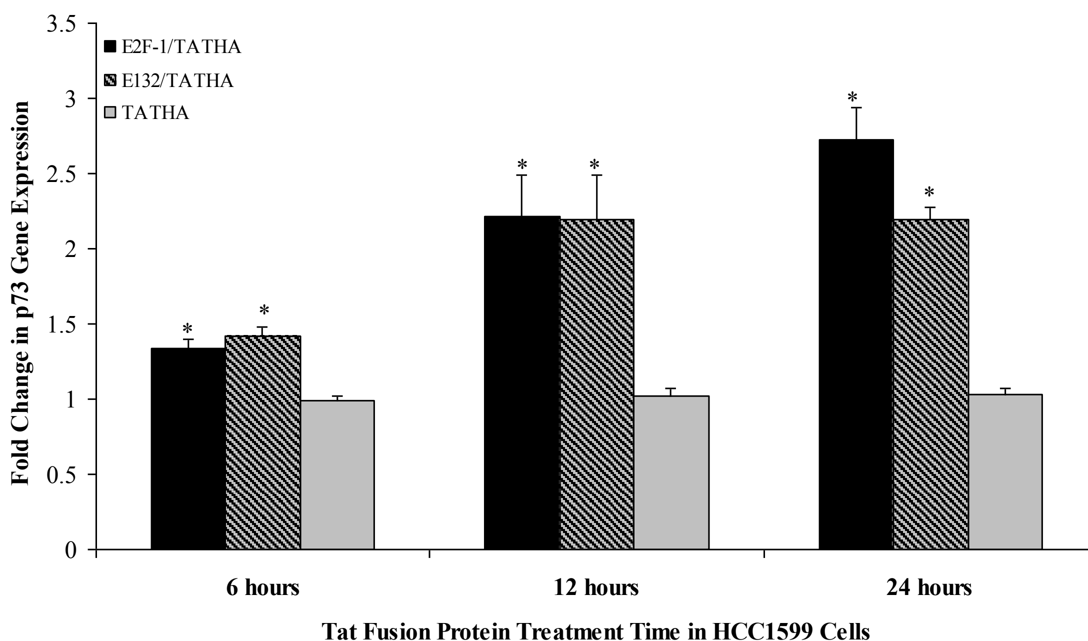

Figure 5.3. Fold difference in p73 gene expression in HCC1599 cells following 6, 12 and 24 hours of treatment with TFPs. HCC1599 cells were seeded at a density of 2×10^5 in 24-well plates for treatment with 2 μM Tat fusion proteins. Following treatment for 6, 12 or 24 hours, RNA was isolated and subjected to RT-qPCR as described under Materials and Methods. Fold increases in p73 gene expression for different Tat fusion proteins are indicated on the chart. The asterisks (*) indicate significant fold increases in p73. The results are means +/- SEM of three separate experiments (6 hour treatment, n=16; 12 hour treatment, n=17; 24 hour treatment, n=18).

Fold Change in p73 Gene Expression in HCC1937 Cells

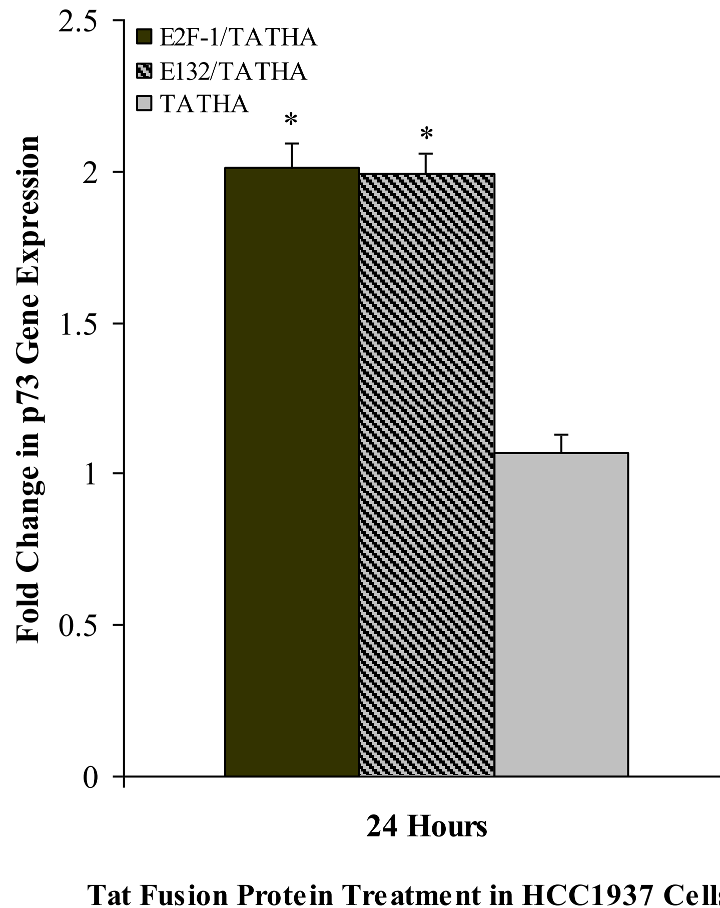

Figure 5.4. Fold difference in p73 gene expression in HCC1937 cells following 24 hours of treatment with TFPs. HCC1937 cells were seeded at a density of 2 x 10^5 in 24-well plates. Cells were allowed 24 hours to adhere to plates prior to Tat fusion protein treatment. Cells were treated with 2 µM Tat fusion proteins for 24 hours, washed, and cells detached from plates using HyQTase (Hyclone, Logan, UT). RNA was isolated and RT-qPCR performed as described under Materials and Methods. The asterisks (*) indicate significant fold increases in p73. The results are means +/- SEM of three separate experiments (n=17).

Fold Change in p21$^{WAF1/CIP1}$ Gene Expression in HCC1599 and HCC1937 Cells

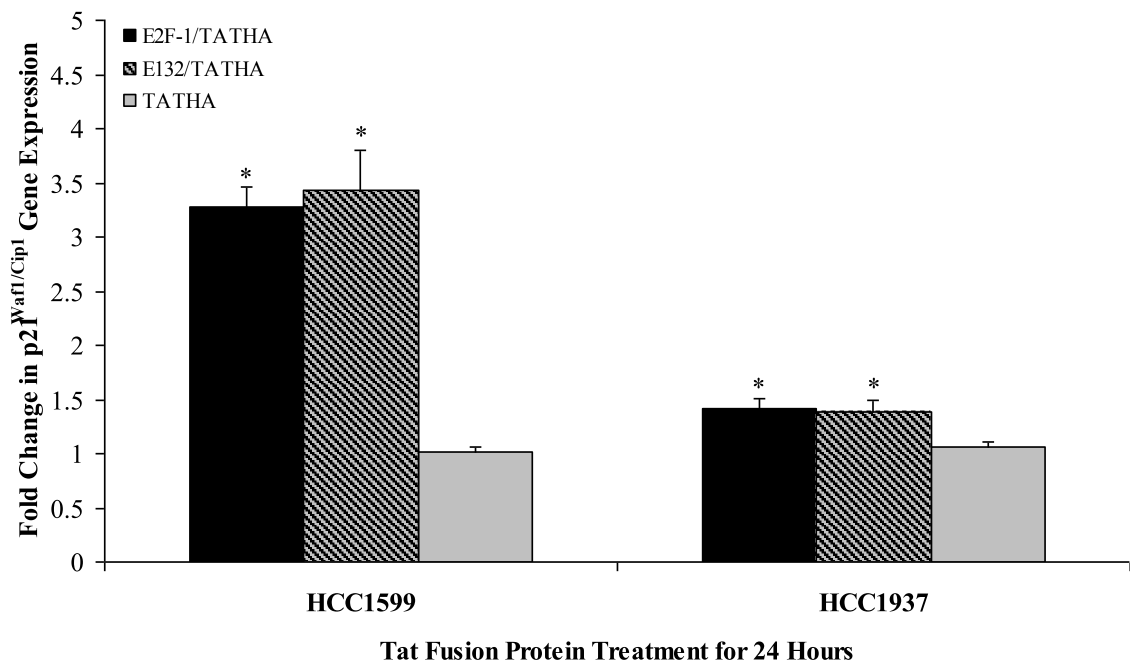

Figure 5.5. Fold difference in p21$^{WAF1/CIP1}$ gene expression in HCC1599 and HCC1937 cells following 24 hours of treatment with TFPs. HCC1599 and HCC1937 cells were seeded at a density of 2 x 10^5 in 24-well plates. HCC1937 cells were allowed 24 hours to adhere to plates prior to Tat fusion protein treatment, and HCC1599 cells were seeded directly into media with TFPs. Cells were treated with 2 μM Tat fusion proteins for 24 hours. RNA was isolated and RT-qPCR performed as described under Materials and Methods. The asterisks (*) indicate significant fold increases in p21$^{WAF1/CIP1}$. The results are means +/- SEM of three separate experiments (HCC1599, n=17; HCC1937, n=16).

Apoptotic Activity in HCC1937 Cells Following 24 Hours of Tat Fusion Protein Treatment

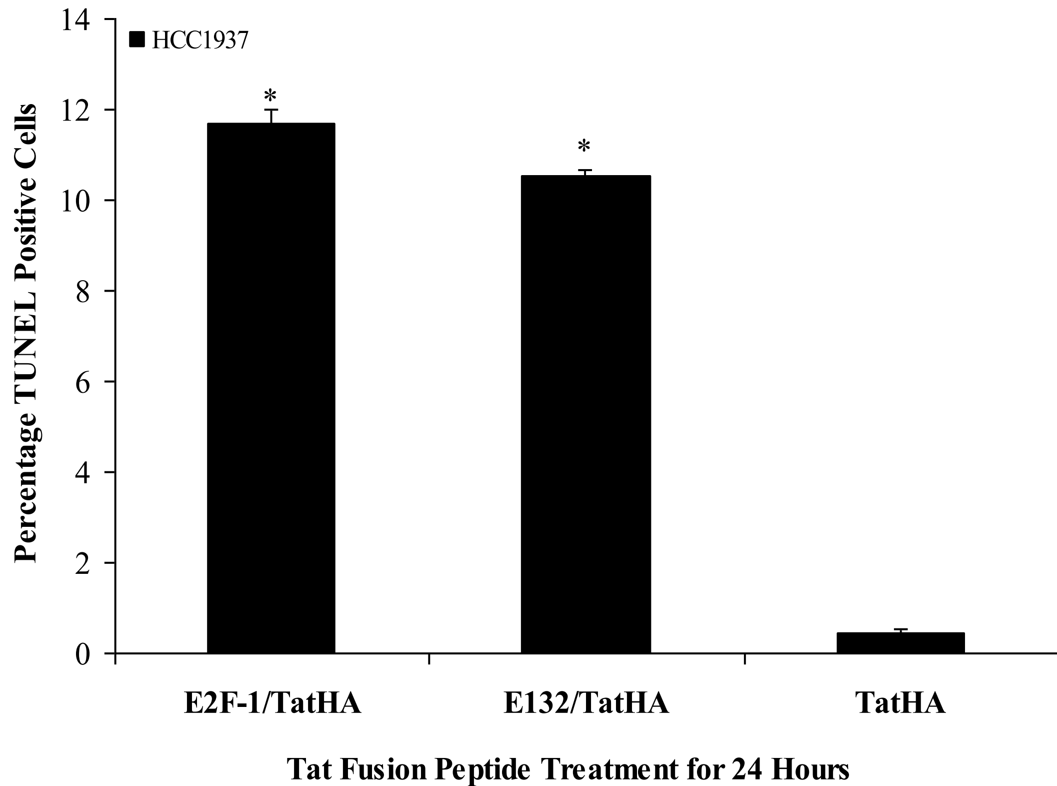

Figure 5.6. Apoptotic activity in HCC1937 cells following treatment with 2 μM TFPs for 24 hours. The percentage of cells undergoing apoptosis is represented by the bar graph. For each treatment, cells that were positive and negative for the TUNEL assay were manually counted in three separate fields of view. The apoptotic activity represents the mean number of TUNEL-positive cells from three independent experiments. The asterisks (*) indicate significant increases in apoptotic activity in E2F-1/TatHA treated cells ($p<0.0001$, $n=9$) and E132/TatHA treated cells ($p<0.0001$, $n=9$) in comparison to TatHA control peptide.

Immunocytochemistry of Apoptotic Activity in HCC1599 and HCC1937 Cells Following 24 Hours of Treatment with Tat Fusion Proteins

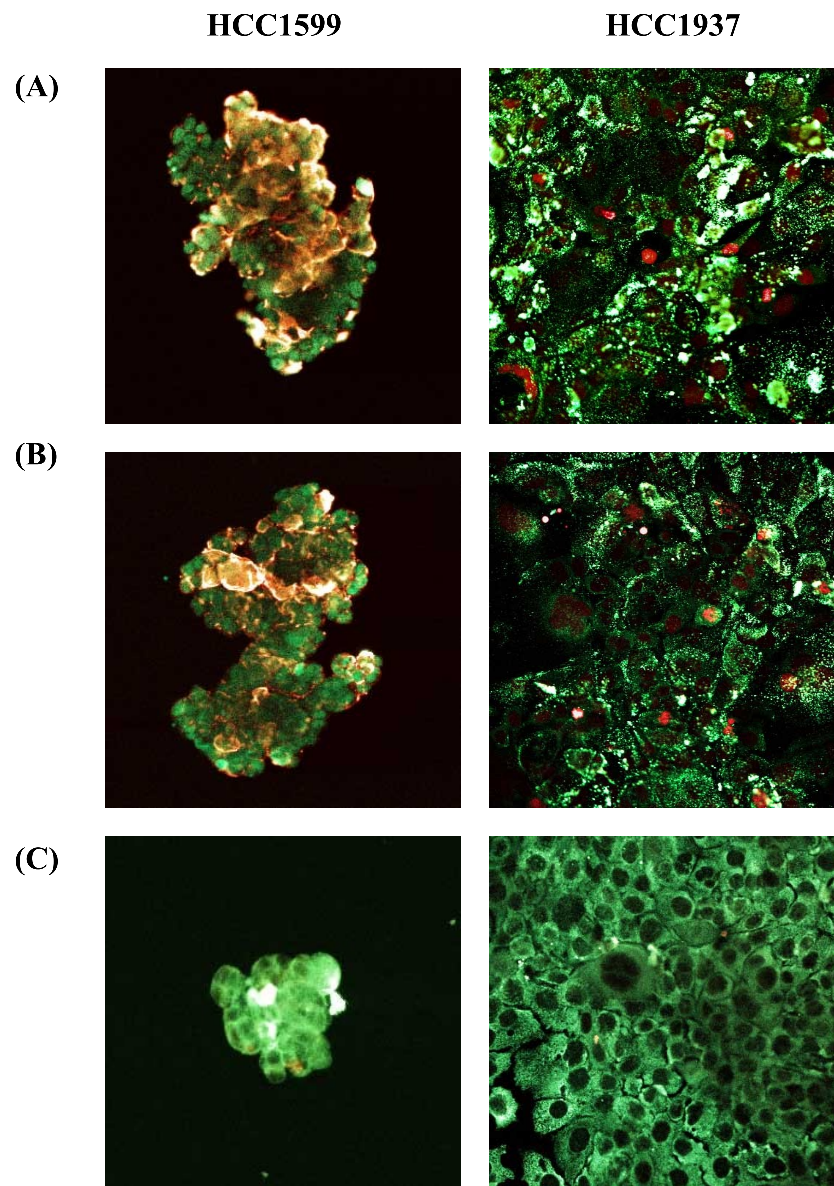

Figure 5.7. Immunocytochemistry and apoptotic activity in HCC1599 and HCC1937 cells after 24 hours of treatment with TFPs. (A) E2F-1/TatHA treated cells. (B) E132/TatHA treated cells. (C) TatHA treated cells. Breast cancer cells were treated with 2 μM Tat fusion proteins for 24 hours. E2F-1/TatHA and E132/TatHA induced apoptosis in both HCC1599 and HCC1937 cells. Apoptotic cells are indicated by the Texas Red fluorescing nuclei. Transduction of the recombinant Tat fusion proteins is indicated by fluorescein-labeled proteins as described under Materials and Methods. Images were captured using a 40X objective with a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope.

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CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

This research has provided valuable information regarding future applications of E2F-1 Tat fusion proteins to augment current chemotherapeutic regimens used to treat primary ductal breast carcinomas. The production and purification of E2F-1 Tat fusion proteins has permitted the investigation of a reversible, protein therapy to target specific genetic alterations contributing to tumorigenesis. Specifically, this research targeted p53-mutated primary infiltrating ductal breast carcinomas. Approximately 80% of all breast tumors are classified as primary infiltrating ductal breast carcinomas, and our goal was to impact this stage of tumor development with our novel therapy. Importantly, this research is the first to report the therapeutic application of E2F-1 Tat fusion proteins in primary infiltrating ductal breast cancer cell lines.

In order to test the therapeutic potential of E2F-1 Tat fusion proteins, the protein constructs were first cloned and optimal protein production conditions determined. A multi-step purification protocol was optimized to produce large quantities of recombinant E2F-1 and E132 Tat fusion proteins. Utilizing 6 M guanidine-HCl in the solubilization buffers permitted complete denaturation of recombinant proteins allowing for isolation on nickel-charged HisTrap™ columns. Conditions were also optimized to

prevent protein precipitation cascades during the purification stage of protein production. An obvious limitation of protein therapy is the ability to produce sufficient quantities of proteins for in vitro and possibly in vivo experimentation. We modified existing protein purification protocols for E2F-1/TatHA and E132/TatHA proteins to yield 10-12 mg of recombinant proteins per 500 ml of bacterial culture. This protocol enabled us to produce sufficient protein for multiple series of in vitro experiments. However, future in vivo studies are potentially limited due to the maximum 0.5 mg/ml concentration of E2F-1/TatHA and E132/TatHA proteins produced using our protocol designed to prevent protein precipitation. In addition, we were unable to use cryopreserved TFPs on our cell lines because the cells were extremely sensitive to all concentrations of glycerol tested. Our experiments with cells were limited to less than a week per batch of purified proteins since protein storage was restricted to a maximum of 5 days to preserve biological activity. Therefore, future investigation into mammalian expression systems to produce the E2F-1/TatHA and E132/TatHA proteins will be investigated in hopes of increasing the solubility and biological activity of our proteins. It is anticipated that mammalian expression vectors could also permit the production of larger quantities of biologically active proteins without the obstacles encountered from our bacterial expression system.

Following purification of E2F-1/TatHA and E132/TatHA fusion proteins, we prepared various cell lines for in vitro testing and to verify protein transduction. The E2F-1/TatHA and E132/TatHA proteins produced in our lab possess the capability to transduce all cell types. The exact mechanism of E2F-1 TFP transduction has not been definitively determined but appeared to parallel the pattern of transduction observed in the study by Yang et al. (2002) where significant nuclear and nucleolar accumulation of

proteins was observed (1). Likewise, it is plausible that internalization of the E2F-1/TatHA and E132/TatHA fusion proteins occurs in the rapid, lipid raft-dependent and receptor independent form of endocytosis not associated with caveolar- and clathrin-mediated endocytosis described by Wadia et al. (2004) (2-4). In all breast cancer cell lines tested, significant cytoplasmic, perinuclear and nucleolar accumulations of proteins were evident after all measured time points (1, 6, 12 and 24 hours). Due to the rapid, substantial perinuclear accumulation of the proteins, their half-lives also seemed to be greatly extended versus the native forms of the proteins. The wildtype E2F-1 protein and the mutant E2F-1 protein, E132, reportedly have biological half-lives of approximately 70 minutes (4). Caron et al. (2004) published that perinuclear accumulation of proteins is suggestive of sequestration of the recombinant proteins within the Golgi apparatus and endoplasmic reticulum (ER) (2). This protein sequestration ultimately results in a sustained release of TFPs to the nuclear target thus appearing to extend the protein half-lives (2). The extended half-lives of the E2F-1/TatHA and E132/TatHA recombinant proteins observed in our studies suggests that the transduction and probable Golgi and ER accumulation in cells results in sustained biological activity of the proteins following 24 hours of treatment.

Upon verification of efficient protein transduction, we assessed the effects of E2F-1/TatHA and E132/TatHA on gene expression in breast carcinoma cell lines. It is important to review the specific genetic alterations in the breast cancer cell lines investigated by this research. First, the HCC1937 and HCC1599 cell lines both contain p53 tumor suppressor gene mutations and are negative for expression of ER/PR receptors and Her2/neu. Second, both cell lines are similar in tumor stage development (TNM

stage IIB, grade 3 and TNM stage IIIA, grade 3 respectively). Third, both cell lines are positive for the expression of the catalytic subunit of telomerase, hTERT (human telomerase reverse transcriptase). Thus, it was anticipated that E2F-1/TatHA proteins would impact both cell lines in a similar manner. Initially, it was expected that E2F-1/TatHA would effectively repress the expression of hTERT in both HCC1937 and HCC1599 cell lines. The E132/TatHA protein (mutated DNA-binding domain) was used as a control in hTERT experiments in addition to a TatHA alone protein. Following treatment of cell lines with E2F-1/TatHA, E132/TatHA and TatHA proteins for 24 hours, our data revealed significant repression of hTERT with E2F-1/TatHA treatment; however, complete repression of hTERT was not achieved. In HCC1937 cells, hTERT was repressed 3.5-fold by E2F-1/TatHA in comparison to E132/TatHA and the TatHA peptide controls. In HCC1599 cells, hTERT was also repressed with E2F-1/TatHA treatment by 4.0-fold when compared to E132/TatHA control. A slightly lower hTERT repression of 3.3-fold was observed with E2F-1/TatHA in the HCC1599 cells when compared to the TatHA control. Overall, these results suggest that transduction of E2F-1/TatHA fusion proteins in vitro is a moderately effective repressor of hTERT expression in the primary ductal breast cancer cell lines HCC1937 and HCC1599. The hypothesized repression of telomerase activity in infiltrating ductal carcinoma cells via E2F-1 repressor proteins is supported by significant hTERT repression in both HCC1937 and HCC1599 cells. However, the ability to achieve complete repression of hTERT via the E2F-1 transcription factor still remains elusive. Future experiments will further investigate the transcriptional repressive effects of E2F-1 and its effects on cell-cycle progression and telomerase repression.

Although real-time RT-qPCR did not reveal significant effects of E132/TatHA on hTERT expression, the cell lines did exhibit visible signs of stress such as cell membrane blebbing and cell detachment in both E2F-1/TatHA and E132/TatHA versus TatHA treated cell lines. This was suggestive of an alternate effect of E132/TatHA on gene transcription via the remaining intact protein domains. Further literature review revealed an important role for the E2F-1 transcription factor in activating the p73 tumor suppressor pathway, and our initial research focus expanded to investigate this alternate pathway to target cell senescence and apoptosis. Multiple studies using E2F-1 adenoviral and retroviral transfection of the E2F-1 gene support the activation of the p73 tumor suppressor pathway in carcinoma cells (5, 6). Additionally, upregulation of p53-responsive genes has been observed in cells following activation of p73 (7, 8). Specifically, stimulation of the p73 tumor suppressor gene has induced $p21^{WAF1/CIP1}$ expression leading to cell growth arrest and apoptosis in tumor cells (8, 9).

Despite the mutation in the DNA-binding domain present in E132/TatHA, the intact marked-box domain enabled this protein to elicit its effect on p73. In our work, we monitored expression of the transcriptionally active form of p73, often referred to as TAp73, which includes both the alpha and beta isoforms of the p73 tumor suppressor protein. Both TAp73α and TAp73β have been found to induce apoptosis in cell lines and activate p53-homologue proteins. The real-time primer sequences utilized in our research were obtained from Marabese et al. in anticipation of achieving comparable elevations in transcriptionally active p73 gene expression with our novel E2F-1/TatHA and E132/TatHA protein therapy. In our experiments, following 24 hours of treatment in HCC1599 cells, the wildtype E2F-1/TatHA protein increased p73 by 2.7-fold versus the

2.2-fold observed following E132/TatHA treatment. This small difference could be attributed to altered biological activity of the E132/TatHA proteins due to the DNA-binding domain mutation affecting protein folding and capacity to bind the p73 promoter. However, in HCC1937 cells, both E2F-1/TatHA and E132/TatHA treatment for 24 hours increased p73 by 2-fold. Interestingly, these results support the biological activity of E132/TatHA proteins in alternate cellular pathways other than transcriptional repression of hTERT, which could explain some of the morphological changes we observed in both E2F-1/TatHA and E132/TatHA treated cell lines. These findings are also consistent with the results published in the study by Marabese et al. (2003). Our results, ranging from 2.0- to 2.7-fold increases in p73 expression following E2F-1/TatHA and E132/TatHA treatment, reflect Marabese et al.'s reported findings of 2.5-fold induction of p73 in the neuroblastoma cell line SH-SY5Y following treatment with doxorubicin (10). Therefore, our results parallel the findings of Marabese et al. (10) and support the ability of E2F-1 and E132 Tat fusion peptide treatment to induce changes in gene expression comparable to the effects elicited by chemotherapeutic agents such as doxorubicin. The chemotherapeutic agent doxorubicin (also referred to as Adriamycin) is a standard chemotherapeutic agent used to treat breast cancer. The similar elevation in p73 expression between our E2F-1/TatHA treatment and doxorubicin supports the feasible role of our protein therapy in breast cancer management in combination to standard chemotherapeutic agents.

As mentioned above, this research primarily focused on breast cancer cell lines with p53 tumor suppressor gene mutations. Due to the high prevalence of p53 mutations in breast carcinomas, alternative pathways must be activated to impact the cell cycle.

This study supports the efficient activation of p73 and induction of p53-responsive genes in p53-mutated cell lines. In HCC1599 cells, E2F-1/TatHA and E132/TatHA treatment increased $p21^{WAF1/CIP1}$ expression by at least 3.3-fold. In contrast, the BRCA1 mutated cell line, HCC1937, demonstrated a significantly smaller 1.4-fold activation of $p21^{WAF1/CIP1}$. Overall, this research supports the ability of E2F-1/TatHA and E132/TatHA proteins to induce expression of $p21^{WAF1/CIP1}$ via p73 overexpression in comparison to TatHA controls. However, an obvious difference in $p21^{WAF1/CIP1}$ gene expression was appreciated in the wildtype BRCA1 cell line, HCC1599, versus the mutated BRCA1 cell line, HCC1937. These results reveal the diminished ability of increased levels of p73 to activate $p21^{WAF1/CIP1}$ expression in breast carcinomas with BRCA1 mutations. These results also stress the importance of wildtype BRCA1 tumor suppressor proteins in the activation of p53-target genes. Apoptotic activity was still present in both cell lines suggesting that additional pathways and target genes may have been induced by TFP treatment. These results suggest that E2F-1 Tat protein treatment of breast cancer cells would be more beneficial in tumors expressing wildtype BRCA1. Future evaluation of gene and protein expression following TFP treatment using microarray technology may reveal additional upregulated and downregulated cellular pathways resulting from E2F-1 TFP treatment to further distinguish the association between $p21^{WAF1/CIP1}$ and BRCA1.

Our data support a significant biological effect of E2F-1/TatHA and E132/TatHA proteins on gene expression of hTERT, p73 and $p21^{WAF1/CIP1}$. In addition to impacting gene expression, significant apoptotic activity was detected in HCC1937 and HCC1599 cells following treatment with E2F-1/TatHA and E132/TatHA proteins for 24 hours.

Fluorescent microscopic evaluation of HCC1937 cells revealed that approximately 11.7% of cells were TUNEL-positive after exposure to E2F-1/TatHA proteins and 10.5% of cells were TUNEL-positive following E132/TatHA treatment. Cells treated with the TatHA control peptide only revealed 0.46% TUNEL-positive cells. Therefore, following exposure to TFPs, significant apoptotic activity was detected in both E2F-1/TatHA and E132/TatHA treated breast cancer cells. The apoptotic activity visualized in HCC1599 cells was difficult to quantitate due to cell clumping, but we estimated that approximately 10% of cells were TUNEL-positive following 24 hours of treatment with E2F-1/TatHA and E132/TatHA proteins. The mechanism of apoptosis resulting from E2F-1/TatHA and E132/TatHA treatment is assumed to be a downstream effect of the upregulated p73 protein acting in a similar manner as p53. As described in Chapter 2, the most likely mechanism of apoptosis is p73-mediated transactivation of BAX, PUMA and Noxa proteins leading to subsequent mitochondrial dysfunction and activation of effector caspases. Likewise, induction of $p21^{WAF1/CIP1}$ contributed to cell cycle arrest following p73 overexpression.

The E2F-1/TatHA and E132/TatHA proteins tested in our experiments impacted gene transcription and induced apoptosis in non-metastatic primary ductal breast cancer cell lines. The metastatic breast cancer cell lines investigated in our preliminary experiments did not reveal significant fold changes in hTERT, p73 or $p21^{WAF1/CIP1}$ expression. However, MDA-MB-231, MDA-MB-435S and MCF-7 already overexpress p73 (11-14) suggesting that advanced, metastatic breast cancer would not be responsive to the E2F-1/TatHA or E132/TatHA treatments. No morphological changes consistent with apoptosis were observed in the MDA-MB-231 or MDA-MB-435S cell lines with

TFP treatment (data not shown). The MCF-7 cells treated with the TFPs did not experience any changes in hTERT, p73 and $p21^{WAF1/CIP1}$ gene expression as detected by real-time RT-qPCR. However, the MCF-7 cells did exhibit morphological changes consistent with apoptosis such as cell detachment, cell shrinkage and membrane blebbing after 24 hours of treatment with TFPs. These changes suggest an alternate pathway may be activated in metastatic breast cancer cell lines that already overexpress p73.

To summarize the results of our experiments, the following points outline the conclusions of our research.

A. E2F-1/TatHA and E132/TatHA proteins can be effectively produced and purified using an optimized protocol combining strong denaturants, FPLC and dialysis.

B. E2F-1/TatHA, E132/TatHA and TatHA proteins transduced greater than 95% of cell lines tested as detected via immunocytochemistry.

C. Following 24 hours of protein treatment, E2F-1/TatHA repressed expression of hTERT by 3.5-fold in HCC1937 cells and by 4.0-fold in HCC1599 cells versus E132/TatHA and TatHA controls. No differences were observed in cell lines with wildtype versus mutant BRCA1 genes.

D. Complete repression of hTERT was not achieved with E2F-1/TatHA treatment versus E132/TatHA and TatHA treatment of breast cancer cell lines.

However, 75% of hTERT expression was inhibited by E2F-1/TatHA treatment in the HCC1599 cell line.

E. Treatment of HCC1937 and HCC1599 cell lines with E2F-1/TatHA and E132/TatHA proteins resulted in greater than 2-fold induction of p73 gene expression.

F. Upregulation of the p53 responsive gene, $p21^{WAF1/CIP1}$, was observed in breast cancer cell lines following E2F-1/TatHA and E132/TatHA treatment due to increased expression of p73. In HCC1937 cells, E2F-1/TatHA and E132/TatHA increased $p21^{WAF1/CIP1}$ gene expression by at least 3.3-fold versus a smaller induction of 1.4-fold in HCC1599 cells.

G. E2F-1/TatHA and E132/TatHA treatments revealed a diminished effect of p73 overexpression to induce $p21^{WAF1/CIP1}$ expression in the BRCA1 mutated cell line, HCC1599, which supports the importance of wildtype BRCA1 tumor suppressor proteins in the activation of p53-target genes. Therapeutic treatment of p53-mutated primary ductal breast tumors with E2F-1 Tat protein therapy would be more effective if tumors express wildtype BRCA1.

H. HCC1937 cells exhibited apoptotic activity following treatment with TFPs as detected by TUNEL-positive cells. E2F-1/TatHA treatment resulted in 11.7% of TUNEL-positive cells and E132/TatHA resulted in 10.5% of TUNEL- positive

cells versus 0.46% of TUNEL-positive cells observed in TatHA control treatment groups. It is estimated that approximately 10% of HCC1599 cells were TUNEL-positive following treatment with E2F-1/TatHA and E132/TatHA.

Although the experiments investigating the effects of E2F-1/TatHA and E132/TatHA on hTERT and p73 have been evaluated separately, recent studies suggest a possible overlap in these pathways. Beitzinger et al. (2006) recently reported that forced expression of p73 via E2F-1 results in downregulation of hTERT (15). This study suggested two dose-dependent mechanisms for hTERT repression: a) high levels of E2F-1 protein results in direct hTERT repression via the mechanisms supported by Crowe et al (2001) via binding two specific sites in the hTERT promoter (16), and b) low levels of E2F-1 protein results in indirect hTERT repression via TAp73 induction and the TAp73 protein binding Sp1 sites in the hTERT core promoter inducing transcriptional repression (15). Beitzinger et al. induced E2F-1 expression via 4-hydroxytamoxifen induction of an ER-E2F-1 plasmid transfected into cell lines (15). Tamoxifen induction of their ER-E2F-1 construct resulted in significant induction of the TAp73 promoter and direct repression of the hTERT promoter (15). Interestingly, although their method of introducing E2F-1 into cells is different from our Tat-mediated E2F-1 transduction, their results are consistent with the findings of our studies.

It is possible that induction of TAp73 could have contributed to hTERT repression in our cell lines via Sp1 binding sites in the hTERT promoter; however, the results from E132/TatHA treatment of cells for 24 hours did not reveal this phenomenon. In E2F-1/TatHA treated cells, hTERT was repressed approximately 4-fold, in other

words, only 25% of hTERT was expressed in E2F-1/TatHA treated cells versus the 100% expression in E132/TatHA and TatHA treated cell lines. E132/TatHA did induce expression of p73 via the intact marked box domain as our studies revealed 2.2-fold (HCC1599 cells) and 2.0-fold (HCC1937 cells) increases in p73 expression. The E132/TatHA induction of p73 did not appear to induce repression of hTERT in the 24-hour treatment period. However, it is conceivable that this effect could be detected following a longer course of treatment for 48 or 72 hours. In HCC1599 and HCC1937 cells treated with E2F-1/TatHA for 24 hours, as previously mentioned, a 2.7-fold (HCC1599) and 2.0-fold (HCC1937) increase in p73 was observed in combination to a 4-fold repression of hTERT. It is possible that the induction of p73 contributed to the E2F-1 repression of hTERT, but it is more probable that this effect would be observed at greater treatment time points since induction of p73 by E132/TatHA treatment for 24 hours did not demonstrate hTERT repression.

In summary, this research is the first to report the effects of biologically active E2F-1 Tat fusion proteins on gene expression in breast carcinoma cell lines. These recombinant proteins rapidly transduced cells and either induced or repressed expression of target genes. We conclude that E2F-1/TatHA is a moderately effective repressor of hTERT. We also determined that both E2F-1/TatHA and E132/TatHA are effective inducers of transcriptionally active p73 tumor suppressor protein expression. Likewise, the overexpression of p73 from E2F-1/TatHA and E132/TatHA is an efficient method of inducing expression of $p21^{WAF1/CIP1}$. Although it is feasible that elevated levels of p73 could impact hTERT and further repress its transcriptional activation, at the 24 hour treatment time point, the hTERT repression in our experiments was only attributed to

high levels of transduced E2F-1/TatHA proteins. Ultimately, treatment of breast cancer cell lines for 24 hours with E2F-1/TatHA and E132/TatHA proteins induced detectable apoptotic activity in approximately 10% of treated cells. Importantly, this work supports our hypotheses that protein transduction with E2F-1 Tat fusion proteins would repress hTERT, induce expression of p73 and $p21^{WAF1/CIP1}$ and ultimately provoke cancer cell death.

It appears that E2F-1/TatHA proteins are marginally effective inducers of apoptosis; however, the primary goal of any cancer therapy is to induce 100% cancer cell death. It is more feasible to consider future investigations of E2F-1/TatHA proteins as part of a combination therapy for breast cancer. The combination of E2F-1 Tat fusion proteins with chemotherapeutic agents could offer a multifaceted approach targeting both cancer cell division and the genetic alterations contributing to the development of immortalized breast tumor cells.

Breast cancer will continue to impact at least 1 out of 8 women worldwide. Therefore, the development of alternative therapies is necessary to advance cancer treatment by targeting the genetic alterations underlying cancer development. The concept of chemoprotein therapy, the combination of chemotherapeutic drugs with protein therapy, will offer a novel modality to reversibly impact gene expression in cancer cells to induce cell senescence and/or apoptosis. Further in vitro and in vivo investigation of E2F-1 Tat fusion protein therapy in combination with common chemotherapeutic agents will spearhead novel approaches in cancer treatment. Clearly, the complex effects of E2F-1 Tat fusion proteins must be further investigated via microarray analysis and further in vitro and in vivo experimentation. Overall, the results

of this work suggest potential applications of E2F-1/TatHA protein therapy in translational research utilizing a chemoprotein therapeutic approach to treat breast cancer.

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VITA

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Doctor of Philosophy

Thesis: TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS INTO PRIMARY INVASIVE DUCTAL BREAST CARCINOMA CELL LINES AND SUBSEQUENT EFFECTS ON GENE TRANSCRIPTION

Major Field: Biomedical Science Emphasis: Molecular Genetics

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Title of Study: TRANSDUCTION OF E2F-1 TAT FUSION PROTEINS INTO PRIMARY INVASIVE DUCTAL BREAST CARCINOMA CELL LINES AND SUBSEQUENT EFFECTS ON GENE TRANSCRIPTION

Pages in Study: 183 Candidate for the Degree of Doctor of Philosophy

Major Field: Biomedical Science

Scope and Method of Study: Breast cancer cells harbor specific genetic alterations that contribute to their immortalized state, including overexpression of telomerase and mutations of the p53 tumor suppressor gene. E2F-1 is a known transcriptional repressor of the catalytic subunit of telomerase (referred to as hTERT). In p53 mutated cells, E2F-1 possesses the ability to transactivate the p73 tumor suppressor pathway and p53-homologues involved in cell cycle arrest such as $p21^{WAF1/CIP1}$. We investigated an alternative method of introducing the E2F-1 transcription factor via Tat-mediated protein transduction to effectively target and reversibly impact gene expression in breast cancer cell lines. Using affinity chromatography and strong denaturants, the E2F-1 Tat fusion proteins were isolated and purified via FPLC and dialysis. Real-time RT-qPCR was utilized to assess the effects of E2F-1 Tat fusion protein treatment on gene expression of select breast cancer cell lines. Specifically, we investigated repression of hTERT and induction of p73 and $p21^{WAF1/CIP1}$ genes in HCC1937 and HCC1599 primary invasive ductal breast cancer cell lines. We also monitored apoptotic activity in carcinoma cells with TUNEL assays following treatment with E2F-1 Tat fusion proteins.

Findings and Conclusions: The E2F-1 Tat fusion proteins effectively transduced greater than 95% of breast cancer cell lines. Protein therapy with E2F-1/TatHA repressed expression of hTERT by 3.5-fold in HCC1937 cells and by 4.0-fold in HCC1599 cells versus control proteins. Treatment of HCC1937 and HCC1599 breast carcinoma cell lines with E2F-1 Tat fusion proteins resulted in a greater than 2-fold induction of p73 gene expression. Upregulation of the p53 responsive gene, $p21^{WAF1/CIP1}$, was also observed in HCC1937 and HCC1599 cell lines following treatment with our E2F-1 Tat protein therapy. A 3.3-fold induction of $p21^{WAF1/CIP1}$ was observed in HCC1937 cells versus a smaller induction of 1.4-fold in HCC1599 cells, which was attributed to the presence of a BRCA1 mutation in the HCC1599 cell line. Following 24 hours of treatment with our E2F-1 Tat fusion proteins, apoptotic activity was detected in approximately 10% of breast cancer cells versus control proteins. Overall, our E2F-1 Tat protein therapy proved to be a moderately effective repressor of hTERT and activator of both p73 and $p21^{WAF1/CIP1}$ resulting in detectable apoptotic activity. This suggests a potential application of E2F-1 Tat protein therapy in cancer therapeutics to modulate gene expression in breast carcinomas.

ADVISER'S APPROVAL: Lee Rickords, Ph.D.