2007

Telomerase Inhibition and Sensitization of Breast Tumor Cells

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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May 2007

Acknowledgement

I would like to thank my advisor Dr. Shawn Holt for his encouragement and direction in this project as well as my other committee members for their advice and assistance: Dr. Colleen Jackson-Cook, Dr. Jolene Windle, Dr. Rob Tombes and Dr. Joy Ware. I would also like to thank Dr. Lynne Elmore for her support and guidance in the completion of this work, as well as the past and current lab members for their advice, assistance, and laughter. To my parents, thank you for your unwavering love and support in everything I do, but especially in graduate school. I would also like to thank my other family members and friends for the constant encouragement, particularly my grandmother, Margaret Greene Rueger, who inspired me to study breast cancer. Finally, I would like to say a special thanks to my fiancé, Travis, for his support, patience, fortitude, and love through everything.

Table of Contents

List of Tables

List of Figures

ABSTRACT

TELOMERASE INHIBITION AND SENSITIZATION OF BREAST TUMOR CELLS

By Kennon R. Poynter, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Major Director: Dr. Shawn E. Holt, Associate Professor, Department of Human Genetics, Department of Pathology, and Department of Pharmacology and Toxicology

Telomerase, a ribonucleoprotein enzyme minimally composed of an RNA template (hTR) and a catalytically active protein subunit (hTERT), synthesizes telomeric repeats onto chromosome ends and is obligatory for continuous tumor cell proliferation, as well as malignant progression of breast cancer cells. Telomerase is an attractive anti-cancer therapeutic target because its activity is present in over 90% of human cancers, including more than 95% of breast carcinomas, but undetectable in most somatic cells. Traditional chemo- and radio-therapies lack the ability to effectively control and cure breast cancer, in part because residual cells are or become resistant to DNA damaging modalities.

While various telomerase inhibition strategies cause cancer cells to undergo apoptosis or senescence, there is often a lag period between administration and biologic effect (Corey, 2002). Our goal in this study was to compare the efficacy of different telomerase inhibition strategies in concert with standard chemotherapeutic agents at triggering senescence and/or apoptosis in cultures of breast cancer cells. We hypothesized that telomerase inhibition strategies will sensitize breast cancer cells to traditional chemotherapies, potentially reducing the lag phase, allowing for more potent anti-tumor effects at lower doses, and therefore ultimately imparting less toxicity to the patient.

We blocked telomerase by targeting hTR and hTERT, individually and collectively utilizing synthetic short interfering RNA (siRNA), short hairpin RNA (shRNA) and a dominant negative form of hTERT (DN-hTERT) in MCF-7 breast cancer cells. We analyzed the efficiency of telomerase inhibition for each strategy alone and then treated the cells with two mainstay chemotherapeutic agents, Adriamycin (AdR) and Taxol. The most effective telomerase inhibition strategies were synthetic siRNA and DN-hTERT, individually. After treatment with various concentrations of AdR or Taxol, breast cancer cells with inhibited telomerase grew significantly slower and exhibited widespread senescence or apoptosis within a much shorter time period and at a dose that is insufficient to trigger cytostasis. In addition, we provide evidence that cells in which telomerase was inhibited were more sensitive to anti-cancer agents, whether the drug inhibited topoisomerase II resulting in DNA damage (AdR) or blocked mitosis via protracted microtubule stabilization (Taxol). Collectively, our data indicate that alone,

anti-telomerase inhibition strategies differ in their efficacy. However, when used in the adjuvant setting with diverse acting chemotherapeutic agents, there is a potent synergy resulting in chemotherapeutic sensitization characterized in part by widespread senescence and/or apoptosis.

Chapter 1

Review of Current Literature

Telomere Structure and Maintenance

Telomeres are the natural ends or caps of linear chromosomes that are composed of both repeated DNA elements and specific DNA-binding proteins. Although size and sequence varies among species, human telomeres are composed of approximately 10-15kb of tandem hexameric repeats, 5'-TTAGGG-3' (Moyzis et al., 1988). Telomeres serve to maintain and protect the ends of chromosomes from end-to-end fusions, as well as double strand breaks (Kirk et al., 1997). Telomeres serve to maintain and protect chromosomes from being recognized as double strand breaks, preventing the nonhomologous end joining that result in telomere fusions (de Lange, 2002). Normal somatic cells continually shorten their telomeres with each cell division.

The actual structure of the telomere is unique because there is a single-stranded 3' overhang, approximately 45-200bp in length that forms a large duplex loop, called the T-loop, by folding back on itself and invading the proximal telomeric sequences (Griffith et al., 1999). This creates the secondary structure of the telomeres, consisting of the larger T-loop as well as the smaller d-loop, which is created by the single-stranded end displacing one of the telomere strands. The configuration is preserved and regulated primarily by a protein complex known as shelterin, which is composed of six proteins: TRF1, TRF2, POT1, TIN2, TPP1, and Rap1 (de Lange, 2007). Telomere Repeat binding Factors 1 and 2 (TRF1, TRF2) and protection of telomeres 1 (POT1) directly bind the

T_2AG_3 repeats, with the first two binding the double-stranded telomeric DNA and the third binding only the single stranded 3' overhang (Baumann, 2001). Therefore, POT1 does not function catalytically but has been suggested to be required for processive elongation by telomerase (Zaug et al. 2005). TIN2, TPP1 and Rap1 serve to connect and interact with the other three DNA binding proteins. The distinctive composition of the telomere prevents the single stranded ends from being recognized as damaged DNA or chromosome breaks that require repair and protects the telomeres from end to end fusions with other chromosomes.

Most normal cells continually shorten their telomeres after each cell division resulting from the inability of DNA polymerases to replicate the ends of linear molecules (Olovnikov, 1971). This event, known as "the end replication problem," occurs when the lagging strand nears the end of the chromosome and a final RNA primer is unable to bind the parental strand for further synthesis due to lack of available sequence (Figure 1). Thus, the very end of the chromosome does not get replicated, and the telomeres progressively shorten.

Approximately 50-100 base pairs are lost with each division (Harley, 1990). This progressive shortening is the primary counting mechanism for lifespan within the cells, first observed by Hayflick in 1961 (Hayflick et al., 1961). As a result, the cells will only proliferate for a certain number of population doublings, followed by cellular senescence (Harley et al., Hastie et al, 1990). In aging cells, the progressive telomere shortening correlates with the decline in proliferative capacity and is thought to induce senescence

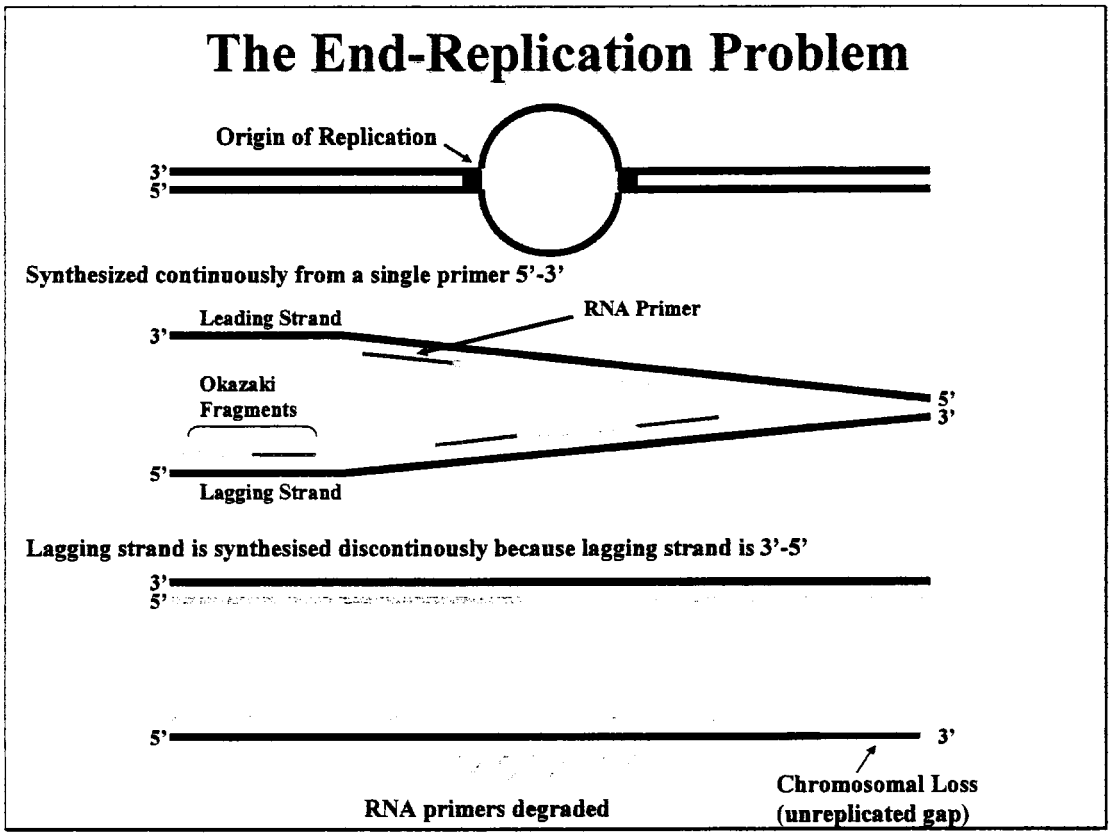


Figure 1: The End-Replication Problem.

During replication, the leading strand is synthesized continuously from a single primer in the 5'-3' direction. The lagging strand, on the other hand, replicates intermittently due to the necessity of additional priming events because synthesis occurs 3'-5', thus creating Okazaki fragments. The problem lies with the final priming event when there is a lack of enough bases for the primer binding and these terminal repeats are lost.

(Figure 2). This stage of the cellular life cycle is referred to as Mortality stage 1 (M1) or senescence, which is controlled by cell cycle regulatory genes and/or tumor suppressor genes including pRB, p53, and p21 (Wright and Shay, 1992). The inception of M1 occurs due to the presence of at least one sufficiently short telomere because telomere shortening is recognized as DNA damage and activates the DNA damage response (Holt et al., 1996). Some cells are capable of inactivating the tumor suppressor proteins, thereby inhibiting senescence and allowing continued cell growth, as well as telomere shortening, until Mortality stage 2 (M2) (Wright and Shay, 1992). The cells in M2 or crisis will activate cell death pathways because of critically short telomeres, which can no longer protect the chromosomes. Therefore, the shortening of telomeres plays a significant role in the control of both M1/senescence and M2/crisis.

The immortalization of cells is accomplished by bypassing both senescence (M1) and crisis (M2). In addition, the cells must develop a mechanism to elongate and re-establish the integrity of the telomeres, which is most often achieved through the activation of telomerase (Kim et al., 1994). Observed in most cancer cells, telomerase is activated upon immortalization of the cells whereupon the cells are able to limitlessly proliferate, suggesting that telomerase is important in the formation and progression of cancer.

The Telomere Hypothesis discussed above was further supported by a study demonstrating that exogenous expression of hTERT in two telomerase-negative normal diploid somatic cell lines, retinal pigment epithelial cells and foreskin fibroblasts, resulted in the activation of telomerase (Bodnar et al., 1998). Consequently, both cell

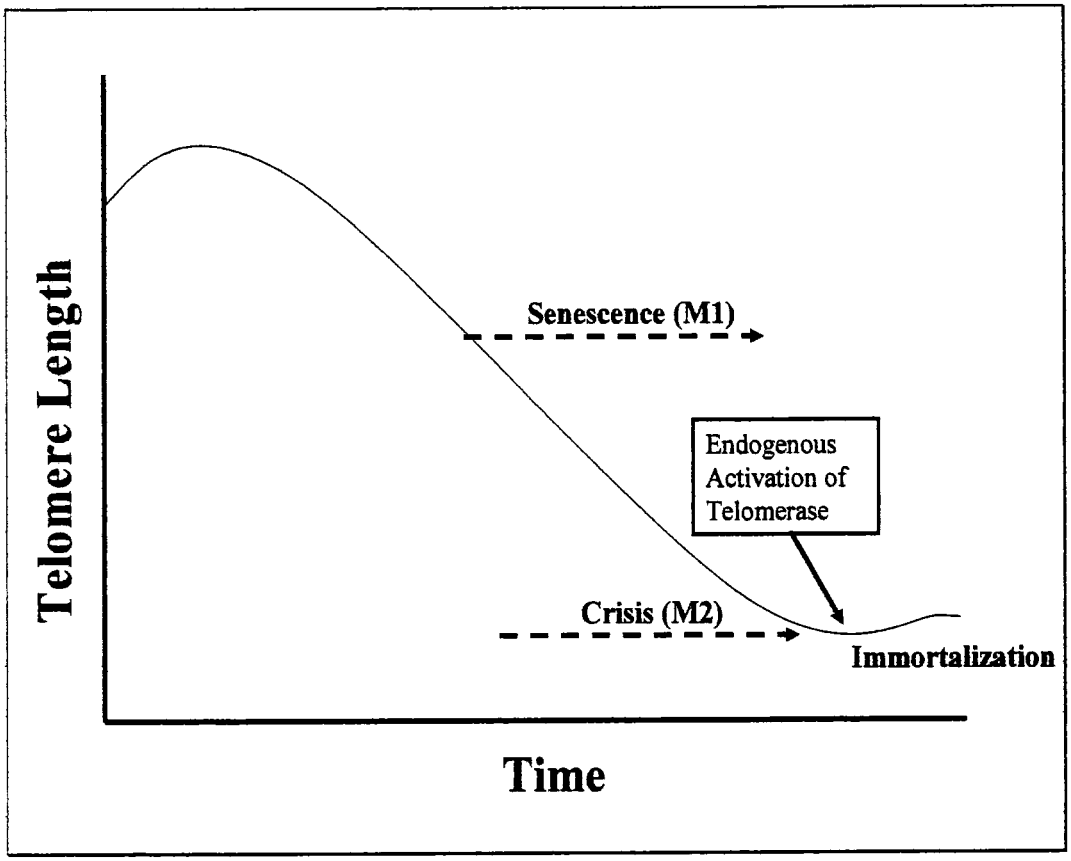


Figure 2: Schematic Representation of Cellular Aging in Terms of Telomere Length. As normal cells age, proliferation occurs for a finite number of population doublings before the presence of one critically short telomere is recognized and the cells will then enter senescence (M1). Inactivation of the tumor suppressor genes, p53 and pRB, controlling this stage, enables continued growth. With this expansion, the telomeres continue shortening and eventually result in the cells entering crisis (M2). At this stage the cells either die or immortalization transpires through the reactivation of telomerase.

lines displayed increases in telomere length and *in vitro* cellular life-span. The cell lines maintained a normal karyotype with advanced age as well as decreased staining for β-galactosidase, a biomarker for senescence, whereas their telomerase-negative counterparts demonstrated telomere shortening and senescence. Another study showed that human bronchial epithelial cells could be immortalized *in vitro* at high rates using only hTERT and cyclin-dependent kinase 4 (Cdk4) expression vectors (Ramirez et al. 2004). In combination, the hTERT functioned to overcome telomere loss and senescence while the Cdk4 overexpression inhibited the upregulation of $p16^{INK4a}$, a cyclin dependent kinase inhibitor, and the resulting premature growth arrest that normally occurs in culture. Both experiments provided the necessary evidence to conclude that telomere attrition in normal cells is one of the primary causes of senescence. Furthermore, the introduction of hTERT into senescent cells was found incapable of reversing cellular aging, but rescue does occur in cell cultures with only a few doublings remaining (Farwell et al. 2000).

Besides allowing normal somatic cells to bypass replicative senescence, exogenous telomerase is also able to extend the lifespan of cancer prone cells such as Li Fraumeni Syndrome (LFS) human mammary epithelial (HME) cells that have been shown to spontaneously immortalize (Elmore et al. 2002). In these cells, the hTERT expression provided protection against the typical hallmarks of malignant cells such as genomic instability and immortalization. This observation suggests hat telomerase may also function as a unique tumor suppressor protein only when expressed prior to genomic instability, since ectopic telomerase may suppress the instability, thereby having anti-

cancer effects. In this instance, the telomerase-expressing LFS cells are clearly different from classically immortal cells. However, in the context of genomic instability and loss of cell cycle checkpoints, telomerase can fuel cancer progression.

Telomerase

In humans, telomerase is a reverse transcriptase containing a catalytic protein component, hTERT (human Telomerase Reverse Transcriptase), and an RNA template, hTR (human Telomerase RNA), and functions to catalyze the addition of telomeric (TTAGGG) DNA repeats onto the chromosome ends (Feng et al., 1995; Weinrich et al., 1997). Additional proteins are associated with telomerase including telomerase associated protein (TEP-1; no known function); RNA (hTR) binding proteins (hnRNPs; snoRNAs) and the hsp90 chaperone complex (p23 and Hsp90; modulate telomerase assembly) (Figure 3). Telomerase elongates telomeres by utilizing the 11 base hTR template to add nucleotides via the catalytic subunit to the 3' overhang at the end of chromosomes, which allows telomere elongation and maintenance through replication by conventional polymerases.

Different species have been shown to share common secondary structures of telomerase RNA, including a template, a 5' template boundary element, a large loop including the template and putative pseudoknot, and a loop-closing helix (Figure 3). However, there are vast differences in sequence and size of the telomerase RNA between species, indicating the importance of secondary structure for function. Only two hTR domains are mandatory for catalytic activity, the pseudoknot domain (nt 1-209), which

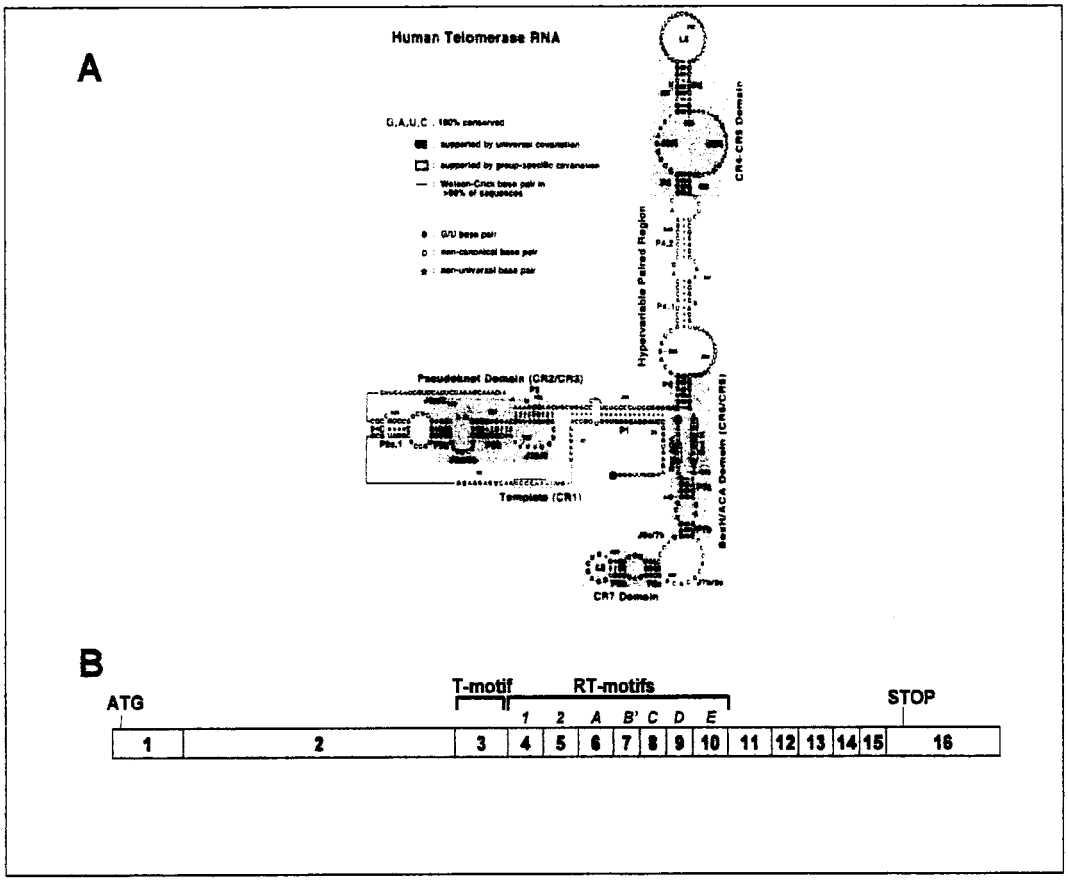


Figure 3: Models of the Core Components of Telomerase.
Proposed secondary structure of human telomerase RNA (Chen et al., 2000) with the five conserved regions is enclosed in blue boxes except for the template region (A). Linear schematic of the hTERT gene with the T-motif as well as the seven conserved RT-motifs labeled above the gene (B).

includes the template region, and the CR4/CR5 domains (nt 241-330) (Tesmer et al., 1999). These domains have been shown to bind to hTERT independently (Chen and Greider, 2003). Within the active telomerase complex, it is proposed that there are two telomerase RNA molecules that are co-dependent and functionally collaborate with one another (Wenz, 2001). While in the nucleus, hTR interacts within small nucleolar ribonuclear proteins (snoRNPs), which have been implicated in maintaining hTR maturation, stability and localization (Dez et. al., 2001; Dragon et al., 2000). hTR also associates with heterogeneous ribonuclear proteins (hnRNPs) C1 and C2. These proteins have been suggested to function at the telomeres with telomerase binding (Ford et al., 2000).

hTERT, the catalytic subunit of telomerase, has a conserved primary structure consisting of four domains. The RT domain, containing all seven of the universally conserved RT motifs, is located centrally in the gene (Figure 3). RID1, a primer binding domain (Bryan et al., 2000), and RID2, an RNA-binding domain, are located in the N-terminal proximal domain to the RT motif (Lee et al., 2003). Lastly, the C-terminal domain is responsible for promoting processive polymerization (Huard et al., 2003). hTERT is distinctive from other reverse transcriptases in that it carries hTR, an intrinsic RNA template, which is essential for *de novo* addition of telomeric sequences (Greider, 1996). Before telomerase can be assembled and active, hTERT must be modified and folded by the Hsp90 chaperone complex, minimally composed of five chaperones: Hsp40, Hsp70, Hsp90, p23 and the heat shock organizing protein (HOP) (Holt et al., 1999). Once assembled, only Hsp90 and p23 remain associated with active telomerase

(Forsythe et al, 2001) (Figure 4). The interaction with the other members of the complex is transient until proper assembly with hTR occurs. Another protein associated with hTERT regulation is 14-3-3, a ubiquitous protein that plays a key role in several pathways such as signal transduction, apoptosis and checkpoint control (Muslin and Xing, 2000). Studies have shown that the 14-3-3 proteins are also responsible for the nuclear localization of hTERT since 14-3-3 inibition, causes hTERT to remain in the cytoplasm (Seimiya, et al., 2000).

Associated with over 90% of malignant breast cancer, telomerase functions to extend and maintain telomere length, thereby enabling indefinite proliferation. Although absent in normal somatic cells, this ribonucleoprotein has been identified in a variety of other cell types, in addition to cancer, including stem cells, bone marrow and epithelial cells (Chiu et al., 1996; Wright et al., 1996; Yasumoto et al., 1996). As such a prominent molecular marker for human cancer, telomerase has proven useful for detection of recurrent disease, as well as a promising target for adjuvant cancer therapy, especially for breast cancer treatment.

RNA Interference

In 1998, Andrew Fire and Craig Mello, using *C. elegans*, characterized a new mechanism for the regulation of gene expression called RNA interference (RNAi). They discovered that injection of dsRNA resulted in the sequence specific silencing of the related gene, occurring post-transcriptionally, at the mRNA level (Fire et al., 1998).

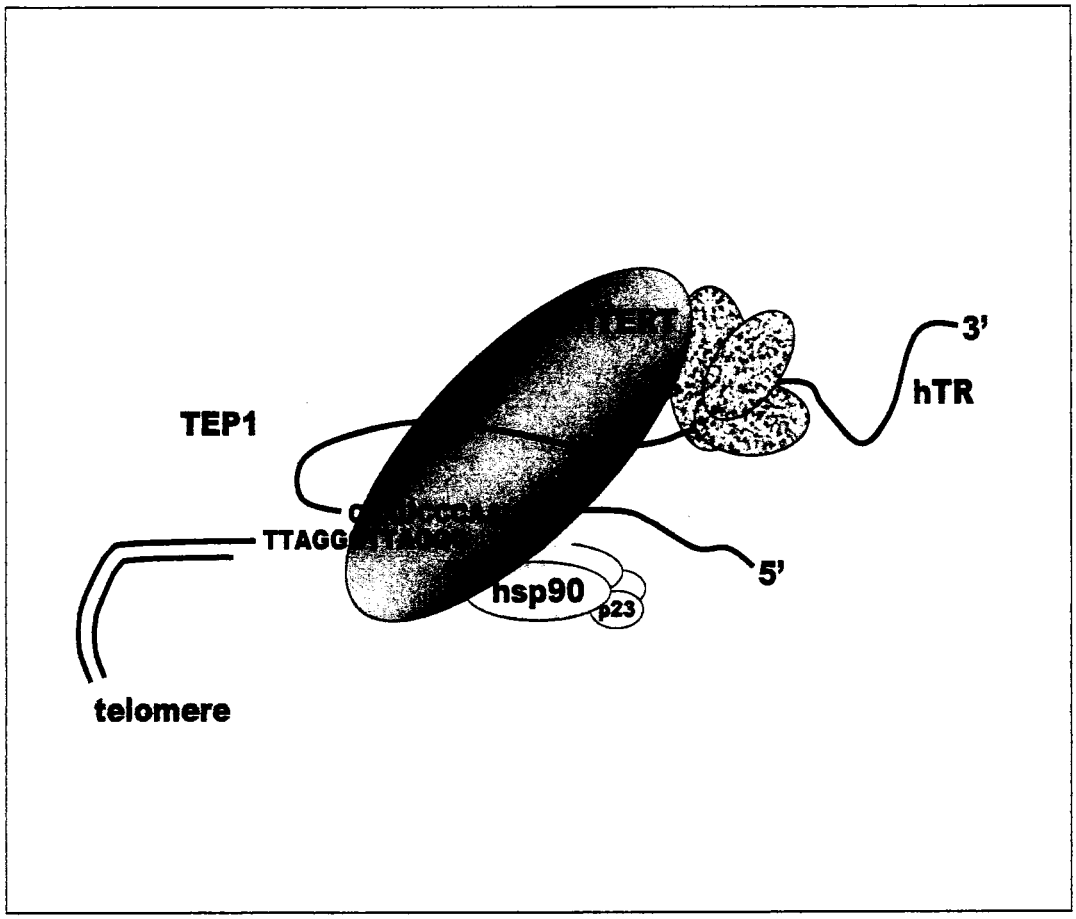


Figure 4: Schematic of Telomerase, its Components, and Functioning at the Telomere. Telomerase is a ribonucleoprotein enzyme that consists of two core components, hTR (template RNA) and hTERT (catalytic subunit). There are additional other associated proteins including telomerase associated protein (TEP-1; no known function); hsp90 chaperone complex (p23 and hsp90; modulate telomerase assembly), and the RNA (hTR) binding proteins (hnRNPs; snoRNAs; in marble). Telomerase elongates telomeres by utilizing the 11 base hTR template to add nucleotides to the 3' overhang at the end of chromosomes.

In *C. elegans*, RNAi is thought to be a protective mechanism in order to maintain genomic stability by preventing endogenous transposon activity (Tabara et al., 1999). Similarly, in humans, RNAi has been suggested to be protective against viral infections and transposons, specifically a naturally occurring cellular defense mechanism.

The RNAi mechanism involves long dsRNA being cleaved by a ribonuclease III enzyme known as DICER into short interfering RNAs (siRNA), which are approximately 21-25 base long with two-nucleotide 3'-overhangs on both ends and are complimentary to both strands of the gene (Elbashir et al., 2001). Studies report that the siRNAs then form a complex with a group of proteins called the RNA-induced silencing complex (RISC), the contents of which are not completely known (Hammond et al., 2001; Martinez et al., 2002; Mourelatos et al., 2002) (Figure 5). The components that have been identified are endonucleases, helicases and argonaute proteins. In order to activate the RISC complex, the siRNA is unwound followed by release of the sense strand, and the antisense siRNA strand then leads this complex to the appropriate complementary mRNA. Once located, the targeted mRNA is cleaved by the endonucleases within the RISC, approximately in the middle of the siRNA sequence around 10 base pairs from the beginning of the target, followed by degradation of the mRNA (Elbashir et al., 2001). In humans, the argonaute protein, Ago2, is responsible for the target mRNA cleavage. Interestingly, Dicer and Ago2 share common PAZ domains, which mediate RNA binding, but only Ago2 also has a PIWI domain for its interaction with Dicer (Sontheimer, 2005).

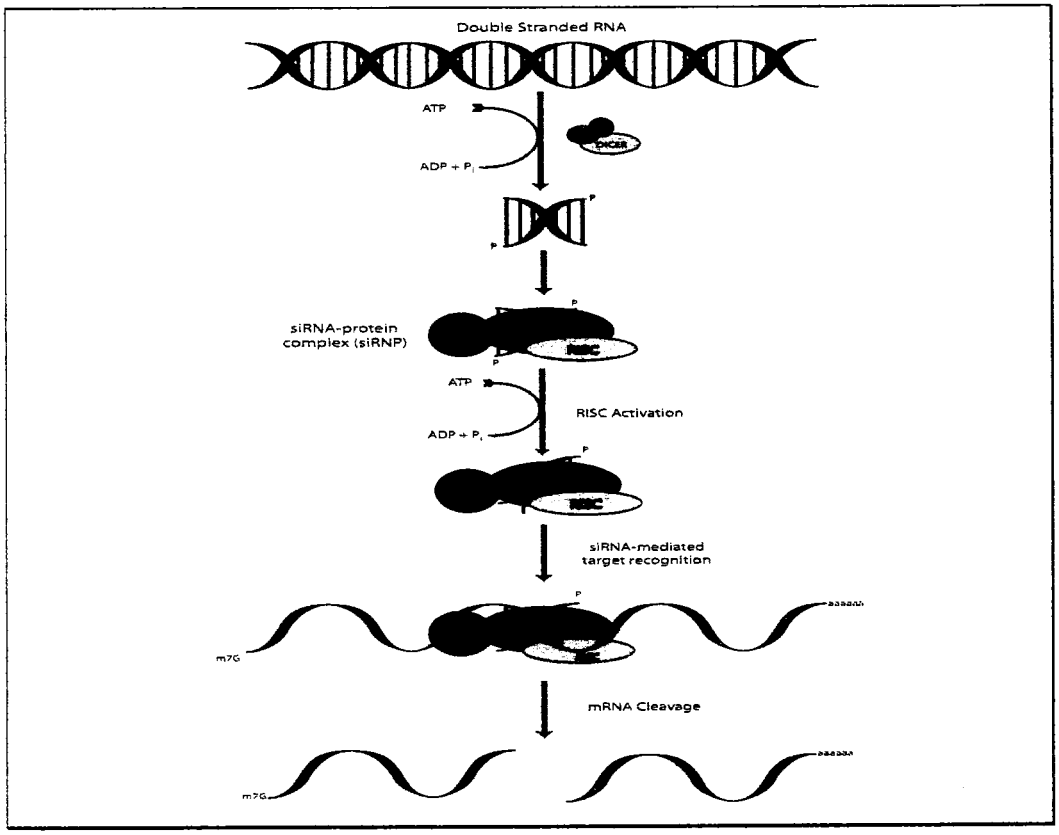


Figure 5: Schematic Representation of RNA Interference within the Cell.
Long dsRNA are processed into 21-25 bases or short interferring RNA (siRNA) by Dicer. The siRNA duplex recruits the RNA-induced silencing complex (RISC), which is then activated by releasing the sense strand of the siRNA. The antisense strand guides the complex to the targeted mRNA, followed by cleavage and degradation within the cell.

This naturally occurring mechanism has been widely exploited as a tool for silencing expression of target genes in mammalian cells. Application of RNAi in mammals, especially as a tool for cancer biology research, has been widespread with the use of a variety of delivery systems for the siRNAs, including synthetic siRNAs, viral vectors (retroviruses, lentiviruses), and DNA expression vectors. Regarding the design of siRNA, rules have been established for predicting optimal targets in the mRNA, such as regions without secondary or tertiary structures. However, other factors can inhibit effective silencing including incorporation into or stability within the RISC; interference of mRNA-associated proteins (Vickers et al., 2003); extremely high levels of mRNA within the cell causing saturation of the system; or blocking of the RISC by ribosomes due to a high rate of translation (Dykxhoorn et al., 2003). There also must be a perfect match of base pairs between siRNA and mRNA for degradation of the target to occur. However, partial complementarity may result in repressed translation (Doench et al., 2003). Therefore, in most cases, several siRNAs are designed for each target in order to determine the most efficient sequence or to combine two or more to effectively knockdown expression (Wilda et al., 2002).

However, there are complications of this technology unrelated to design or delivery that have to be considered, such as the activation of dsRNA-responsive pathways. Activation of at least two major stress response pathways occurs upon intracellular recognition of dsRNA longer than 30bp, through RNAse L or protein kinase PKR, an interferon (IFN)-inducible serine/threonine protein that regulates protein synthesis. (Silverman, 1997; Williams, 1999). Specifically, 2'-5' oligoadenylates are

synthesized which bind to and activate RNase L, a ribonuclease that causes non-specific degradation of both cellular and viral RNA, thereby leading to protein synthesis inhibition and eventually, apoptosis (Gil and Esteban, 2000). In the other pathway, upon recognition of long dsRNA, PKR is autophosphorylated and then phosphorylates the small subunit of the eukaryotic initiation factor 2 (eIF2), resulting in a cessation of translation followed by cellular apoptosis (Stark et al., 1998). However, activated PKR also functions as a signaling transducer, resulting in the production of interferons, which are cytokines that protect the cell from viral infection. Interferons then activate a signaling cascade including tyrosine kinases, Jak1 and Tyk2, as well as signal transducers STAT 1 and 2 (Haque and Williams, 1998). Also, upregulated within the nucleus is the transcription factor IRF9 and ultimately, interferon-stimulated genes which mediate proliferation as well as apoptosis (Stark et al., 1998).

Dominant Negative hTERT

The rate-limiting component of the telomerase complex is the levels of hTERT, while hTR is constituitively expressed. Most somatic cells express little or no hTERT and therefore lack telomerase activity, whereas the majority of tumorigenic cells have telomerase activity because they express hTERT (Feng et al., 1995). Numerous studies have shown that telomerase is vital for the sustained growth of malignant cells by stabilizing telomere length, and is not merely a secondary marker of the transformed state. This is supported by a study that ectopically expressed hTERT which resulted in restored telomerase activity, lengthened telomeres and indefinite proliferation (Bodnar,

1998). In 1999, Hahn et al., created a protein that abolished the catalytic function of hTERT and acted as a dominant negative (DN-hTERT) in order to ascertain how prevention of the catalytic function of hTERT would affect growth. This was attained by substituting two amino acids at positions 710 and 711 in the third reverse transcriptase (RT) motif of hTERT, specifically an aspartic acid and valine with alanine and isoleucine, respectively. Introduction into mammalian cells resulted in telomere shortening, limited growth and in some instances, apoptosis (Hahn et al., 1999).

Basically, DN-hTERT retains the ability to interact with the same elements as the wild-type protein, but the mutation within the protein prevents some part of its function and adversely affects the endogenous wild-type gene product. In this instance, there are several theories regarding how the DN-hTERT protein works. One theory involves oligomerization of hTERT at the telomere. Studies have shown that human telomerase functions as a multimer with two cooperating hTR molecules and presumably, two hTERT molecules (Tessmer et al, 1999; Wenz et al., 2001). Therefore, if telomerase functions as a dimer at the telomere, the DN-hTERT retains the dimerization domain but because of the mutations, one or both of the protein dimers would be missing one of the functional or catalytic domains. Specifically, within each cell there would be heterodimers of mutant/wild-type telomerase as well as homodimers of mutant/mutant telomerase and wild-type/wild-type telomerase (Rahman, et al., 2006). Because there are few molecules of telomerase within every cell, less than 100, and of those complexes approximately only 10-20% assemble and function properly, the DN-hTERT is able to highly decrease if not completely block telomerase activity when ectopically over-

expressed. Another theory suggests that the DN-hTERT blocks function of telomerase by simply binding to the components of telomerase, resulting in the sequestering of the telomerase-associated proteins and telomerase and preventing the telomerase complex from functioning at the telomeres. However, there is not one proven supposition of how the DN-hTERT operates within the cell.

Telomerase and Breast Cancer

Cancer is the second leading cause of death, and the occurrence of cancer is strongly tied to advanced age (Gu, et al., 2005). Genomic instability, including telomere dysfunction, is thought to be a major contributory factor in aging and progression to cancer (Harley, et al., 1990). Last year approximately 211,000 new cases of invasive breast cancer occurred among women in the United States (American Cancer Society Annual Report, 2006). Currently, the primary treatment for breast cancer consists of surgery and adjuvant therapies including chemotherapy, hormone therapy, and localized radiation. Chemotherapeutic drugs have major drawbacks for the patient in that they are highly toxic and lack selectivity. Therefore, despite the initial success of these clinical approaches, the frequent recurrence of breast cancer indicates that resistance to therapy is common in breast tumors and is one of the main causes of failure of cancer treatment. Affecting almost 50% of breast cancer patients, metastatic disease is one of the major challenges for breast cancer treatment today (Hendersin et al., 1998). Despite advances in diagnosis, staging and management, metastic disease is still essentially

incurable resulting from its evasion of traditional chemotherapy and acquired drug resistance.

The pathogenesis of breast carcinoma proposes that hyperplasia progresses to atypical hyperplasia, which subsequently progresses to ductal carcinoma *in situ* (DCIS) and invasive carcinoma. Studies have demonstrated a correlation with increased tumor aggressiveness and concurrent elevation in telomerase levels (Mokbel, 2000; Hoos, 1998). However, telomerase activity does not increase in adjacent normal tissues. In 95% of breast carcinomas telomerase is up-regulated and with this association researchers have discovered several different markers frequently involved in breast cancer that affect telomerase activity. One such oncogene is c-MYC, which functions to activate hTERT expression through its binding to the E-box promoter region (Greenberg, 1999). The breast cancer susceptibility gene 1 (BRCA1), a negative regulator of telomerase, binds the protein c-MYC, thereby inhibiting hTERT activation within the cell (Zhou, et al, 1999). In addition, hTERT transcription has been shown to be stimulated by estrogen, and upstream of the hTERT promoter two estrogen-responsive elements have been identified (Kyo, et al., 2003).

The anthracyline antibiotic Adriamycin (AdR), a topoisomerase II inhibitor, is a core drug in the treatment of breast cancer. Using a well characterized model system for breast cancer, one study found a direct relationship between p53 activity and telomere dysfunction in the induction of senescence in MCF-7 breast cancer cells. Specifically, MCF-7 cells were stably infected with ectopic hTERT, resulting in an increase in telomerase activity followed by telomere lengthening (Elmore et al. 2002).

After an acute treatment with AdR, a matching senescence response, as with controls, was observed in these cell lines with elongated telomeres. Senescence occurred within days and therefore, without sufficient time for telomere shortening to occur. In addtion, the cells displayed high numbers of karyotypic anomalies involving the telomeres as compared with interstitial abnormalities, such as end-to-end fusions, end breaks, ring chromosomes and radials. Thus, indicating preferential targeting of the telomeres by AdR for DNA damage causing a lack of protection of the chromosome ends because of single and double strand breaks amassing, ultimately leading to senescence. They also demonstrate that the knock-down of p53 in MCF-7 cells resulted in a delayed apoptosis instead of the typical senescence response after treatment with AdR, thereby signifying the importance of this protein to the AdR-induced senescence phenotype.

As such a ubiquitous marker of a majority of cancers and accordingly, therapeutic opportunities would be greatly improved if a synergism between telomerase inhibition and established anti-tumor strategies could be shown. The data demonstrate that telomerase inhibition is a slow method utilized to cause apoptosis in cancer cells in that there is a lag of a few weeks, depending on the length of the telomeres, between administration and observable phenotype (Corey, 2002). This delayed effect has been observed in knockout mice (Blasco et al., 1997; Liu et al., 2000), as well as after treatment with oligonucleotide inhibitors (Herbert et al., 2001) and dominant negative hTERT (Hahn et al., 1999; Zhang et al., 1999).

Our goal in this study was to compare the efficacy of different telomerase inhibition strategies in concert with standard chemotherapeutic agents at triggering

senescence and/or apoptosis in cultures of breast cancer cells. We predicted that telomerase inhibition strategies will sensitize breast cancer cells to traditional chemotherapies, potentially reducing the lag phase, allowing for more potent anti-tumor effects at lower doses and therefore ultimately imparting less toxicity to the patient. We blocked telomerase by targeting hTR and hTERT, individually and collectively utilizing synthetic siRNA, shRNA and DN-hTERT in MCF-7 breast cancer cells. We analyzed the efficiency of telomerase inhibition for each strategy alone and then treated the cells with two mainstay chemotherapeutic agents, Adriamycin (AdR) and Taxol. The most effective telomerase inhibition strategies were synthetic siRNA and DN-hTERT, individually. After treatment with various concentrations of Adr or Taxol, breast cancer cells with inhibited telomerase grew significantly slower and exhibited widespread senescence or apoptosis within a much shorter time period and at a dose that alone is insufficient to trigger cytostasis. In addition, we provide evidence that cells in which telomerase was inhibited were more sensitive to anti-cancer agents, whether the drug inhibited topoisomerase II resulting in DNA damage (AdR) or blocked mitosis via protracted microtubule stabilization (Taxol). Collectively, our data indicate that alone anti-telomerase inhibition strategies differ in their efficacy, however, when used in the adjuvant setting with diverse acting chemotherapeutic agents there is a potent synergy resulting in chemotherapeutic sensitization characterized in part by widespread senescence and/or apoptosis.

Chapter 2

Materials and Methods

Materials

Adriamycin and Taxol were kindly provided by David Gewirtz, VCU. All lentivectors were kindly provided by Elizabeth H. Blackburn, UCSF

Cell Culture

All cells were cultured in RPMI 1640 containing 5% fetal bovine serum (FBS) and 800ng/ml of gentamycin. The human breast adenocarcinoma cell line, MCF-7 used in these studies has been well characterized (Elmore et al. 2002). All cells were mycoplasma free, as assessed by the mycoplasma T.C. Rapid Detection System, (Glen-Probe, San Diego, CA). Cells were washed, trysinized and resuspended in 10mls of growth media at each cell passage. The cells were counted using a hemocytometer and population doublings were computed using the formula [$\log_{10}$ (number of cells counted/number of cells plated)/log2.

Isolation of sub-clones

Isolation of sublones was conducted as follows: cells were counted, 500 total cells seeded and subsequently, monitored for approximately two weeks at which time clones were selected depending on which selectable marker was present within the vector utilized. Specifically, the selection was random in clones containing the puromycin

marker or by visualization on an OLYMPUS 1X 70 fluorescent microscope (Optical Elements Corporation) with the clones having the green fluorescent protein (GFP) marker. The individual clones were isolated using sterile cloning cyclinders dipped in sterile grease and placed over each colony. Following trypsinization, each clone was placed into a 48-well plate and grown to confluency. Cells were then passaged into larger plates and this method was repeated until a sufficient number of cells had grown so that a stock of each clonal cell line could be stored frozen (liquid nitrogen), as well as used for subsequent assays.

Design of siRNAs

Two of the short interfering RNA (siRNA) sequences targeting hTR were from other researchers (hTR-2 and hTR-3) and the other two were novel designs developed for this study (hTR-T and hTR-1 (Li et al. 2005). Similarly, the hTERT sequences were from others (Nakamura et al. 2005; Masutomi et al. 2003). These sequences were utilized for transient transfections (Lipofectamine RNAiMAX Transfection Reagent, Invitrogen), as synthetic siRNA, as well as permanent insertion into the genome (Fugene 6 Transfection Reagent, Roche), specifically with the retroviral vector pSUPER.retro (SUPpression of Endogenous RNA) system (Oligoengine). Briefly, a 19 nucleotide target sequence specific to hTR or hTERT was identified and used to synthesize a pair of 64-mer oligonucleotides with the target sequence in the sense and anti-sense orientation separated by a 9 nucleotide spacer region (TTCAAGAGA). The synthesis of the 64-mer in this manner allows for the formation of a hairpin and therefore, initiating RNA

interference within the cell by dicer (Figure 5). Linkers containing 5'Bgl II (GATCCCC) and 3'Hind III sites (TTTTTGGAAA) allowed the directional cloning of this oligonucleotide into the pSUPER.retro vector. The complementary oligonucleotides were annealed according to the manufacturers' instructions prior to cloning into the pSUPER.retro vector. The siRNA target sequences were synthesized as shown in TABLE 1. The numbers bracketing the siRNA sequence represents the cDNA nucleotide number.

TABLE 1. siRNA sequences for hTR and hTERT

Name	**hTR Target Sequence (5'-3')**	**Target Region**
hTR-T	48-***AACCCUAACUGAGAAGGGC***-66	Template
hTR-P	175-***AAUGUCAGCUGCUGGCCCG***-193	Pseudoknot
hTR-2	44-***GUCUAACCCUAACUGAGAAUU***-62	Template
hTR-3	165-***GCAAACAAAAAUGUCAGCUUU***-184	Pseudoknot
	hTERT Target Sequence (5'-3')	**Target Region**
hTERT-1	2659-***UGAUUUCUUGUUGGUGACA***-2677	RT motif
hTERT-2	3114-**TTTCATCAGTCAAGTTTGGA**-3134	Exon 14

Prior to transfecting cells with the siRNA containing vectors, the presence of the correct insert was confirmed by a restriction endonuclease digestion and running the results on a 1% agarose gel to confirm that the correct size was obtained.

All lentivectors targeting the template region of hTR were kindly provided by

Elizabeth H. Blackburn, UCSF (Li et al. 2004). They include:

1. pHR'CMVGFPWSin18(ΔClaI->LS29/30): Vector Control
2. pHR'CMVGFPWSin18-U6siRNAnew: hTR shRNA
3. pHR'CMVPuroWSin18(ΔClaI->LS29/30): Vector Control
4. pHR'CMVPuroWSin18-U6siRNAnew: hTR shRNA

Transient Transfection

Since the cell line being manipulated is MCF-7 cells, the Lipofectamine RNAiMAX (invitrogen), which is specifically developed for the transfecation of siRNA duplexes into eukaryotic cells, recommends a reverse transfection. So for a 6-well plate, 500 μl of Opti-MEM I Reduced Serum Medium (Invitrogen) was added to each well followed by a total of 150pmole of synthetic siRNA duplexes either singularly or in combination. This was mixed gently and then 6.25 μl of Lipofectamine was added to the diluted RNAi molecules. After mixing gently again, the solution was incubated at room temperature for 10-20 minutes and then approximately 250,000 cells in 2.5ml of media were added to each well, giving a final RNA concentration of 50nM. The plate was then rocked back and forth, and incubated at 37°C (with CO_2) allowing at least 24 hours before samples were assessed or treated with chemotherapeutic agents.

Generation of Retroviral Cell Lines, Lentiviral Cell Lines and Infection

The pSUPER.retro and DN-hTERT vectors were transfected using Fugene 6 Transfection Reagent (Roche) into the 293T competent packaging cell lines but had to be

co-transfected with two other additional vectors for packaging proteins (pCl and VSVG) (Naldini et al. 1996). The resulting retroviral supernatant was collected at 24, 48, and 72hrs, filtered with a 0.45μM filter and incubated with MCF-7 cells for 24hrs for infection. The cells were then allowed to recover for 24hrs with normal media followed by selection of cells stably infected with the vector with puromycin (1mg/ml). Isolation of individual clones was described above.

Similarly, the lentivectors targeting only hTR were transfected using FUGENE Reagent into 293T cells and also co-transfected with two added vectors for supplementary packaging proteins (pPAX and pMD2G) (Naldini et al. 1996). The resultant lentiviral supernatant was collected at 2, 4, and 6 days later. After filtration with a 0.45μM filter, the supernatant was incubated with MCF-7 cells for 24hrs for infection. The cells were then allowed to recover for 24hrs in normal media followed by selection of cells stably infected with the vector with puromycin 8μL/10mL of media (stock: 1mg/ml). However, two of the lentivectors have a green fluorescent protein marker and in those cell lines no drug selection occurred and cell lines were manipulated upon recovery.

SA-β-galactosidase

Cells were washed twice in PBS, fixed for 3-5 minutes at room temperature with 2% formaldehyde/0.2%glutaraldehyde in PBS and washed twice more with PBS. After that the cells were incubated overnight (~12-16 hours) at 37°C (without CO_2) with fresh senescence associated β-gal stain solution consisting of: 20mg/ml x-gal in

dimethylformamide, 0.2 citric acid/Na phosphate buffer (pH 6.0), 100mM potassium ferrocyanide, 100mM potassium ferricyanide, 5M sodium chloride, 1M magnesium chloride, and water. The next day cells were washed two times with PBS followed by storage in fresh PBS at 4°C. Representative images were captured using an OLYMPUS 1X 70 fluorescent microscopes (Optical Elements Corporation).

Telomeric Repeat Amplification Protocol (TRAP Assay)

Using the TRAP-eze detection kit (Chemicon), telomerase activity with the cells was determined according to the manufacturers' protocol. Cell pellets of approximately 100,000 cells were lysed in CHAPS lysis buffer for 30 minutes on ice, followed by centrifugation and removal of the supernatant. The substrate oligonucleotide (TS), which represents a telomere, is end-labeled with γ-^{32}P-ATP and incubated with the cell lysates (~250 cells) for 25 to 30 minutes at room temperature. After extension of the TS by telomerase, products were amplified using a PCR based assay in 27 cycles (94°C for 30s, 60°C for 30s). In order to assess activity, the products were run on a 10% polyacrylamide gel, placed on a PhosphorImager cassette (Molecular Dynamics, Sunnyvale CA) for exposure. The resulting image was analysed and quantitated by the ImageQuant software (Molecular Dynamics) which calculates the ratio of the internal control (36bp) to the laddered telomerase products.

Western Blot Analysis

Cells pretreated with Adriamycin or after stable integration of siRNA, lentivectors, or DN-hTERT were harvested at ~80% confluency, washed in PBS, trypsinized and pelleted. The cell pellets were then lysed for 30 minutes on ice in RIPA buffer (50mM Tris pH 7.4, 150mM NaCL, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, 1% Aprotinin and 100mM DTT). Total cellular protein (10-50μg) was denatured for 10 minutes at 85°C followed by electrophoresis on a 12% SDS-PAGE gel at 120V for 1.5 hours. The proteins were then transferred to nitrocellulose membrane (BioRAD) at 85V for 1.5 hours. The membrane was then blocked for one hour using a solution containing 1X PBS with 0.1% Tween-20 and 5% milk (blocking buffer) at room temperature. The membranes were then incubated with α-p53 (Pharmagen; 1:5000), α-p21 (Pharmagen; 1:5000), α-β-actin (Sigma; 1:5000) and/or α-hTERT (Rockland; 1:1000) in the blocking buffer for 1 hour; washed 2 times for 5 minutes each (1XPBS with 0.1% Tween-20); incubated with mouse or rabbit secondary antibody conjugated with horseradish peroxidase (BioRAD) for 1 hour (1:5000) in blocking buffer and washed twice more for 5 minutes each. Using the Pierce ECL detection reagents, the protein bands were detected after exposure to Kodak X-OMAT film. Semi-quantitation was achieved by calculation of the signal from the protein of interest relative to β-actin signal using spot densitometry software (ChemiImager 4400) on Alpha Innotect Imaging System (Alpha Innotech Corporation, CA).

Telomere Amount and Length Assay (TALA)

The measurement of telomeres was conducted using solution hybridization (Gan et. al., 2001). First, a telomere specific probe $(TTAGGG)_4$ was labeled using γ-^{32}P-ATP and polynuclotide T4 kinase (Gibco BRL) for 30 min at 37°C followed by purification using the Qiaquick Nucleotide Removal Kit (Qiagen). Then 2.5μg genomic DNA was digested using the Hae III, Hha I and Hinf I restriction enzymes for 4hr at 37°C in ReACT 2 buffer (Gibco BRL). Approximately 1.5ng of ^{32}P-labeled probe was added to this mixture, heated or denatured at 98°C for 6 minutes, hybridized for 18hr at 55°C and cooled to 4°C for at least 5 minutes. Samples were then loaded onto a 0.8% agarose gel and run for 18hr at 60V in 0.5X TBE (0.045 M Tris-borate, 0.001 M EDTA). The resulting gel was then dried, placed on a PhosphorImage cassette for 24hr, and photographed using a PhosphorImager. The resulting image was analyzed by the ImageQuant software (Molecular Dynamics) using the area-under-curve method and calculations of telomere length were performed according to Gan et al (2001). Briefly, the point representing 50% of the area-under-curve was the mean telomere length.

TdT-Mediated d-UTP-X Nick End Labeling (TUNEL) Assay

Cells were made into a dilution of 20,000 cells in a 100μL of PBS and centrifuged directly onto microscope slides using the Shandon Cytospin 2 (800 rpm for 10 min). The cells were then fixed in 4% formaldehyde in PBS solution for 10 minutes at room temperature and then washed twice in PBS for 5 minutes each. Next, the cells were fixed with an acetic acid:ethanol (2:1) solution for 5 minutes at -20°C and washed two more

times with PBS to remove the fix from the previous step. The subsequent steps were executed in a humidified chamber covered with aluminum foil. The slides were blocked for 30 minutes in a BSA(1mg/ml)/PBS solution at room temperature; washed twice with PBS and incubated with 20μL of an enzyme mix for 60 minutes at 37°C under light sensitive conditions. The enzyme mix consists of: 4μL terminal transferase (Roche), 80μL 5X reaction buffer, 40μL 25mM $CoCl_2$, 8μL Fluorescein 12-dUTP (Boehinger Mannheim), and 268μL water. Afterward, cells were washed twice in PBS for 5 minutes, mounted in Vectashield (Vector Labs), and stored at 4°C. Representative images were captured using an OLYMPUS 1X 70 fluorescent microscopes (Optical Elements Corporation).

Colony Forming Efficiency Assay

In order to compare growth after acute treatment with Adriamycin (AdR), approximately 2000 cells were plated in 10cm dishes. The next day a range of concentrations of AdR was administered (0, 0.2, 0.08, 0.25, and 0.75μM/L) as an acute exposure for 2 hours and then replaced with normal media after the cells were washed twice with PBS. Six different cell lines were tested (MCF7 parental, DN2, DN6, DN9, pBABE2, pBABE3) with each drug concentration per cell line completed in triplicate. The cells were maintained for 10 days followed by fixing and staining. Cultures were fixed using a 3.7% formaldehyde solution for 10 minutes at room temperature, washed twice with PBS, stained with a crystal violet solution for 2-3 minutes, washed with water, and allowed to air dry. Colonies were counted using the mammalian cell counter

ColCount (Discovery Technology International, LLP). Statistical siginificance was determined using a two-tailed t-test.

RT-PCR Analysis of hTR and hTERT

RNA isolation from approximately $1x10^6$ cells was performed using the RNeasy Mini kit with QIAshredders (both Qiagen). The RETROscript RT-PCR kit (Ambion), a 2-step RT-PCR reaction, was utilized to assess the levels of RNA according to the manufacturers' instructions. Approximately 1-2μg of total RNA was used in each reaction. In step 1 the reverse primer R3c (hTR specific) is employed to capture fragments of hTR for reverse transcription, but in those reactions seeking hTERT, random decamers were used. In addition to the primers, there are dNTPs, RNase inhibitors and a reverse transcriptase, which are incubated for 1 hour at 44°C followed by 10 minutes at 92°C, in order to inactivate the reverse transcriptase. Step 2 is the PCR amplification of the cDNA wherein a mixture of 2.5μl of the RT reaction, dNTPs, target specific primers and thermostable DNA polymerase is used. Samples were denatured at 94°C for 2 minutes and for 26 cycles PCR (94°C for 30sec, annealing temp. for 30sec, 72°C for 40sec) in the reactions for hTR and 18S but for hTERT there was 33 cycles of PCR. In order to analyze, an aliquot of the resultant PCR samples were run on a 2.5% agarose gel in the presence of ethidium bromide and visualized under UV light on the Imaging System (Alpha Innotech Corporation).

Chapter 3

Genetic Inhibition of hTR and Sensitization of MCF-7 Cells

Inhibition of Telomerase Activity by Synthetic siRNA Targeting hTR

Telomerase is expressed in the majority of human cancers, approximately 90%, as well as being highly elevated in over 70% of immortalized cell lines, thus making its detection extremely promising for aiding in cancer diagnosis and prognosis, as well as viable therapeutic target. (Kim et al. 1994; Shay and Bacchetti, 1997). However, in normal adult somatic cells telomerase is not active, but the RNA component hTR is ubiquitously expressed. Investigators have shown that the full gene dosage of hTR is highly important within the cell, as seen in patients with the autosomal dominant form of non-X-linked dyskeratosis congentia, which in one family pedigree, was caused by haploinsufficiency or one nonfunctional copy of the hTR gene. These patients have progressive bone marrow failure and die in early adulthood or middle age (Vulliamy et at. 2001; Comolli et at. 2002). This suggests that inhibition of telomerase by reducing hTR levels may have deleterious effects on cancer cells.

Attempts to inhibit telomerase activity usually result in gradual telomere shortening followed by a lag period, ultimately resulting in cellular death or senescence. Since the telomerase holoenzyme is composed of multiple components, there are many potential targets for achieving telomerase inhibition, but in this chapter we will focus only on the human telomerase RNA. Here we describe an important genetic method for sensitization of the breast tumor cell line, MCF-7, by using siRNAs that target hTR as a

pretreatment to traditional chemotherapeutic treatment, adriamycin and taxol. This approach is especially appealing in that the chemotherapies utilized here are shown to be effective at lower dosages, which will ultimately cause less toxicity to the patient and this sensitization effect within the cells occurs within a much shorter time frame in which there is no lag period.

Using the proposed secondary structure of human telomerase RNA (Chen et al., 2000), we designed 4 target sequences within hTR for RNAi (hTR-T, hTR-1, hTR-2, hTR-3) (Figure 6). All targets are located within the template and pseudoknot domains because these regions have been shown to be vital for telomerase activity due to their interaction with hTERT within the telomerase complex. Sequences were synthesized in the sense and antisense direction individually, approximately 21 nt long, and were then annealed together using a duplex buffer followed by transfection into the MCF-7 breast tumor cells. To assess the consequences of telomerase inhibition after transfection with the siRNAs hTR-T and hTR-1, samples were taken 6, 24, 48 and 72 hours post-transfection for use in a TRAP assay (Figure 7). The only significant reduction detected was with hTR-1 (pseudoknot) at the 72 hour time point. We then tested the ability of the second set of siRNA to inhbit telomerase. The synthetics were transfected into the cells, but in order to optimize knock-down of telomerase, we used three different concentrations for the siRNAs, hTR-2 and hTR-3 (Figure 8), and samples were taken for TRAP 1-3 days post-transfection. We found high levels of telomerase inhibition at all concentrations using the hTR-2 siRNA with the most effective inhibition of telomerase at

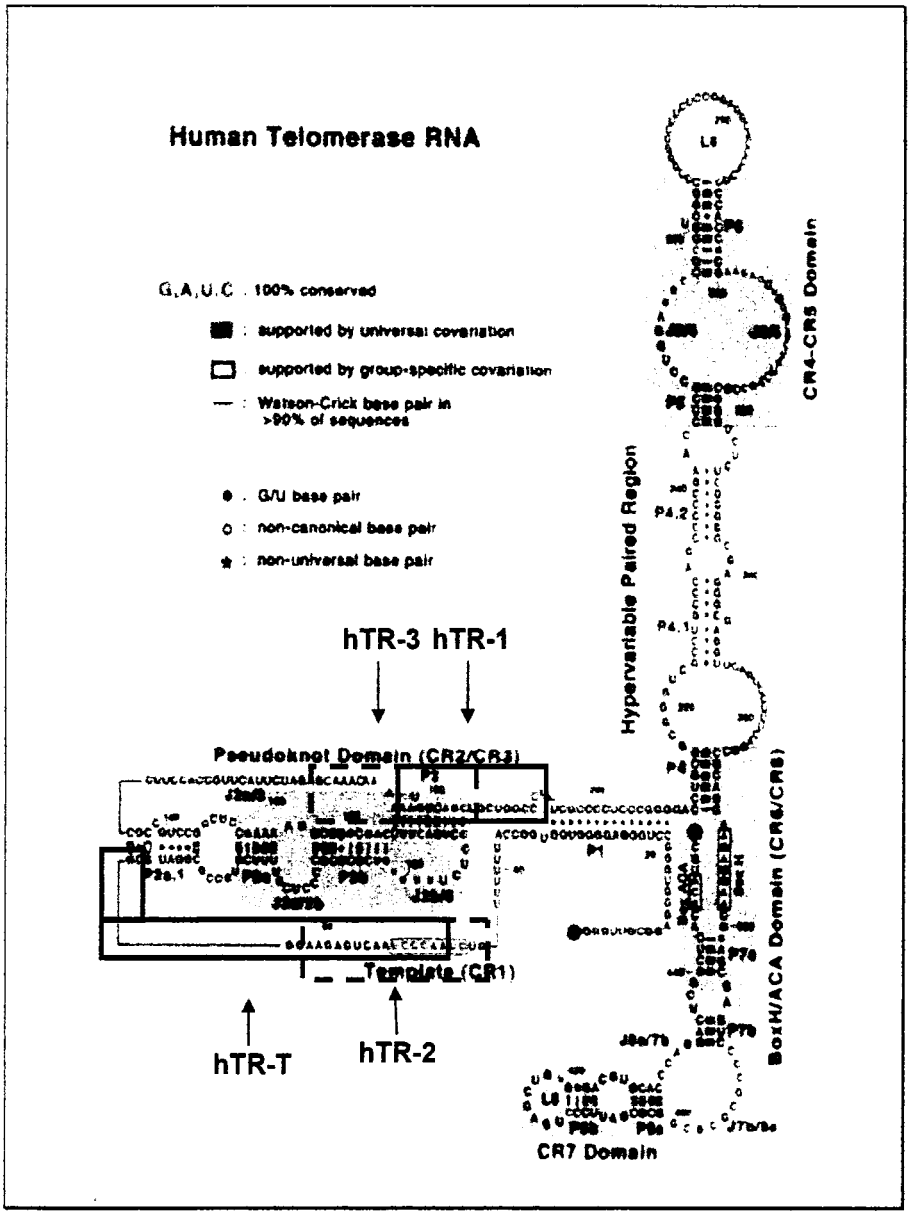


Figure 6: Human Telomerase RNA and siRNA Targets.
Proposed secondary structure of human telomerase RNA (Chen et al., 2000) with the five conserved regions enclosed in boxes except for the template region. Also labeled, within the black boxes, are the siRNA target sequences (hTR-T, hTR-1, hTR-2, and hTR-3) with the template and pseudoknot regions respectively.

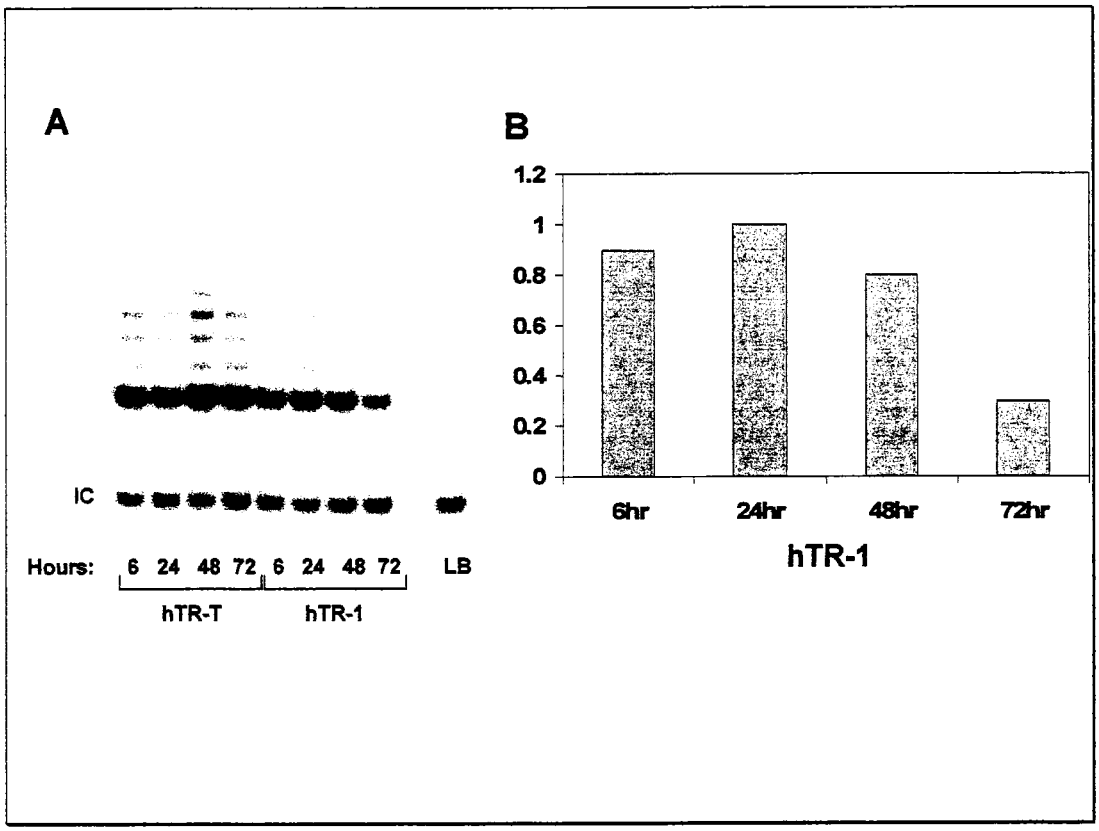


Figure 7: Decline in Telomerase Activity using siRNA Targeted Against hTR. Using the synthetic hTR-T (template) and hTR-1 siRNA (psuedoknot) targeting specific domains of hTR, cells were transfected, harvested and tested for telomerase activity. **A.** Representative telomere repeat amplification protocol (TRAP) showing 250 cell equivalents per sample at various time points is shown. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC), which normalizes sample to sample variation. We observe a reproducible 3 to 4-fold decline in activity after 3 days of treatment.

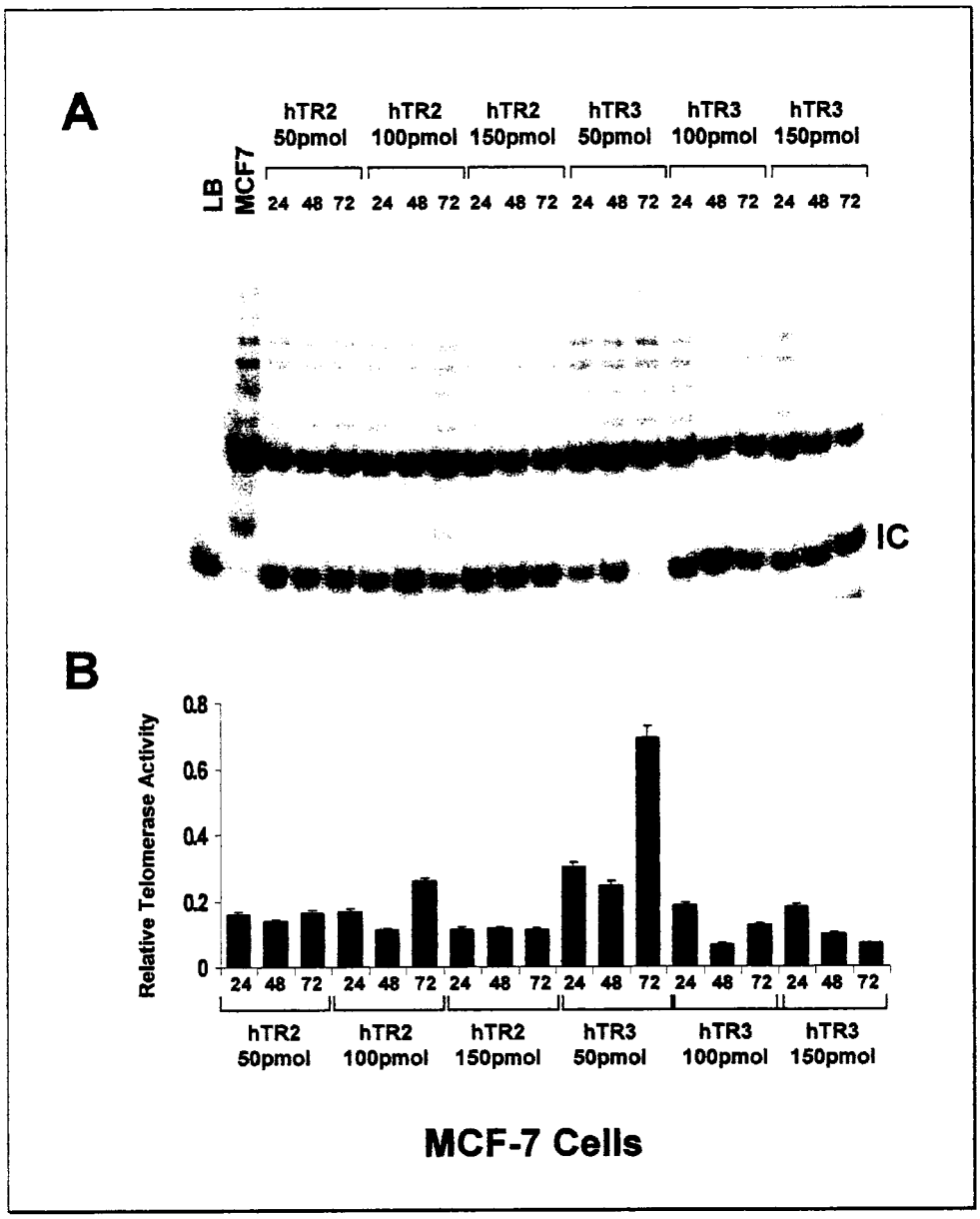


Figure 8: Optimization of Telomerase Inhibition using siRNAs.
A. Representative TRAP assay showing 250 cell equivalents. Synthetic siRNAs, hTR-2 and hTR-3, were transfected into MCF-7 cells at three different concentrations (50, 100, and 150pmol) with samples taken at 24, 48, and 72 hours post-transfection. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC). Each sample was also normalized to the MCF7 cell line and shown as a percent of activity in relation to the MCF-7 cell line. We determined the greatest knockdown of activity occurs at siRNA concentrations of 150 pmol.

90% for 150pmol, which was maintained at each time point. Similarly, with the hTR-3 siRNA, the most efficient concentration of siRNA was 150 pmol with the maximal knockdown at 94%, but at lower concentrations (50 and 100 pmol) inhibition was not as significant or consistent as compared to the hTR-2 siRNA.

Our next goal was to assess if treatment with combinations of siRNAs together produced an even greater inhibition of telomerase than individually. Cells were transfected with the siRNAs (hTR-2 and hTR-3; 150pmol) individually or together, for comparison purposes, into the MCF-7 cells with samples for TRAP analysis and RT-PCR taken 1-3 days post-transfection. We observed high levels of knockdown with the siRNAs in all of the transfected cell lines (Figure 9) and found the use of the hTR-2 siRNA alone works the best in that there is a sustained knock-down of telomerase maxing at 96%. However, hTR-3 produces the greatest knockdown at 98% but this was not consistent for each time point. Both siRNAs together (hTR-2/3) provided successful inhibition at 93%, but this appeared to be a less consistent inhibition than with the other individual siRNAs. We then measured the levels of hTR using RT-PCR in order to determine if the decreased telomerase activity corresponded with a reduction of RNA levels. As compared to the MCF-7 parental samples, there were lower expression levels of hTR in all three combinations (Figure 10). The cells transfected with the hTR-2 siRNA displayed the least amount of hTR followed by hTR-3 and hTR2/3 respectively, thereby showing a correlation of telomerase inhibition and hTR levels.

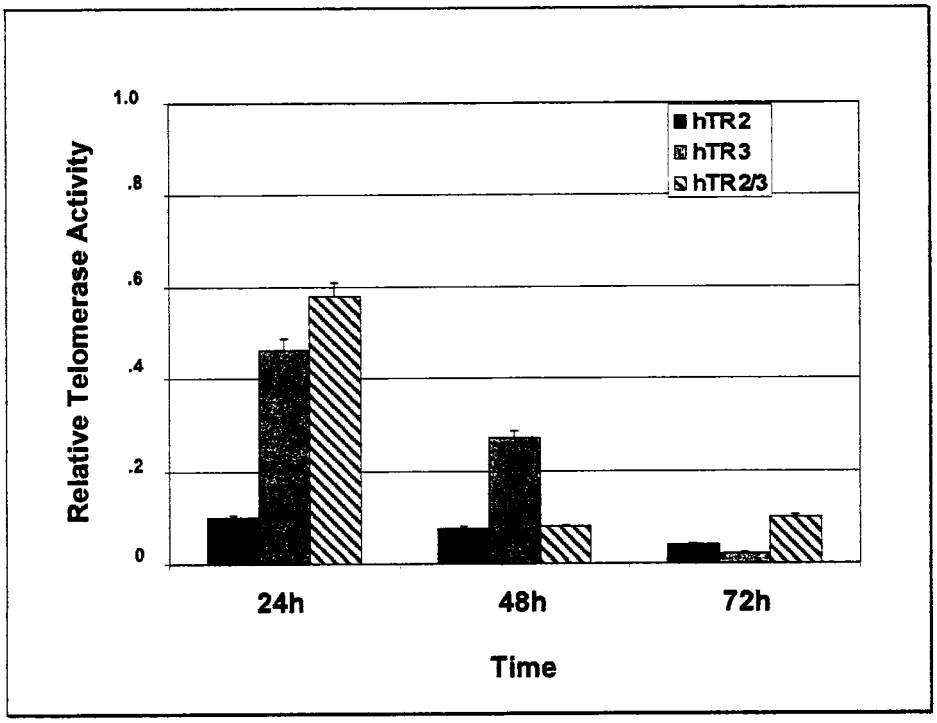


Figure 9. Knockdown of Telomerase Activity using siRNAs Targeting hTR. Treatment of MCF-7 cells with siRNAs hTR-2 and hTR-3 singularly and in combination at 150pmol concentration with samples taken at 24, 48, and 72 hours post-transfection. Quantitation of the relative telomerase activity was accomplished as stated in Figure11. We determined the greatest and most consistent telomerase inhibition occurs with the hTR2 siRNA.

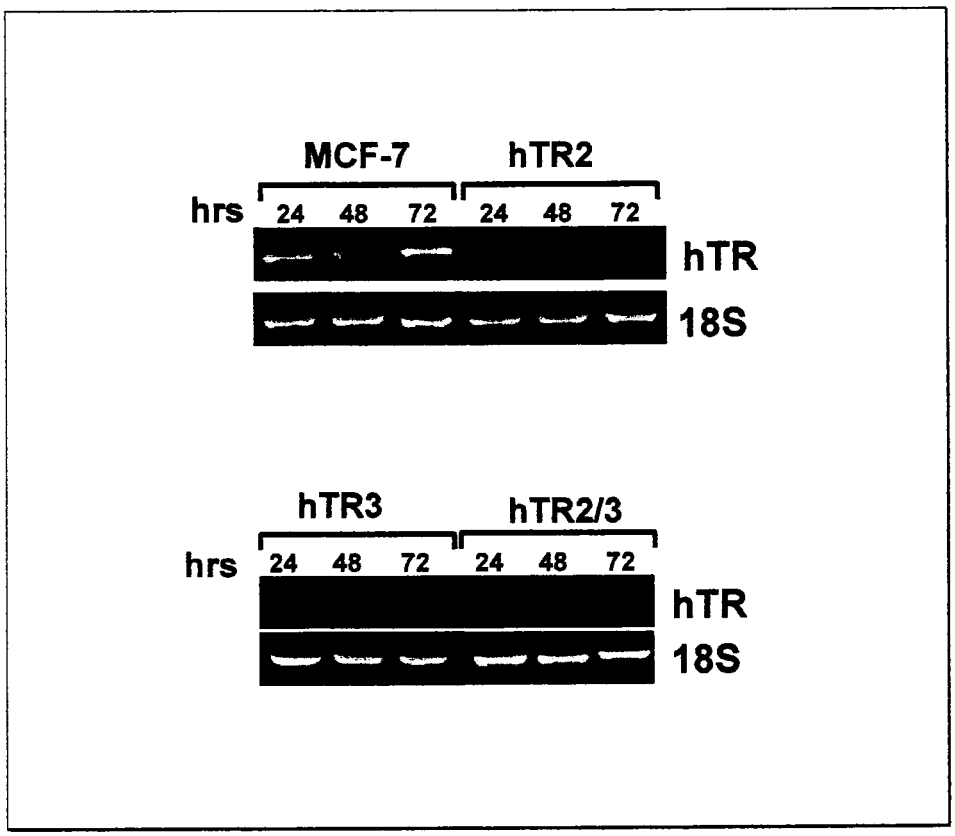


Figure 10. Decreased hTR Expression after Transfection with siRNAs. Treatment of MCF-7 cells with siRNAs hTR-2 and hTR-3, singularly and in combination at 150pmol concentration. RT-PCR was conducted to determine the expression levels of hTR 24, 48, and 72 hours post-transfection. We showed the most stable knock-down of hTR occurs with the hTR2 siRNA.

Sensitization of Breast Tumor Cells using siRNA targeting hTR as a Pretreatment

Now that significant telomerase inhibition has been established using RNAi we determined sensitization of these cells using chemotherapy. One of the older chemotherapeutic drugs that have been in use for decades for the treatment of a variety of cancers, including breast cancer, is Adriamycin (AdR) or Doxorubicin, an anthracycline antibiotic and topoisomerase II inhibitor. Adriamycin functions by blocking replication in that it stabilizes the topoisomerase II, which unwinds the DNA by breaking one strand, and DNA complex thereby preventing the DNA double helix from being resealed or ligated. Treatment with AdR has been shown to cause apoptotic death in a variety of tumor cell lines other than breast, such as human and murine leukemia cell lines (Ling et al. 1993; Zaleskis et al. 1994; Jaffrezou et al. 1996).

The siRNA hTR-2 produced the most effective inhibition of telomerase; therefore, this was the only means of a pre-treatment within the MCF-7 breast cancer cell line utilized for these experiments. The cells were transfected with hTR-2 siRNA (150pmol) and allowed to grow for three days in order for sufficient and sustained knock-down of telomerase to occur. An acute AdR treatment at various concentrations for 2 hours was given to the cells with samples taken 2 and 4 days post-AdR. Growth of the cell lines was calculated to ascertain if pre-treatment with the siRNA followed by AdR produced a growth effect (Figure 11). No difference as compared to the control MCF-7 cells was observed at day 2, but by day 4 cells had significantly slowed growth in the cells treated with the siRNA hTR-2 at all concentrations except for 0.75μM.

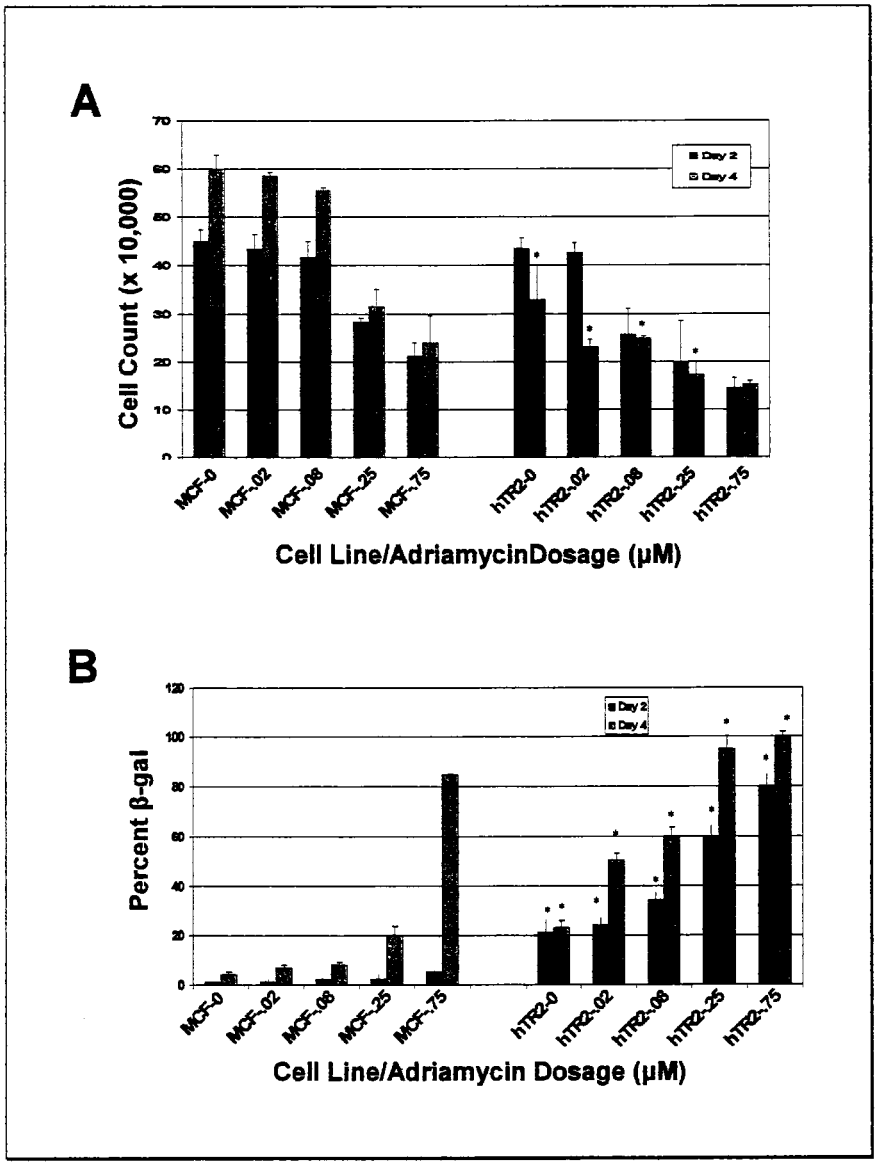


Figure 11. Decreased Growth and Increased Senescence in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the synthetic hTR2 siRNA (150pmol). Three days later, cells were treated with various concentrations of AdR, and samples were then taken 2 and 4 days post-treatment with AdR. **A.** Cell numbers were counted using a hemocytometer and samples were done in duplicate. By day 4 cell growth had slowed significantly, as compared with controls, at dosages lower than clinically relevant. **B.** Cells were fixed and incubated overnight with a β-gal staining solution. At day 2, cells transfected with hTR2 siRNA had significantly higher levels of senescence at all AdR concentrations. Columns represent the calculated mean from three representative fields of 100 cells; bars, SD. Significance for both graphs was determined by a two-tailed t-test and displayed on the graphs. *, $P < 0.05$.

In order to determine if senescence could be induced regardless of telomerase activity or telomere length, we quantitated cellular senescence in the cells post-treatment with AdR. The senescent phenotype is illustrated by a viable cell that is incapable of responding to proliferation or apoptotic signals. Senescence is further characterized by flattened and enlarged cell morphology as well as by increased levels of the biomarker, senescent-associated beta galactosidase (SA-β-gal) (Lee et al. 2006). We detected levels of SA-β-gal in cells using the chromogenic substrate X-gal, which stains the cells blue when activated at pH 6. Significantly elevated levels of senescence were found by day 2 in all cell lines as compared to the MCF-7 control cells regardless of the concentration of AdR administered (Figure 11). Other studies have shown induction of senescence in MCF-7 cells 3-4 days after exposure to the clinically relevant dosage of 1μM AdR (Elmore et al. 2002). Therefore, generation of such high levels of senescence 2 days post-treatment and at concentrations of AdR as low as 0.02μM is extremely significant, revealing definite sensitization of those cells to AdR and providing further support that the senescence response is telomere length independent.

Lastly, we examined the percent apoptotic cells after treatment with AdR using the TUNEL assay, in order to determine how apoptosis was affected by pre-treatment with hTR-2 siRNAs and subsequently, AdR (Figure 12). Apoptosis, or programmed cell death, is characterized within the cell by several phenotypes, some of which include cell shrinkage, nuclear fragmentation, condensation of the nuclei and cytoplasm, as well as loss of cell surface structures. In order for this process to occur, several signaling pathways must be activated, which in turn specifically activate a group of proteases or

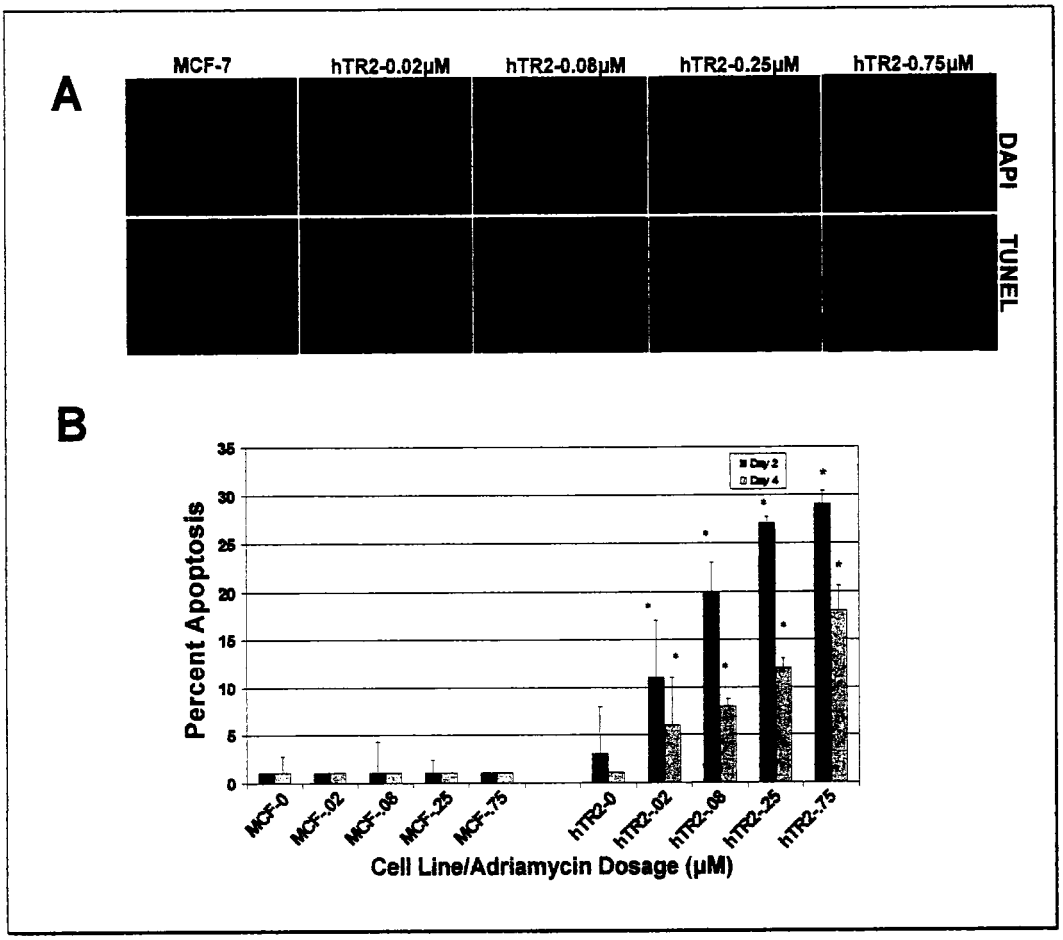


Figure 12. TUNEL Staining for Apoptosis in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the hTR-2 siRNA construct. Three days later, cells were treated with various concentrations of AdR. Samples were then taken 2 and 4 days post-treatment. **A.** At day 4, increased apoptosis was evident. The DAPI (blue) stains the nucleus and the Fluoroscein 12-dUTP stains the cells positive for apoptosis (green). **B.** Quantitation of TUNEL assay revealing, at the concentrations of 0.02-0.75 μM AdR were the levels of cell death appreciably higher at days 2 and 4 than the controls. Columns represent the calculated mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

enzymes known as the caspases. Ultimately, caspase activation leads to the cleavage of DNA into <200bp fragments, which can then be detected using the TUNEL assay. Specifically, cells are fixed and incubated with fluoroscein 12-dUTP, which is transferred onto the ends of the fragmented DNA by a terminal deoxynucleotidyl transferase. Positive cells are stained intensely green, which is judged against the DAPI staining of the same set of cells in order to compare the nuclear staining and the TUNEL positive cells, as well as confirm that a positive TUNEL signal is in fact a cell and not debris. As compared to the control MCF-7 cell line, we found increased incidences of apoptosis in all samples except the untreated cell lines containing only hTR-2 siRNA. So from exposure to AdR at dosages as low as 0.02μM, a 9-fold difference to the clinically relevant dosage of 0.75μM, the hTR-2/MCF-7 cells underwent cell death at significantly higher quantities than the control cell lines. Other studies have shown that MCF-7 cells will only undergo senescence after treatment with AdR because these cells are p53 positive, but we provide evidence that apoptosis does occur at clinically relevant dosages of AdR (Elmore et al. 2002).

As a result, we wanted to assess levels of p53 in the cells to determine how the cells responded to the chemotherapeutic treatments. We transfected cells with either hTR-2 or a scrambled siRNA shown to have no effect within the cell, and 3 days later the cells were treated with various concentrations of AdR for two hours (Figure 13). Samples for western blots were taken 6 and 24 hours post-treatment to assess protein levels. Constituitively, p53 levels were slightly higher in the hTR-2 siRNA cell lines

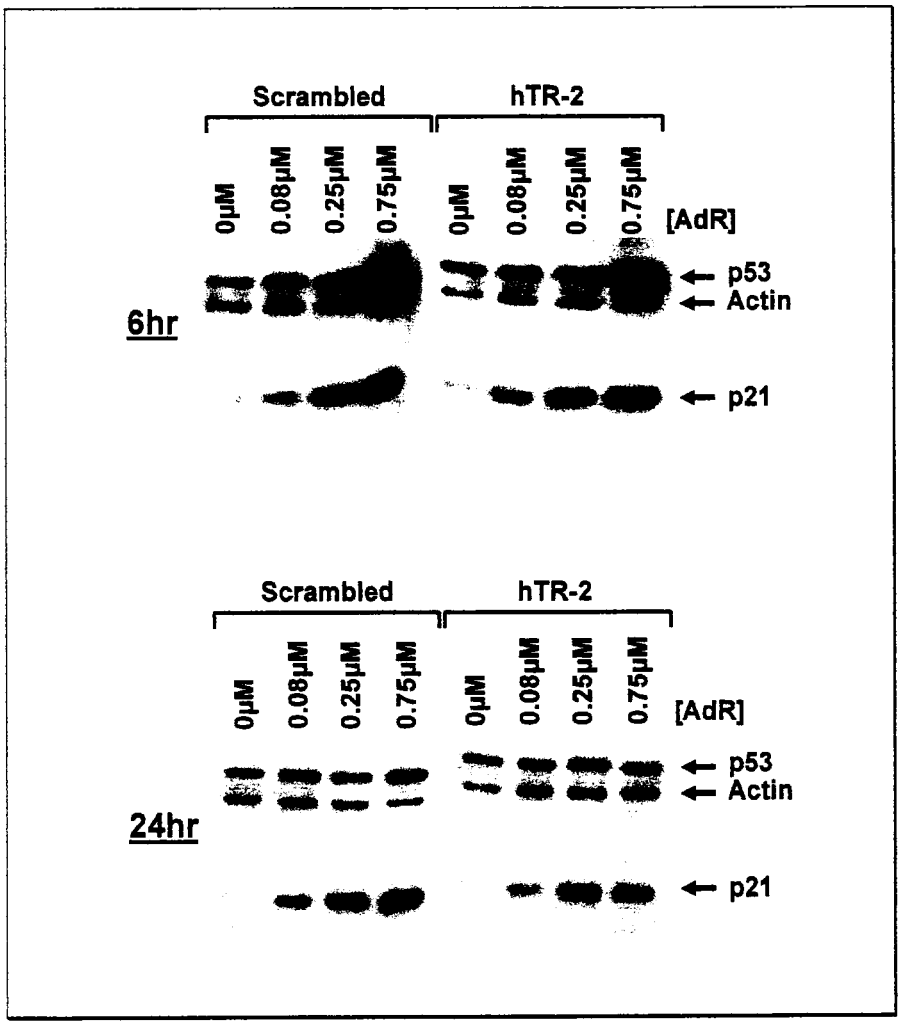


Figure 13. Induction of DNA Damaging Proteins after Treatment with Adriamycin. After transfection with the hTR-2 siRNAs and scrambled siRNAs, the MCF-7 cells were treated with a 2 hour acute dose with various concentrations of AdR. Samples were taken 6 and 24 hours post-treatment, cell pellets were collected, and 15μg of total protein lysate was subject to Western analysis. Immunoblots were probed with anti-p53, anti-p21, and anti-actin as a loading control.

than those found in the scrambled siRNA control cell lines, suggesting a possible increase in DNA damage after telomerase inhibition. As expected, there was an up-regulation of p53 and therefore, p21 activated by p53, after treatment with AdR at all concentrations at the 6 hour time point. However, by 24 hours post-treatment, the elevated levels of p53 had decreased back to normal. In both cell lines, there were no real differences in regulation of p53 during this comparison.

Another mainstay in the treatment of breast cancer, early stage and metastatic, is the chemotherapeutic drug known as Taxol or paclitaxel, which is a member of the group of taxanes (Jemal et al. 2005). Taxol binds to and promotes the formation of mitotic splindle microtubules and then stabilizes them, thereby preventing depolymerization during cellular division (Horwitz et al. 1993; Rao et al. 1995). As a result, the segregation of the sister chromatids is also prevented and the cells are blocked at G2/M phase of the cell cycle. The destruction of the normal dynamic reorganization of the microtubule network, as well as the block in the cell cycle, ultimately leads to cell death (Milross et al. 1996; Yvon et al. 1999).

In order to compare sensitization of MCF-7 breast tumor cells to different types of chemotherapeutic drugs, we again utilized the synthetic siRNA hTR-2 as a pretreatment as it produced the most effective inhibition of telomerase and the same experimental method. Briefly, cells were transfected with hTR-2 siRNA (150pmol) and allowed to grow for 3 days. An acute taxol treatment at various concentrations for 2 hours was administered to the cells, followed by samples harvested at 2 and 4 days post-treatment. The first time the experiment was conducted the range of taxol (0.0-1.5μM/L) was too

high in that the toxicity of the drug caused no significant differences between the hTR-2 and MCF-7 cell lines because neither cell line grew as shown in the day 2 samples and even less growth was seen by day 4 (Figure 14). Therefore, the experiment was repeated with a lower range of taxol dosages (0.0-0.15μM). The control cell lines grew at a steady rate, and the MCF-7/hTR-2 cells lines had significantly slower growth at every taxol concentration compared to controls.

We then evaluated the levels of senescence to assess how taxol affects senescence after telomerase is inhibited. We found at day 2 post-treatment, all cell lines revealed significantly high levels of senescence regardless of the concentration of taxol administered (Figure 15). However, by day 4 only taxol concentrations from 0.0-0.015 μM displayed considerably elevated levels of senescence, implying the higher drug concentrations of 0.05 and 0.15 μM produced similar results as the MCF-7 control cell lines and sensitization only occurred at the lower two dosages.

Finally, we assessed levels of cell death to determine the effects of taxol post-treatment with the siRNA hTR-2. Apoptosis increased significantly for samples taken on both days, especially on day 4 when the percent cell death ranged from 35-50%, but only for the drug concentrations of 0.015-0.15 μM taxol (Figure 15). Other researchers have shown apoptosis induction by taxol treatment in MCF-7 cells by 2 days at dosages of .010 μM, but in that study, taxol treatment was chronic (for the entire 48 hours), whereas our treatment was acute (for only two hours) (Wu et al. 2006).

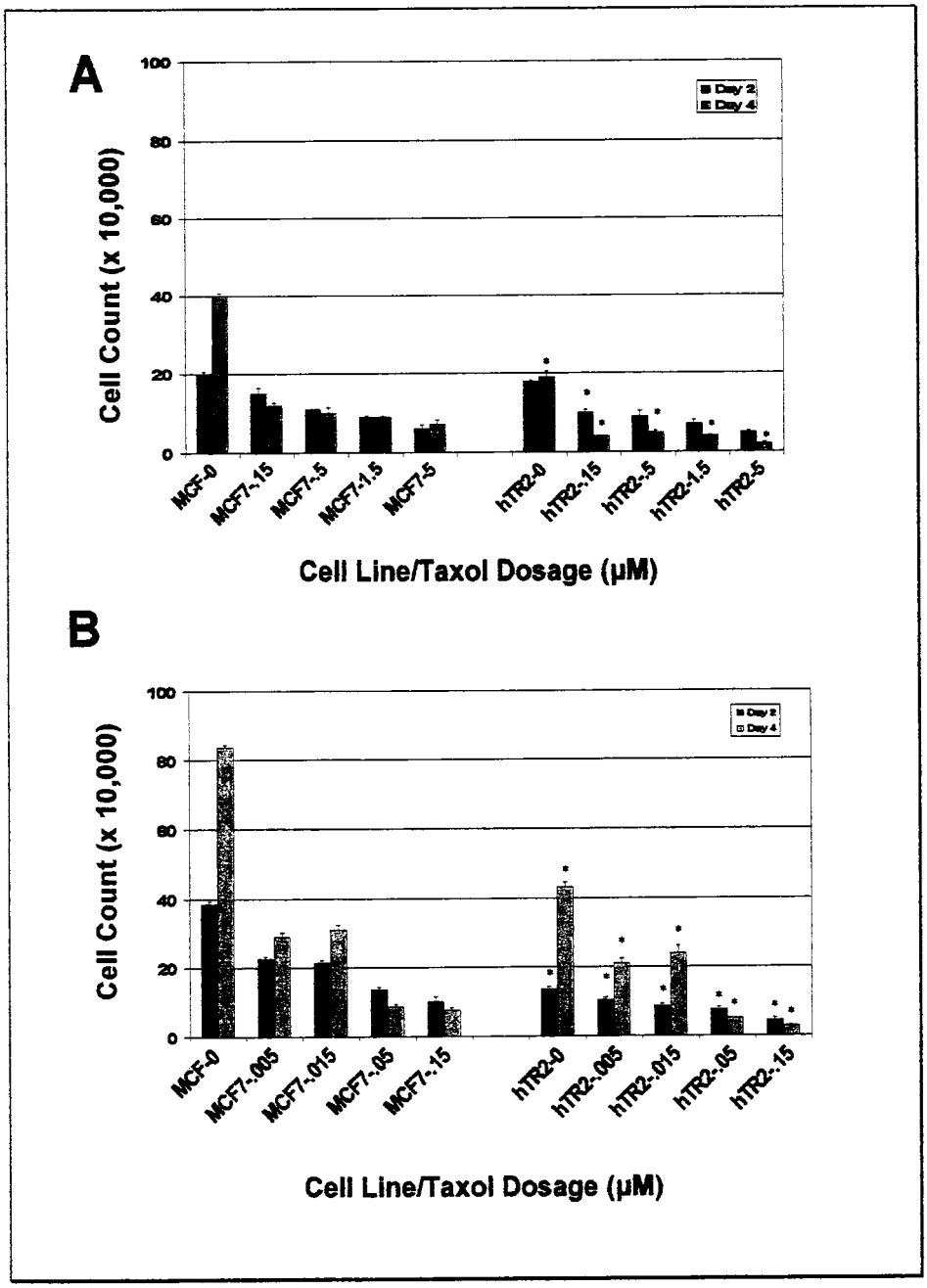


Figure 14. Growth of MCF-7 Cells Post-Treatment with Synthetic siRNAs and Taxol. Cells were transfected with the hTR2 siRNA construct. Three days later, cells were treated with various concentrations of taxol. Samples were then taken 2 and 4 days post-treatment. Cell numbers were in duplicate. The higher taxol (A) concentrations were too toxic for both the control and siRNA cell lines, compared to lower (B) range of concentrations. Columns represent the calculated mean from three representative fields of 100 cells; bars, SD; *, $P < 0.05$.

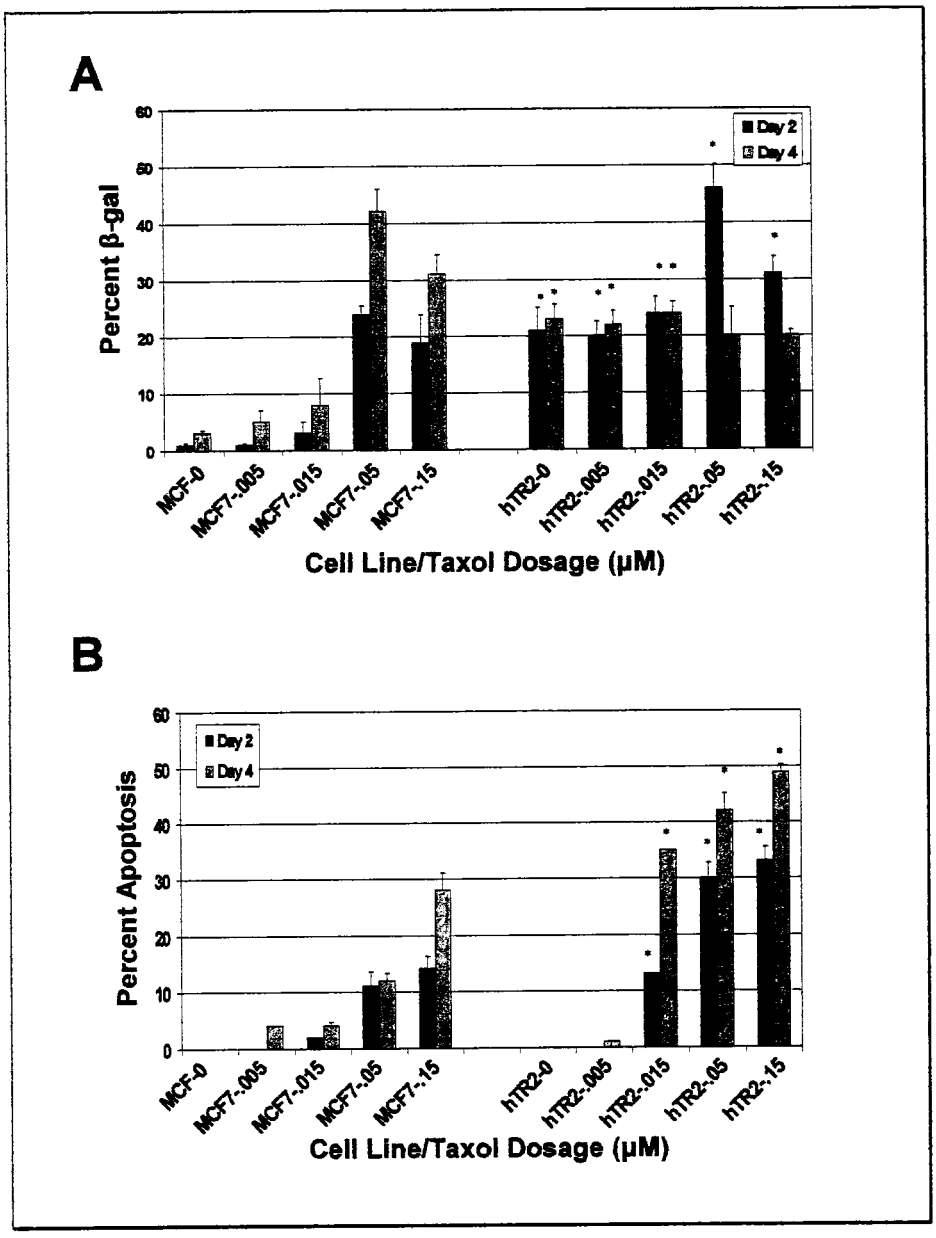


Figure 15. Cells Treated with Synthetic siRNAs and Taxol Showed Increased Sensitivity to Apoptosis. Experimental method is the same as in Figure 17. **A.** Cells were in fixed and incubated overnight with a β-gal staining solution. Differences were observed in senescence were seen in day 2 but only at taxol concentrations from 0.0-0.015 μM on day 4. B. TUNEL assay showing percent apoptosis and levels were considerably higher in the cells treated with 0.015-0.15 μM Taxol on both days as compared to MCF-7 cells. Columns mean from three representative fields of 100 cells; bars, SD; *, $P < 0.05$.

Chronic Inhibition of Telomerase using RNAi Directed at hTR in MCF-7 Cells.

Now that we had established loss of telomerase and sensitization with transient expression of siRNA, we wanted to assess the long-term affects after permanent insertion of the siRNA sequence into the genome using a retroviral vector. Studies have shown the pSUPER.retro vector to be highly efficient at stable gene suppression and functional inactivation of gene expression through the production of siRNA within MCF-7 breast cancer cells (Brummelkamp et al. 2002) (Figure 16).

Utilizing the specific hTR siRNAs, oligonucleotides were synthesized for cloning into the vector with the sense and antisense sequences on the same 64nt oligo separated by a 9nt spacer sequence (Figure 16). This enables the folding and formation of a hairpin loop within the cell, which will activate the RNAi pathway (see Figure 5), and cleavage by DICER to create functional siRNAs with the 19 nt target sequence plus the necessary 2-nt 3' overhangs of UU. Vectors were then infected into the MCF-7 cells followed by selection with puromycin (800ng/mL) for five days and clonal populations were grown, approximately two weeks. These experiments were performed prior to the creation of the hTR-2 siRNA sequence, so only hTR-T and hTR-1 were used. Telomerase activity was analyzed using the TRAP assay in the clonal isolates (Figure 17). Seven clones were chosen with samples taken weekly over the course of 1 month. We found that introduction of the siRNAs, hTR-T and hTR-1, caused significant reduction of telomerase activity in only some of the MCF-7 clonal populations with significant variability among the clones with the greatest knockdown of activity found in hTR-T

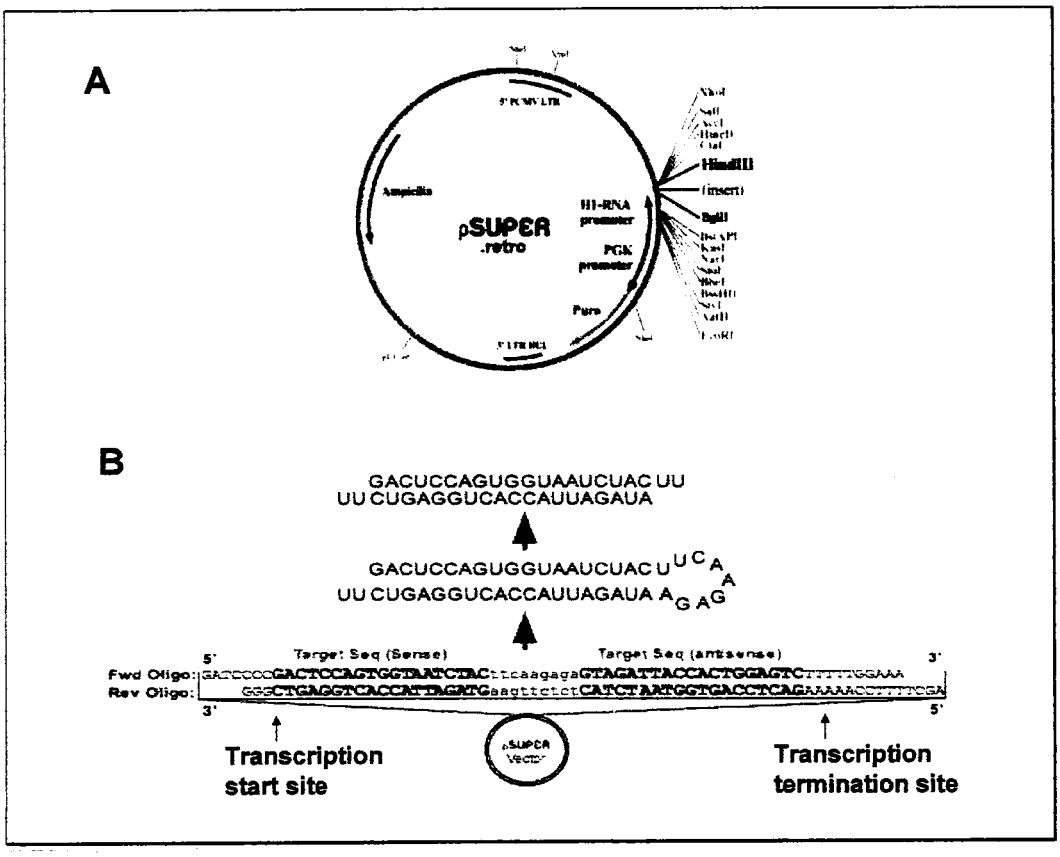


Figure 16. Schematic of the pSUPER.retro Vector. A. Map of the retroviral vector revealing the H1-RNA promoter as well as the location of the insert between the BglII/HindIII sites and puromycin selection tag. **B**. Depiction of how the siRNA sequences were synthesized for insert into the vector with the sense and antisense sequences on the same 64nt oligo separated by a 9nt spacer sequence. This enables the folding and formation of a hairpin loop within the cell, which will activate the RNAi pathway and cleavage by DICER to create functional siRNAs with the 19 nt target sequence plus the necessary 2-nt 3' overhangs of UU.

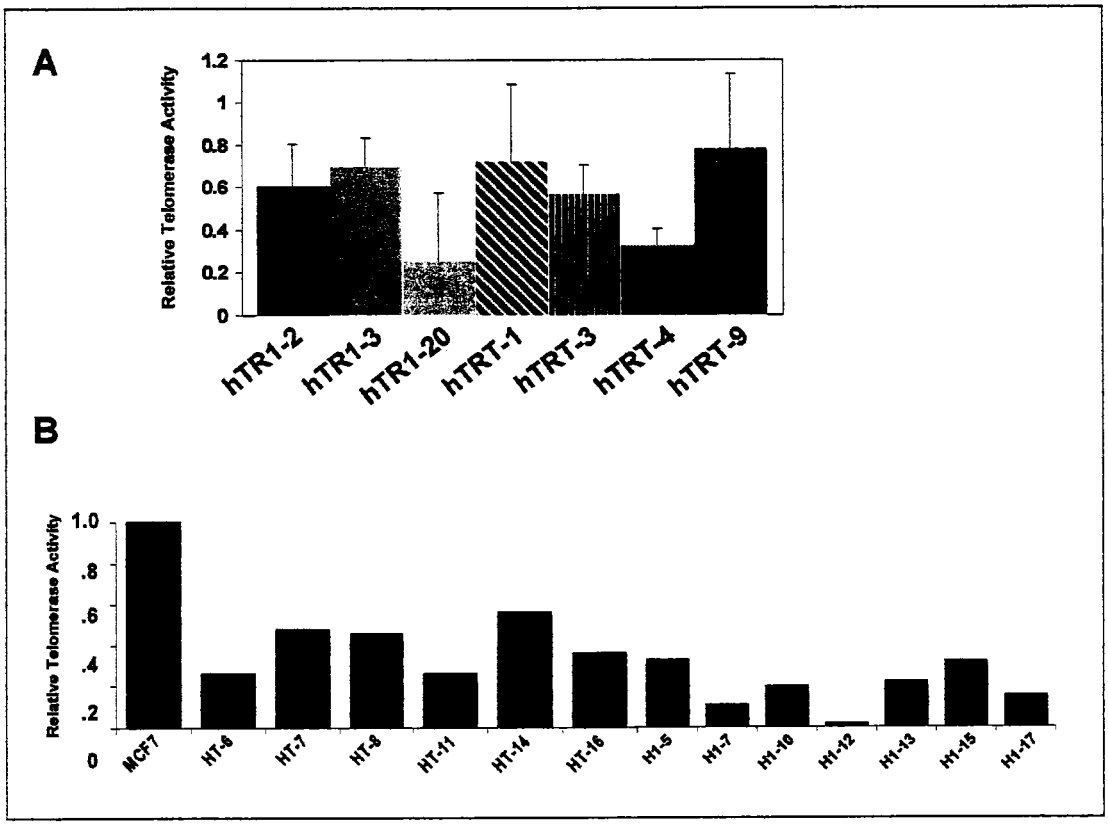


Figure 17. Decline in Telomerase Activity using siRNAs Targeted against hTR is Inconsistent Over Time. The sequences used previously as synthetic siRNA(hTR-T and hTR-1) were then inserted into the pSUPER.retro vector. Cell were infected, selected and grown up into clonal populations. Cells were harvested once a week for a month and tested for telomerase activity using the TRAP assay. **A.** Quantitation of the various clonal populations with vast variability within all clones as seen by the standard deviation. **B.** Other clonal populations were also tested with only one sample per cell line and a mostly insignificant knockdown of telomerase activity.

clone 4 and hTR-1 clone 20. Other clones were also screened for low telomerase activity levels but there was immense inconsistency among the clones.

Due to the variability seen using the pSUPER.retro, we acquired a lentiviral system (Li et al. 2004) containing the hTR-2 sequence targeting the template region of hTR. Here we show that a lentiviral system efficiently delivers and maintains expression of the siRNA sequences. The map of the lentiviral construct (Figure 18) reveals U6 promotor that expresses the short hairpin RNA (shRNA), which will activate the RNAi pathway and thus, DICER within the cell. We utilized two different cassettes with distinict markers, puromycin resistance marker (PURO) or green fluorescent protein (GFP). Because lentiviruses infect both dividing and non-dividing cells at an extremely high efficiency, we did not grow clonal isolates. Therefore, cells were infected with the lentivector cassettes followed by samples taken every two days beginning on day 4 post-infection as with the GFP cassette or the cells were infected with the PURO cassette followed by selection with puromycin (800ng/mL) for five days. After selection, samples were taken every two days starting with day 8. To assess the ability of the lentivectors to knockdown hTR in MCF-7 cells, we measured hTR levels using RT-PCR at the various timepoints on whole cell populations, showing decreases in hTR levels found in cell lines with both lentivectors (Figure 18).

To determine if the decreased RNA levels corresponded with a reduction in telomerase activity, we conducted a TRAP assay of both cell lines at every time point (Figure 19). Again, samples were taken from cell populations and not clonal isolates. The cells infected with the GFP lentivector displayed telomerase activity inhibition

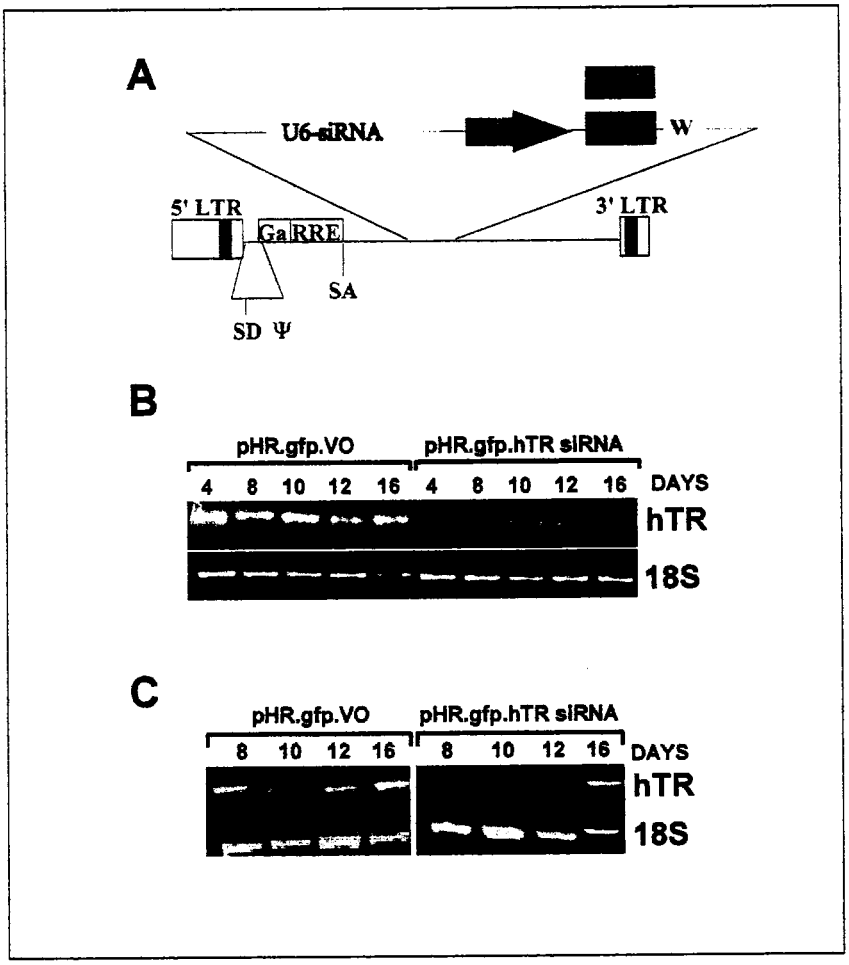


Figure 18. Schematic of the Lentivector Expressing siRNA and Resultant Decline of hTR Expression Levels. A. Short Hairpin RNA (shRNA) construct targeting the template region of hTR that expresses the siRNA from the U6 promoter as well as two alternative markers, puromycin resistance marker cassette or green fluorescent protein. **B.** MCF-7 cells were infected with the lentivectors, and samples were taken at every 2 days post-infection (pHR.gfp) or post-selection (pHR.puro) for 16 days. RT-PCR revealing hTR RNA levels at the various timepoints. Decreases in hTR levels were found in cell lines with both lentivectors.

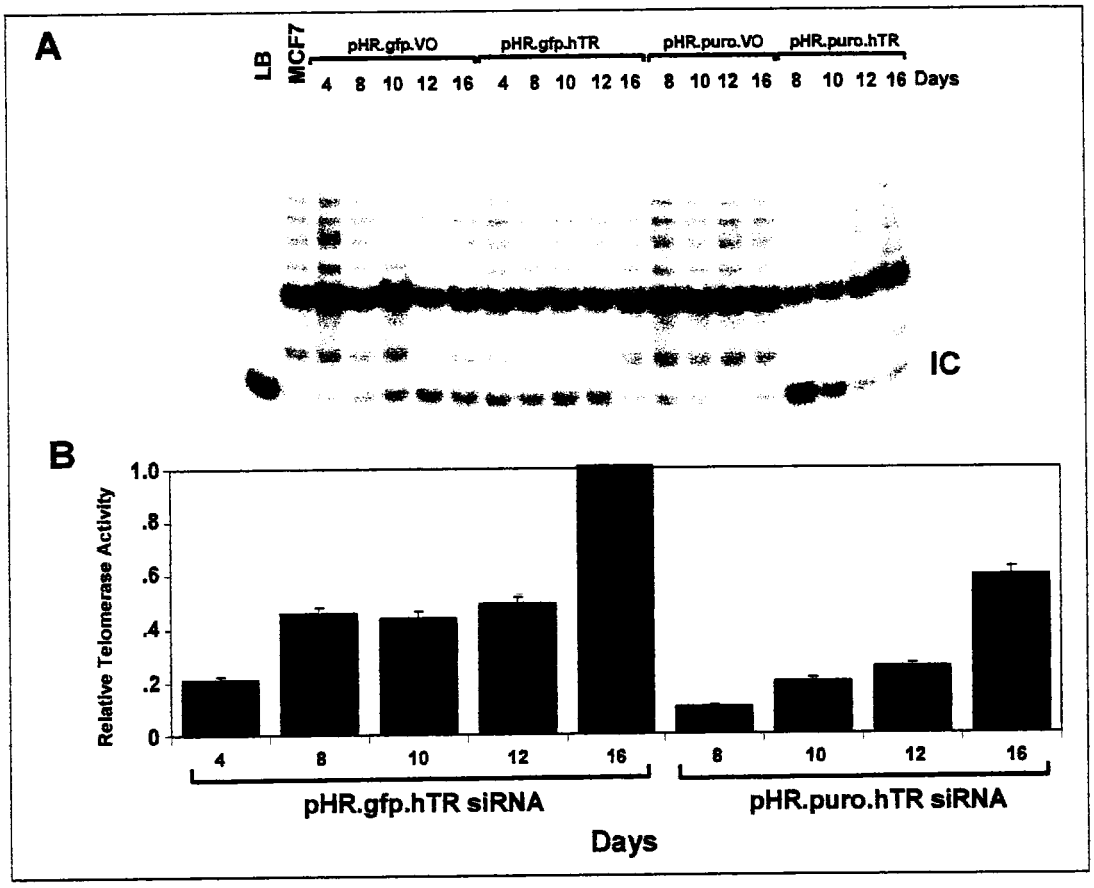


Figure 19. Telomerase Inhibition using a shRNA and Lentiviruses.
A. Representative TRAP assay showing 250 cell equivalents. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC). Each sample was also normalized to the MCF7/pHR.VectorOnly (VO) cell line and shown as a percent of activity in relation to the MCF-7/pHR.VectorOnly cell line. Both constructs produced efficient knockdown of telomerase activity, however, the puromycin lentivector appears to be slightly superior at maintaining the knockdown.

ranging from 80-50% from day 4 to day 12. However, with the PURO vector inhibiton of telomerase was maintained up to day 16 from 90-40% with the greatest decrease of telomerase seen at day 8. The spike in telomerase activity seen on day 16 in the PURO infected cell lines was also observed with the increased hTR levels in the RT-PCR (Figure 18).

Sensitization of MCF-7 Cells after Chronic Inhibition of Telomerase.

Now that significant telomerase inhibition has been established over time using RNAi targeting hTR, we determined sensitization of these cells using chemotherapy. Because the PURO vector produced the most effective knockdown of telomerase, we utilized this vector for all of the sensitization experiments. Briefly, MCF-7 cells were infected with the PURO cassettes, vector only (VO) and hTR siRNA, followed by selection with puromycin (800ng/mL) for five days. On day 8, the cells were administered an acute 2 hour AdR treatment and samples taken 2 and 4 days post-treatment. Growth of the cell lines (Figure 20) was calculated to ascertain if pre-treatment with the siRNA followed by AdR produced an effect. By day 4, the cells containing the hTR siRNA had significantly slower growth than that of the empty vector control MCF-7 cells (VO) except at AdR concentrations of 0.75μM, which equaled that of the VO cells.

Our next step was to determine senescence within the cells in order to see if increased levels resulted from treatment with AdR. At day 2 there were significant increases in the percent of senescent cells as compared to the VO cells, for those treated

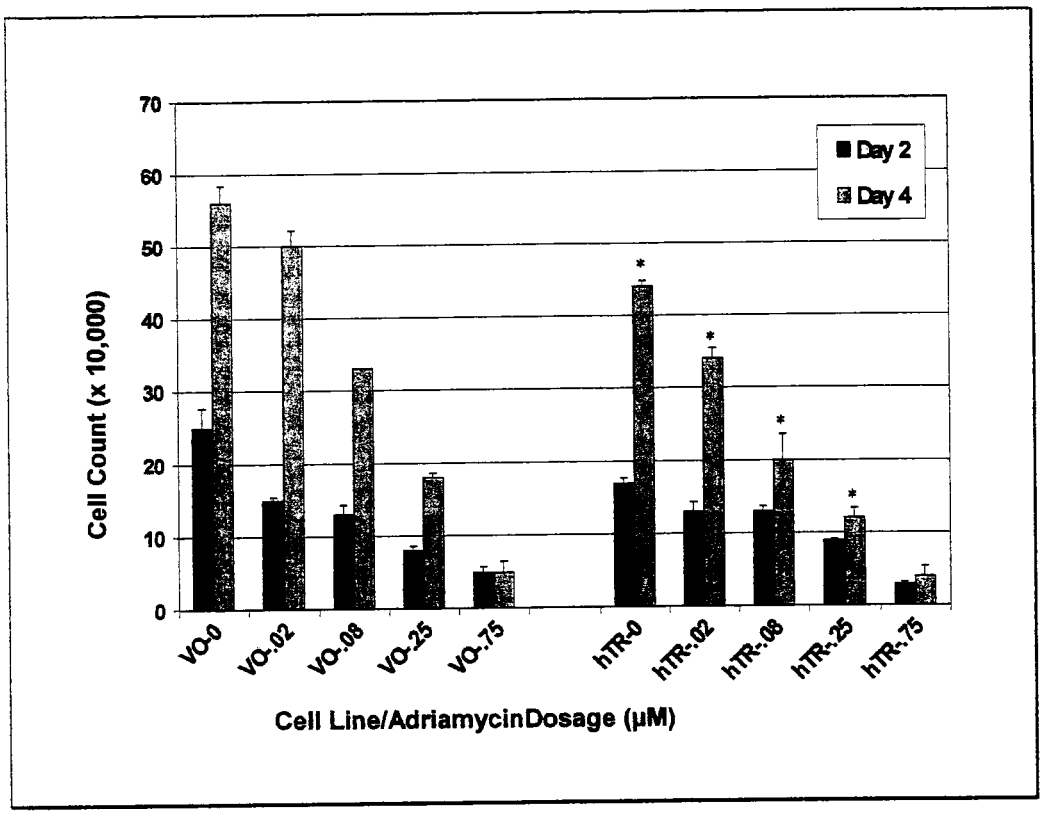


Figure 20. Decreased Growth in MCF-7 Cells Post-Treatment with shRNA and Adriamycin. MCF-7 cells were infected with the lentivectors, and selected with puromycin. On day 10 post-infection, the pHR.puro lentivector cell lines were treated with various concentrations of AdR. Samples were then taken 2 and 4 days post-treatment with AdR. Cell numbers were counted using a hemocytometer and samples were done in duplicate. By day 4 cell growth had slowed significantly, as compared with controls, at dosages lower than clinically relevant. Significance was determined by a two-tailed t-test and displayed on the graphs. *, $P < 0.05$

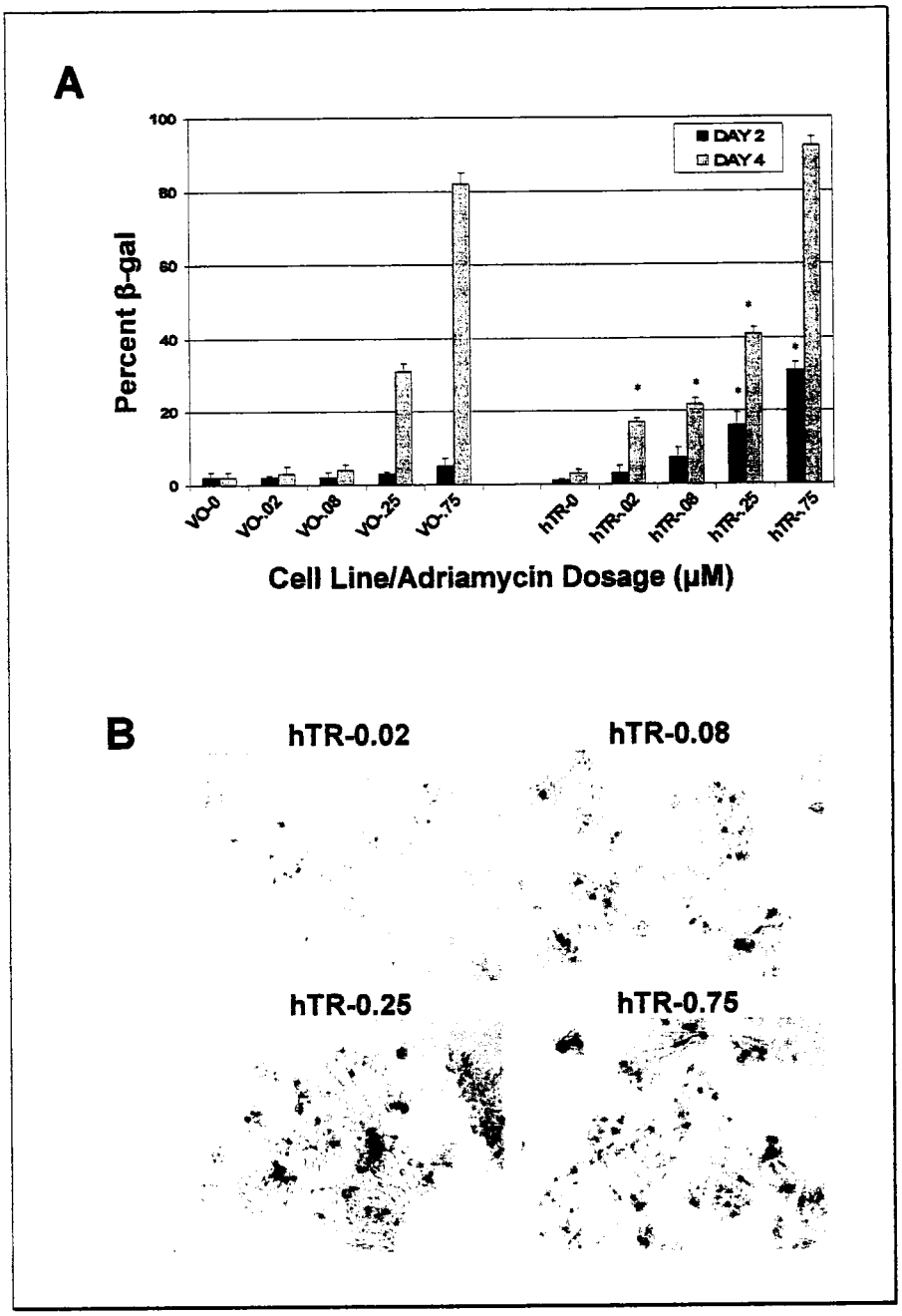


Figure 21. Senescence Occurs by Day 4 at Very Low Levels of Adriamycin. A. Cells were fixed and incubated overnight with a β-gal staining solution. **A.** Quantification of β-galactosidase staining. Significant increases in positive staining for senescence is demonstrated by day 4 at 0.02 and 0.08 μM AdR. Columns are the mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$. **B.** Positive staining of senescent cells four days post-treatment with AdR with various concentrations.

with the higher concentrations of AdR, 0.25 and 0.75μM (Figure 21). However, by day 4 significant increases in senescence were observed in the hTR shRNA cells after treatment with dosages as low as 0.02 μM. Thereby, our results show sensitization of the cells 4 days after treatment at lower dosages than clinically relevant. We also tested the percent apoptosis post-treatment with AdR and found no significant difference in the amount of cell death between the VO control and MCF-7 /hTR siRNA containing cells (not shown).

Consequently, we wanted to assess levels of p53 in the cells to determine how they responded to the chemotherapeutic treatments. Briefly, MCF-7 cells were infected with the PURO cassettes, VO and hTR siRNA, followed by selection with puromycin (800ng/mL) for 5 days. On day 8, the cells were administered an acute 2 hour AdR treatment, and subsequently, samples for Western Blot were taken 6 and 24 hours post-treatment to assess protein levels (Figure 22). p53 levels were higher in the hTR-2 siRNA cell lines than those found in the VO control cell lines without treatment as before (see Figure 13). At 6 hours, p53 was up-regulation and therefore, p21 was activated but only in the hTR siRNA cells treated with 0.75 μM AdR. However, by 24 hours post-treatment, the elevated levels of p53 had decreased back to constituitive levels. However, also occurring at 24 hours was increased p53 levels at the very low concentration, of 0.08μM AdR.

In addition, we compared sensitization using the chemotherapeutic drug taxol. The PURO lentivectors were also utilized for these sensitization studies. Briefly, MCF-7 cells were infected with the PURO cassettes, VO and hTR siRNA, followed by selection with puromycin (800ng/mL) for 5 days. On day 8 the cells were administered an acute 2

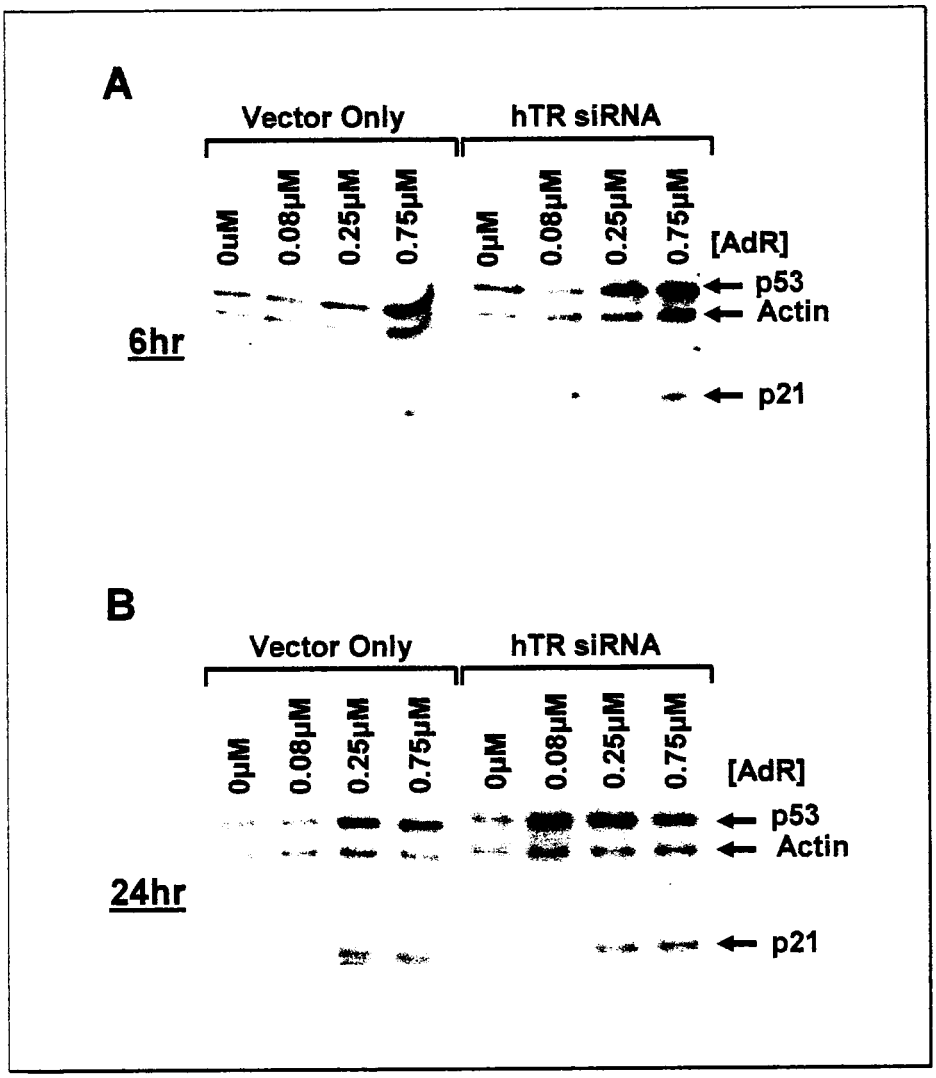


Figure 22. Induction of DNA Damaging Proteins after Treatment with shRNA and Adriamycin. A., B. MCF-7 cells were infected with the lentivectors, and selected with puromycin. On day 8 post-infection, the pHR.puro lentivector cell lines were treated with a 2 hour acute treatment with various amounts of AdR. Samples were taken 6 and 24 hours post-treatment. Cells were pelleted and 15μg of protein lysate was subject to Western Blot analysis. Immunoblots were probed with anti-p53, anti-p21, and anti-actin as a loading control. At 24 hours, up-regulation of p53 is observed at a lower concentration, 0.08μM, than the control cell line.

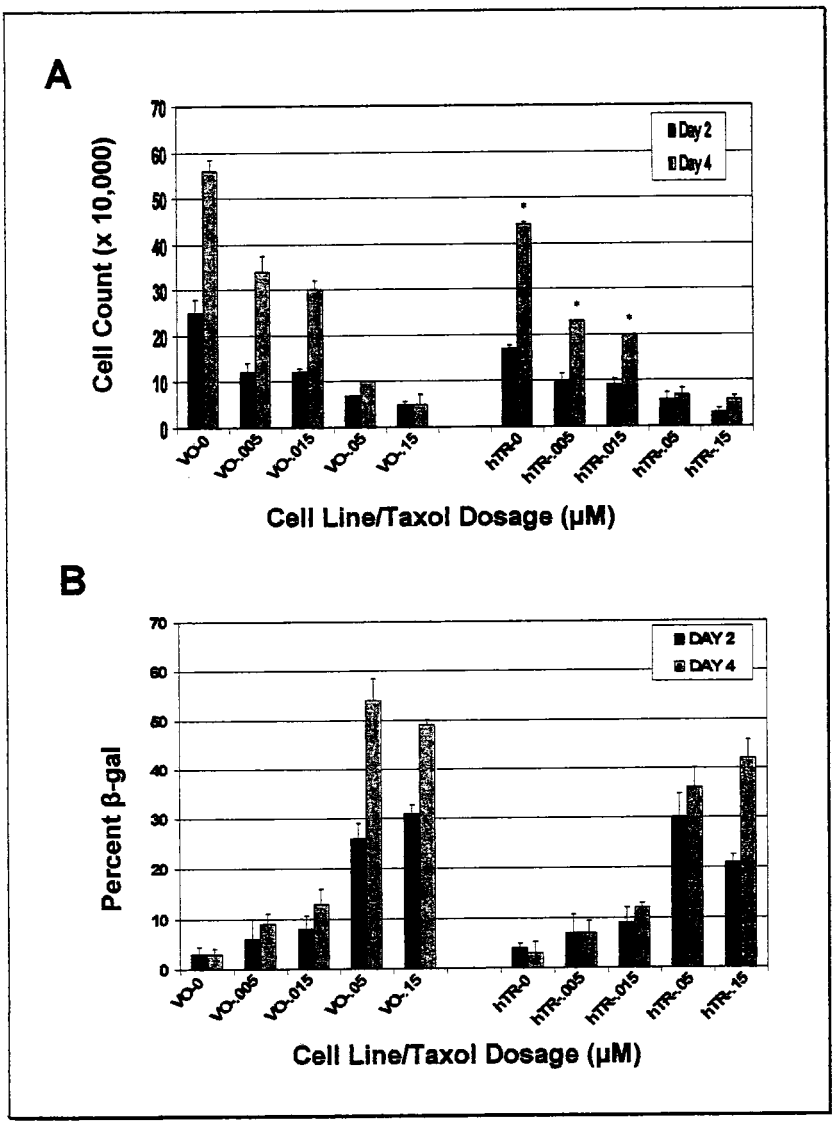
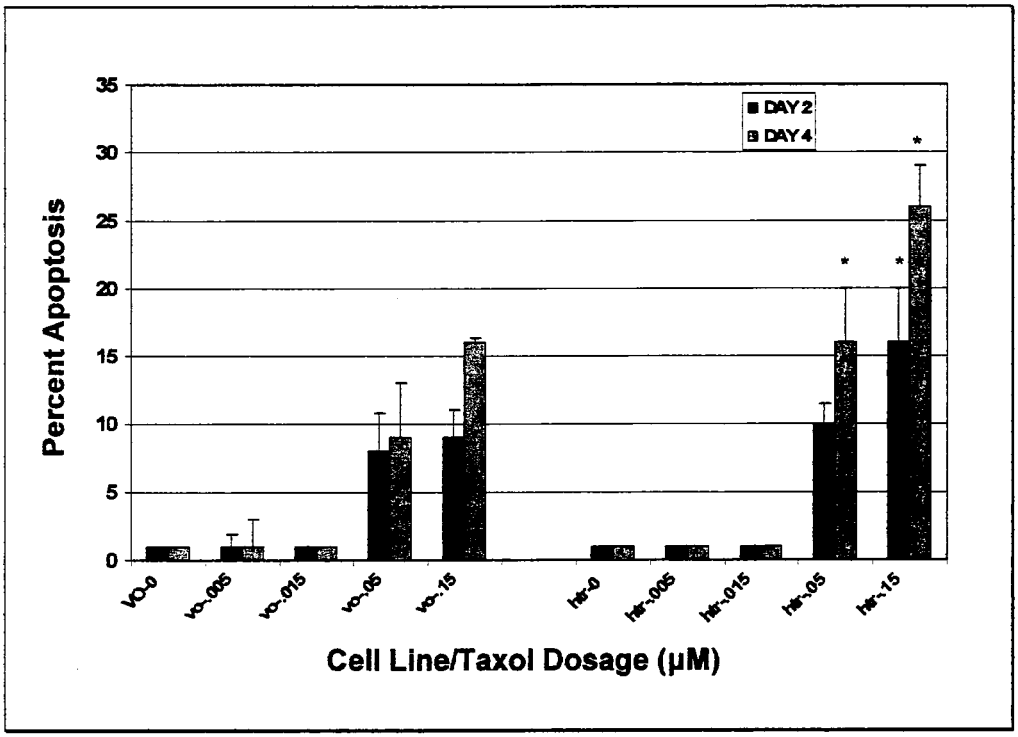


Figure 23. Taxol Causes Decreased Growth in MCF-7 Cells with shRNA But No Evidence of Elevated Senescence. MCF-7 cells were infected with the lentivectors, and selected with puromycin. On day 10 post-infection, the pHR.puro lentivector cell lines were treated with various concentrations of Taxol. Samples were then taken two and four days post-treatment with AdR. **A.** Cell numbers were counted in duplicate. By day 4, cell growth had slowed significantly, as compared with controls, at dosages lower than clinically relevant, 0.005μM and 0.015μM. **B.** Quantitation of percent of cells that stained positively for β-gal. No Differences were seen between shRNA and empty vector cell lines. Columns represent the calculated mean from three representative fields of 100 cells; bars, SD; *, $P < 0.05$.

hour taxol treatment, and samples were taken 2 and 4 days post-treatment. Growth of the cell lines was calculated to ascertain if pre-treatment with the siRNA followed by AdR produced an effect (Figure 23). By day 4, the cells containing the hTR siRNA hadsignificantly slower growth than that of the empty vector control MCF-7 cells but only for the 0.0-0.08μM taxol. As for levels of senescence, no differences were observed for the hTR siRNA compared to the VO control. We also examined increases in apoptosis post-treatment with taxol and found increases in cell death on day 2 but only at 0.15 μM while on day 4 cells treated with 0.05 and 0.15 μM displayed significantly elevated levels of apoptosis (Figure 24).



Figrure 24. Sensitization of hTR shRNA cell lines to Taxol was not detected. Experimental design is the same as seen in Figure 24 and the TUNEL was accomplished as in Figure 15. The quantitation of the TUNEL assay indicates that no significant differences in the response of the lentivector cells to the various dosages of taxol in terms of posistively stained apoptotic cells. Columns represent the calculated mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

Chapter 4

Genetic Inhibition of hTERT and Sensitization of MCF-7 Cells

Inhibition of Telomerase Activity by Synthetic siRNAs Targeting hTERT

Together with the hTR templating RNA, the other key component of telomerase is hTERT, which must be activated in order for telomerase activity to be restored in human malignancies, making this protein a promising target for cancer therapy. Other studies have attempted to target hTERT in a chronic fashion and inhibit telomerase using a variety of strategies including ribozymes, peptide nucleic acids, and antisense oligonucleotides, as well as combining these strategies with chemotherapy (Hao et al. 2005; Kraemer et al. 2004). However, here we show that telomerase can be inhibited in breast tumor cells, transiently using RNAi and then sensitization of these cells occurs rapidly post-treatment with chemotherapeutic drugs.

As seen in Figure 25, hTERT is composed of two very important features, namely, the telomerase specific motif T specific for telomerases, as well as the seven reverse transcriptase (RT) motifs (1, 2, A, B', C, D, and E) that are conserved among the family of reverse transcriptases (Nakamura et al. 1997). RNAi target sequences for hTERT were acquired (Nakamura et al. 2005; Masutomi et al. 2003), followed by synthesis in the sense and antisense direction individually, approximately 21 nt long. Using duplex buffer, the oligonucleotides were then annealed followed by transfection into the MCF-7 breast tumor cells. One of the hTERT siRNA targets is located within the RT motif 2 or exon 5, and the other sequence lies outside of the conserved RT region

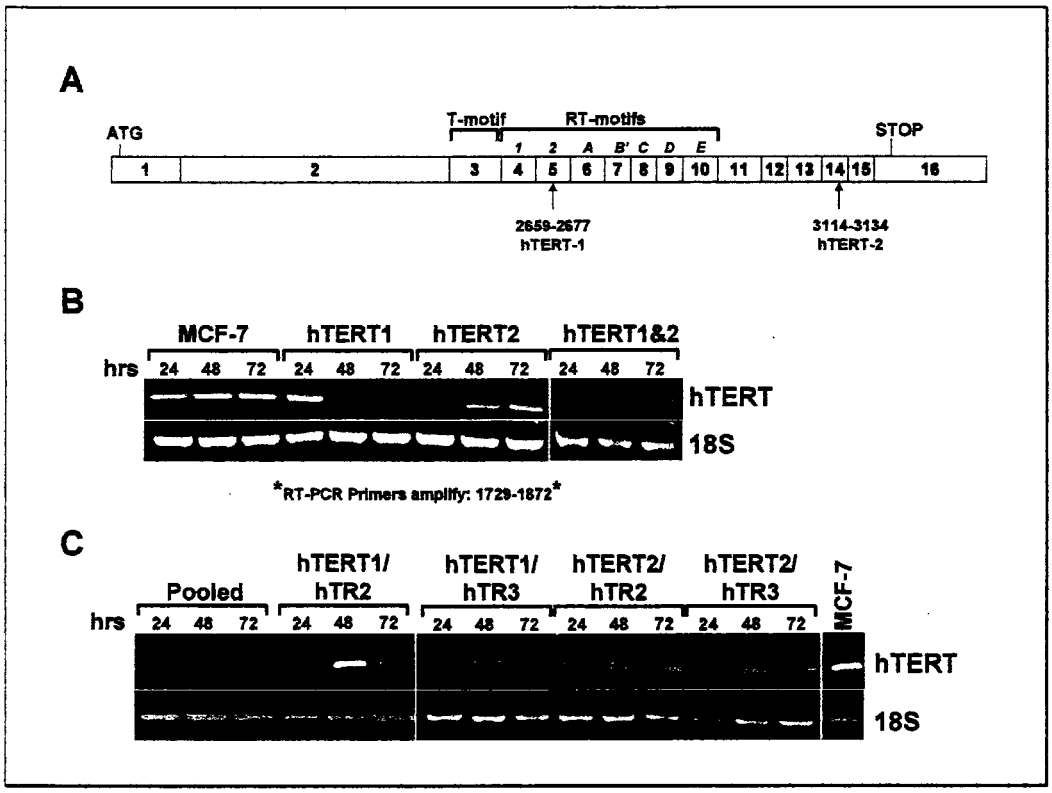


Figure 25. Schematic of the hTERT Gene and Knockdown of hTERT RNA Levels using Synthetic siRNAs. A. Linear schematic of the hTERT gene with the T-motif as well as the seven conserved RT-motifs labeled above the gene. Also labeled are the siRNA target sequences. **B.** Treatment of MCF-7 cells with siRNAs targeting hTERT only (150pmol). RT-PCR was conducted to determine the expression levels of hTERT 24, 48, and 72 hours post-transfection. We showed the hTERT1 siRNA caused the most stable knockdown. **C.** RT-PCR of MCF-7 cells after treatment of different combinations of siRNAs targeting hTERT and hTR, which revealed the Pooled siRNAs (hTR2, hTR3, hTERT1, and hTERT2) to produce the most efficient inhibition of hTERT expression. The location of the sequence amplified by the primers in the RT-PCR experiments is located below the gel in part **B.**

in exon 14. To assess the expression levels of hTERT after inhibition with the siRNAs (hTERT-1, hTERT-2, hTR-2, and hTR-3), either singularly or in various combinations, samples were taken 24, 48 and 72 hours post-transfection for use in RT-PCR (Figure 25). As compared to the MCF-7 control cell line, significant decreases in hTERT RNA were only observed post-transfection with the siRNAs hTERT-1, hTERT-1/2 and Pooled, which is all four siRNAs together. Variable knock-down of hTERT RNA was shown when hTERT-1 was utilized in combination with hTR-2 or hTR-3. The siRNA combinations that did not produce consistent decreased hTERT RNA levels were hTERT-2, as well as hTERT-2 with either hTR-2 or hTR-3 indicating that hTERT-2 is not the most efficient siRNA for use in telomerase inhibition. Interestingly, it seems that the combination of hTERT-1 and -2 caused a significant and immediate decrease in hTERT, which is consistent with the hTERT-2 having an immediate effect and hTERT-1 having a more gradual but pronounced effect (Figure 25). However, the primer set utilized for detecting hTERT amplifies a region located upstream of both hTERT target sequences, which might have caused the variability seen in the hTERT RNA levels.

Because reduction of hTERT mRNA was confirmed within the breast tumor cells, TRAP samples were collected 24, 48 and 72 hours post-transfection, to assess telomerase activity after inhibition with the siRNAs. In the first analysis, cell lines containing the siRNAs targeting hTERT plus the Pooled siRNAs were tested for telomerase (Figure 26). Telomerase activity was highly decreased in all of the cell lines with the hTERT-1 siRNA, which produced the most effective levels of inhibition at 98% by day 3. On the other hand, hTERT-2 by day 1 showed 93% inhibition but this telomerase activity

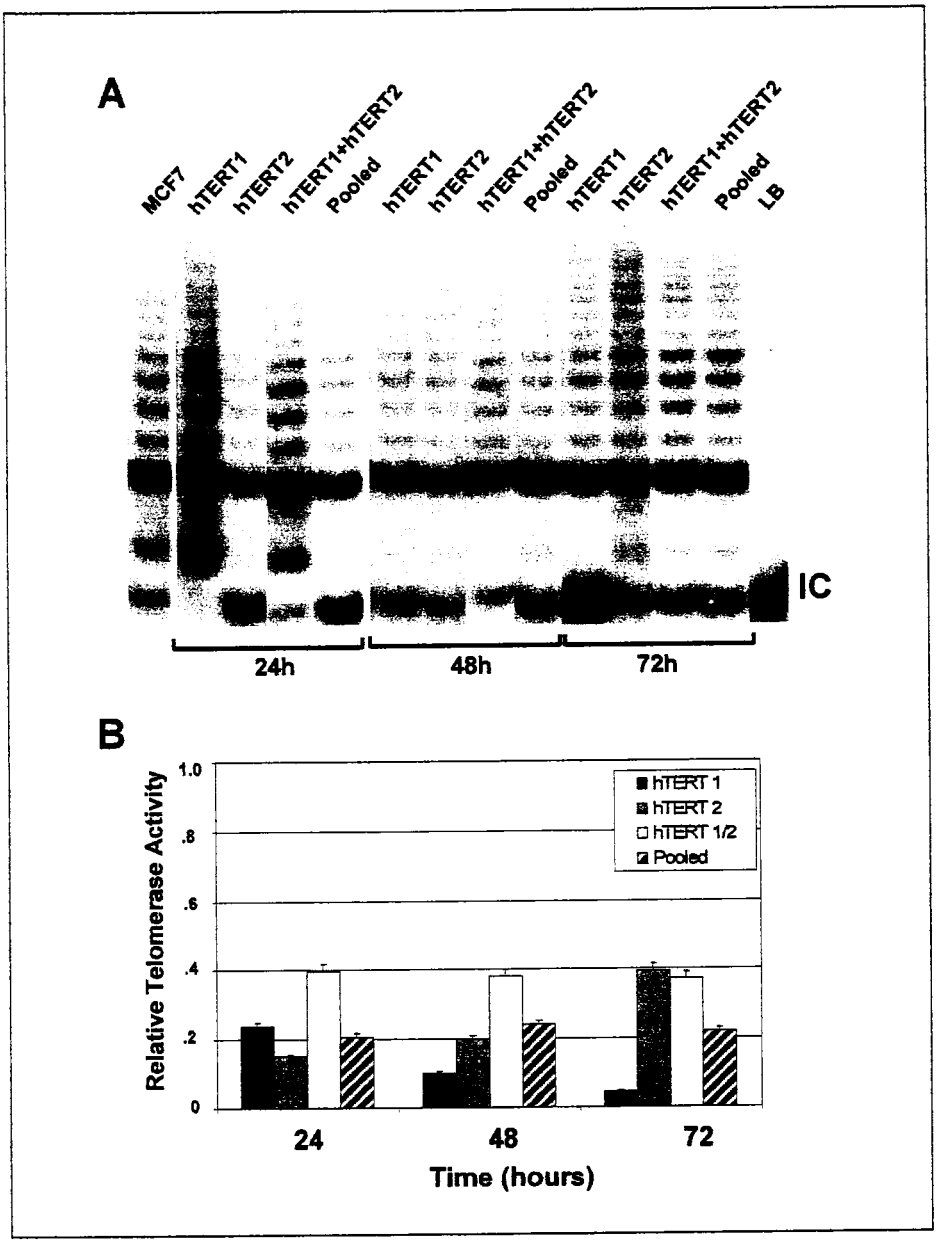


Figure 26. Knockdown of Telomerase Activity using siRNAs Targeting hTERT. Treatment of MCF-7 cells with siRNAs singularly and in combination at 150pmol concentration with samples taken at 24, 48, and 72 hours post-transfection. **A.** Representative TRAP assay using 250 cell equivalents. **B.** Quantitation of the relative telomerase activity was accomplished as stated in Figure 11. The greatest knockdown of activity was determined to occur with hTERT-1 while the most consistent telomerase inhibition occurs with the Pooled siRNAs (hTERT-1, hTERT-2, hTR-2, and hTR-3).

increased with each sample and inhibition dropped to 80% by day 3. Taken together, these results are consistent with the RNA knockdown shown in Figure 25. The Pooled siRNA and hTERT-1 and -2 both displayed consistent amounts of knockdown among the samples at 90% and 81%, respectively.

Our second study analyzed telomerase inhibition after transfection with the different combinations of the hTERT and hTR targeting siRNAs together (Figure 27). The percent inhibition of telomerase that most corresponded with the previous study was found after transfection with hTERT-1/hTR-2 together and maximal levels of knockdown of telomerase activity was 98%. However, when hTERT-1 and hTR-3 were transfected together, the levels of telomerase activity progressively decreased with each sample taken ranging from 65-91%. Similarly, with hTERT-2/hTR-3 the levels of telomerase activity gradually decreased but the scope of inhibition was 0-66%. With the last combination of siRNAs, hTERT-2/hTR-2, the telomerase activity levels as seen on day 1 were the lowest and then grew larger with every sample so the percent inhibition from 24 to 72 hours went from 82% to 62%. It is important to note here that although the telomerase ladder is visible in each lane (even with a 98% reduction in quantifiable activity levels); the quantification is done by taking a ratio of the telomerase ladder to the internal control, which is amplified in a semi-conservative manner. Thus, even though activity is detected, it is substantially reduced after siRNA knockdown of telomerase components.

Significant decreases in telomerase activity were found even in those samples where hTERT RNA levels were not diminished, which could be partially due to the primer set used for the RT-PCR. These primers were the original set used by Feng et al.

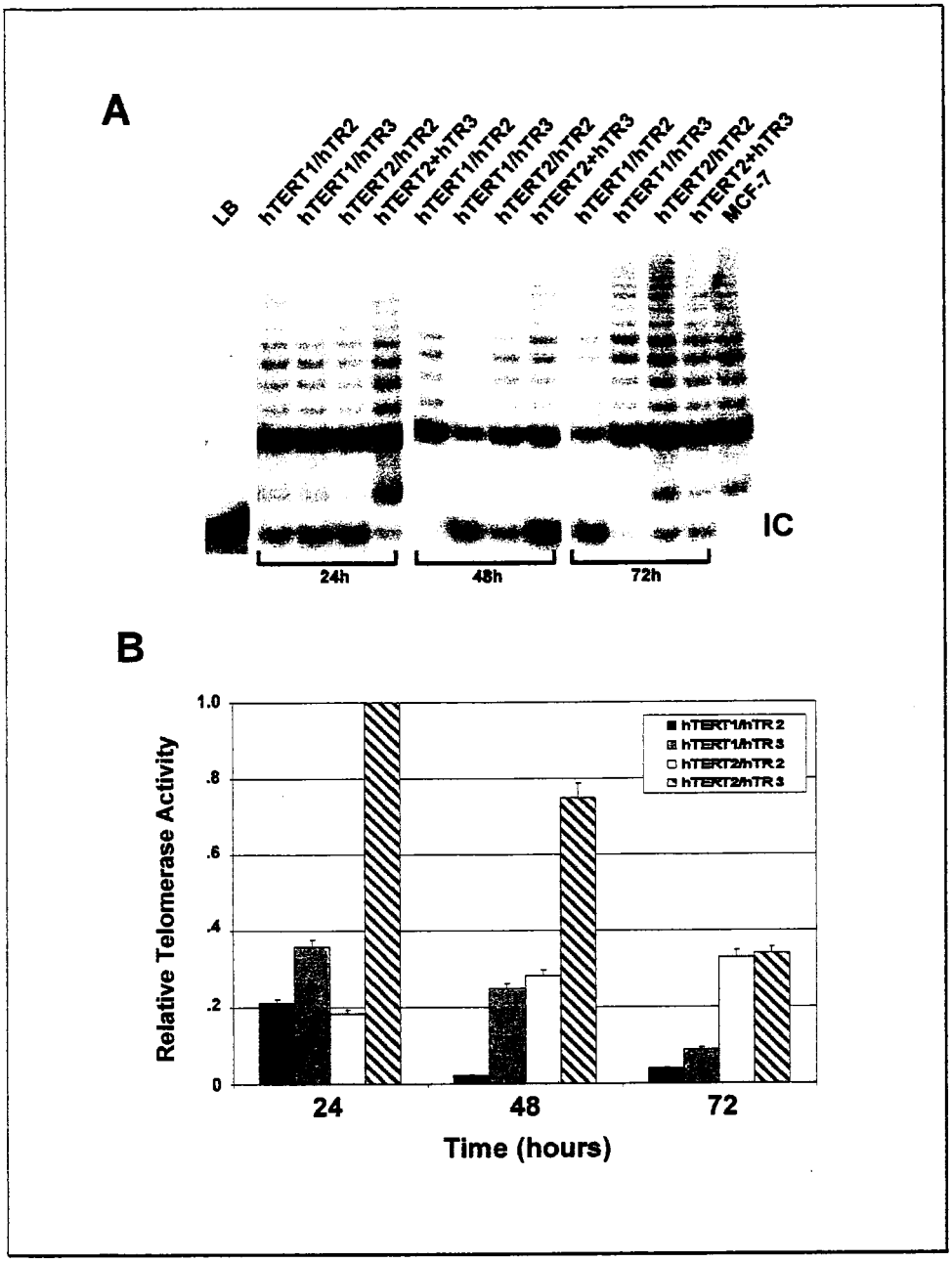


Figure 27. Telomerase Inhbition using siRNAs Targeting hTERT and hTR Simultaneously, as outlined in Figure 26. **A.** Representative TRAP assay showing 250 cell equivalents. **B.** Quantitation of relative telomerase activity was accomplished as stated in Figure 11. When knocking down both hTERT and hTR, the largest inhibition was observed with the combination of hTERT-1 and hTR-2 siRNAs. The other synthetic siRNA combinations also reduced telomerase activity to varying degrees.

(1995), which are not near the site of siRNA complementation. As such, a more appropriate primer set was designed to encompass the region(s) of siRNA knockdown. The primers amplified the region that technically would be cut by the hTERT-1 siRNA (Figure 28). Therefore, RT-PCR was repeated on the same cells containing siRNA targeting hTERT. As compared to the MCF-7 control, knockdown of hTERT RNA was observed in all samples, which confirmed the capability of the hTERT-1 siRNA to cause degradation of hTERT mRNA. Interestingly, we also saw a slight decrease in the levels of hTERT post-treatment with the hTERT-2 siRNA.

We also wanted to investigate the effects of RNAi using different combinations of hTERT siRNAs on the levels of hTR. The RNA levels within the cells transfected with siRNA targeting both hTERT and hTR is shown in Figure 29. We found high levels of hTR knockdown in the cell lines with the hTR-2 siRNA but not with hTR-3 regardless of the hTERT siRNA also used, indicating the template region of hTR is a more efficient target of hTR than the pseudoknot region. Another study has shown that the hTERT protein functions to stabilize hTR in that the steady-state hTR levels increase with increased expression of endogenous hTERT (Yi et al. 1999). Therefore, we wanted to determine if the reverse was true, that a decline in hTERT would cause a decline in hTR levels. Using RT-PCR, we found a slight decrease in hTR levels after day 1 that was maintained for the 2 and 3 day samples (Figure 29), suggesting that hTERT is capable of stabilizing hTR as shown previously (Yi et al., 1999).

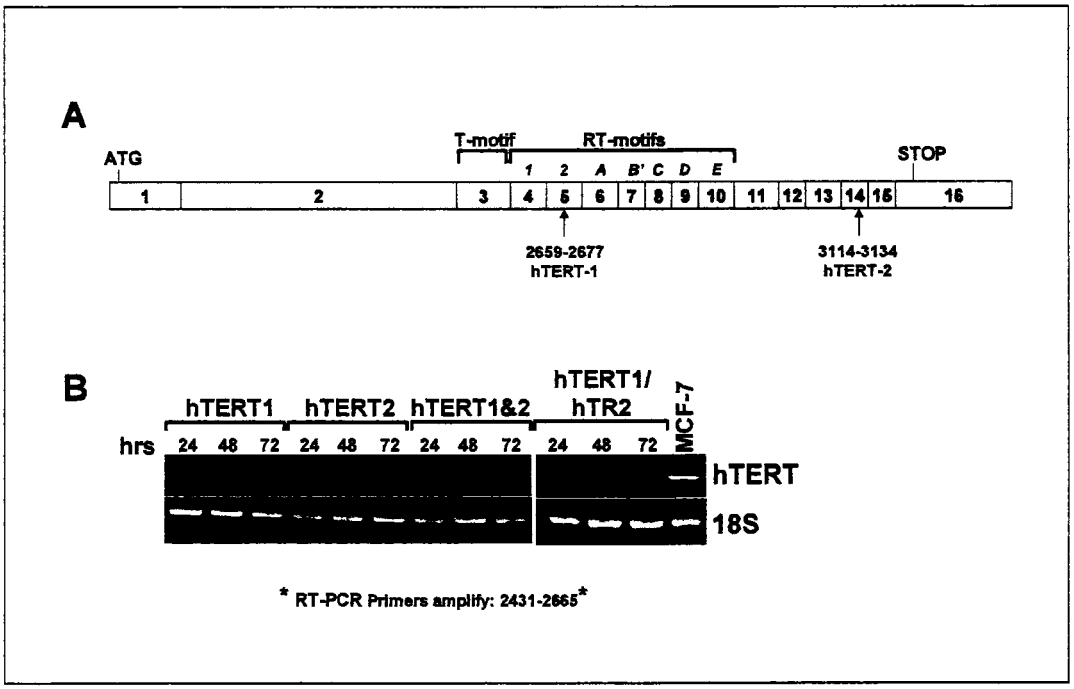


Figure 28. Schematic of the hTERT Gene and Knockdown of Telomerase RNA Levels using Synthetic siRNAs. A. Linear schematic of the hTERT gene with the T-motif as well as the seven conserved RT-motifs labeled above the gene. **B.** Transfection of MCF-7 cells with siRNAs targeting hTERT and hTR (150pmol). RT-PCR was conducted to determine the expression levels of hTERT 24, 48, and 72 hours post-transfection with primers that enclose the region targeted by the hTERT1 siRNA. We showed a decrease in the expression of hTERT with all of the siRNAs, singularly or in combination, except hTERT2 siRNA. The location of the sequence amplified by the primers in the RT-PCR experiments is located below the gel.

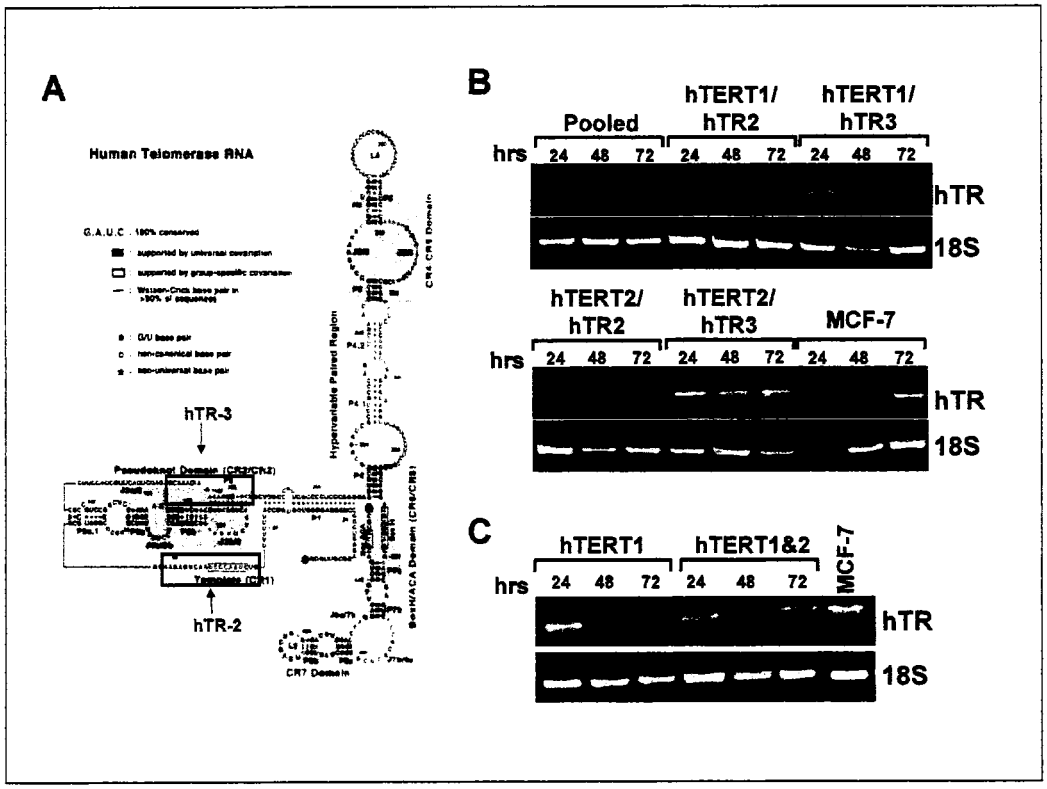


Figure 29. Decreases in hTR levels after Treatment with Combinations of synthetic siRNAs Targeting Telomerase. A. Schematic of the proposed secondary structure of hTR (Chen et al., 2000) and the siRNA target sequences, hTR-2 and hTR-3, within the template and pseudoknot regions, are identified within the black boxes. **B.** RT-PCR of MCF-7 cells after treatment of the assorted combinations of siRNAs targeting hTERT and hTR, which indicated the hTERT-1/hTR-2 and hTERT-2/hTR-2 to generate the greatest inhibition of hTR RNA levels. **C.** RT-PCR of MCF-7 cells after treatment with hTERT-1 and hTERT-1 and -2 in order to determine hTR levels were affected by knocking down hTERT levels.

Chemosensitization of Breast Tumor Cells After siRNA Silencing of hTERT

In order to provide the most comprehensive coverage of sensitization using knockdown of hTERT, we utilized three sets of siRNAs (hTERT-1, hTERT-2, and Pooled) as a pre-treatment to exposure to Adriamycin (AdR). The cells were transfected with the siRNAs (150pmol each) and allowed to grow for 3 days in order for sufficient and sustained knockdown of telomerase to occur. An acute AdR treatment at various concentrations (0.0-0.75μM) for 2 hours was done, followed by sample harvest at 2 and 4 days post-treatment. Growth of the cell lines was calculated to determine if pre-treatment with the siRNAs followed by AdR produced an effect (i.e. sensitized the cells) (Figure 30). As compared to the control MCF-7, cells transfected with the hTERT-1 siRNA displayed signficantly slowed growth by day 2 at all concentrations of AdR administered and this continued with the day 4 sample. As for those cells transfected with the hTERT-2 siRNA, considerably reduced levels of growth were observed at every concentration of AdR except 0.75μM. Finally, for those cells treated with the Pooled siRNA, differences in growth when compared to MCF-7 cells occurred on day 2 only at concentrations of 0.08 and 0.25μM AdR, but by day 4, sensitization of cells was seen at concentrations as low as 0.02μM AdR as well as 0.08 and 0.25μM AdR

We also measured cellular senescence occurred within the cells post-treatment in order to determine if senescence could be induced regardless of telomerase activity or telomere length. We found high levels of senescence in the cells transfected with hTERT-1 siRNA for both days and at every concentration of treatment (Figure 30). The hTERT-2 transfected cell lines only showed significant levels of senscence at day 2 with

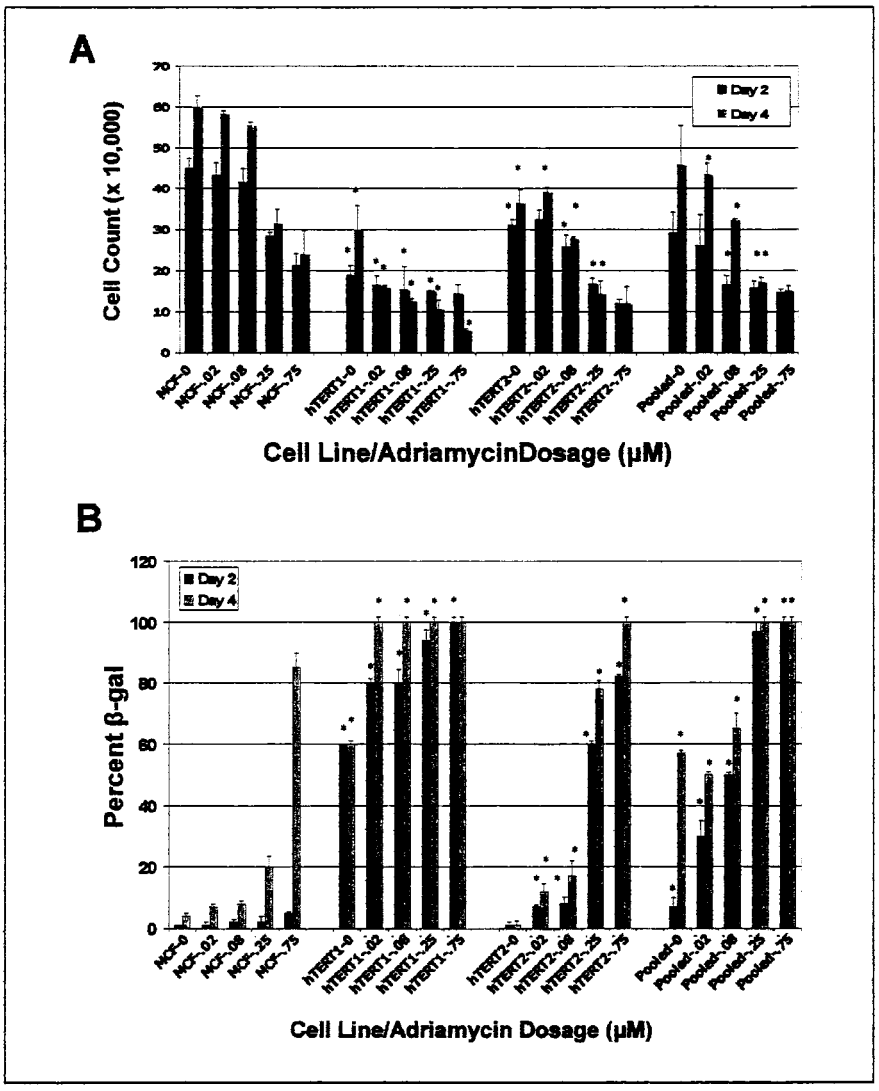


Figure 30. Decreased Growth and Increased Senescence in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the siRNA constructs (150pmol). Three days later, cells were treated with various concentrations of AdR. Samples were then taken two and four days post-treatment. **A.** Cell numbers were counted in duplicate. In the hTERT1 and hTERT2 transfected cell lines growth had slowed significantly, as compared with controls, at all dosages tested with hTERT-1 siRNA cell line revealing the most severe reduction of growth. **B.** The hTERT-1 transfected cells displayed the greatest percentage of senescent cells on both days and at every drug concentration. While not as successful, the Pooled siRNA cell line did show significant levels of senescence on day 2 at concentrations as small as 0.08µM AdR. Columns represent the mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

AdR concentrations of 0.25 and 0.75μM as well as at day 4 at 0.25μM. Similarly, the MCF-7 cells containing Pooled siRNAs revealed elevated levels of senescence at day 2 with AdR concentrations of 0.25 and 0.75μM but by day 4 cells treated with 0.08μM AdR also showed significant levels of senescence.

Sensitization of the cells was also tested by measuring levels of apoptosis (Figure 31). Cells transfected with any of the three sets of siRNA showed elevated levels of cell death by day 2 and continued on day 4 with all concentrations tested 0.0-0.75μM AdR with the largest percent apoptosis found in the hTERT-1 siRNA cell line followed by hTERT-2 and Pooled siRNA cell lines. Thus, regardless of the siRNA or combination of siRNAs utilized to inhibit telomerase, higher levels of cell death as compared with the MCF-7 cell controls were observed.

In order to compare sensitization of MCF-7 breast tumor cells to different types of chemotherapeutic drugs, we again utilized three sets of siRNAs (hTERT-1, hTERT-2, and Pooled) as a pre-treatment to exposure to taxol. The same experimental method was utilized as with AdR. Briefly, the siRNAs (150pmol) were transfected into the cells, and 3 days later, the cells were administered a 2 hour acute taxol treatment at various concentrations. Growth of the cell lines was calculated to investigate the effect pre-treatment with the siRNAs had on taxol treated MCF-7 cells (Figure 32). The first time the experiment was conducted the range of taxol (0.0-1.5μM) was too toxic, as before with the hTR siRNA experiments (see Figure 14). Therefore, the experiment was

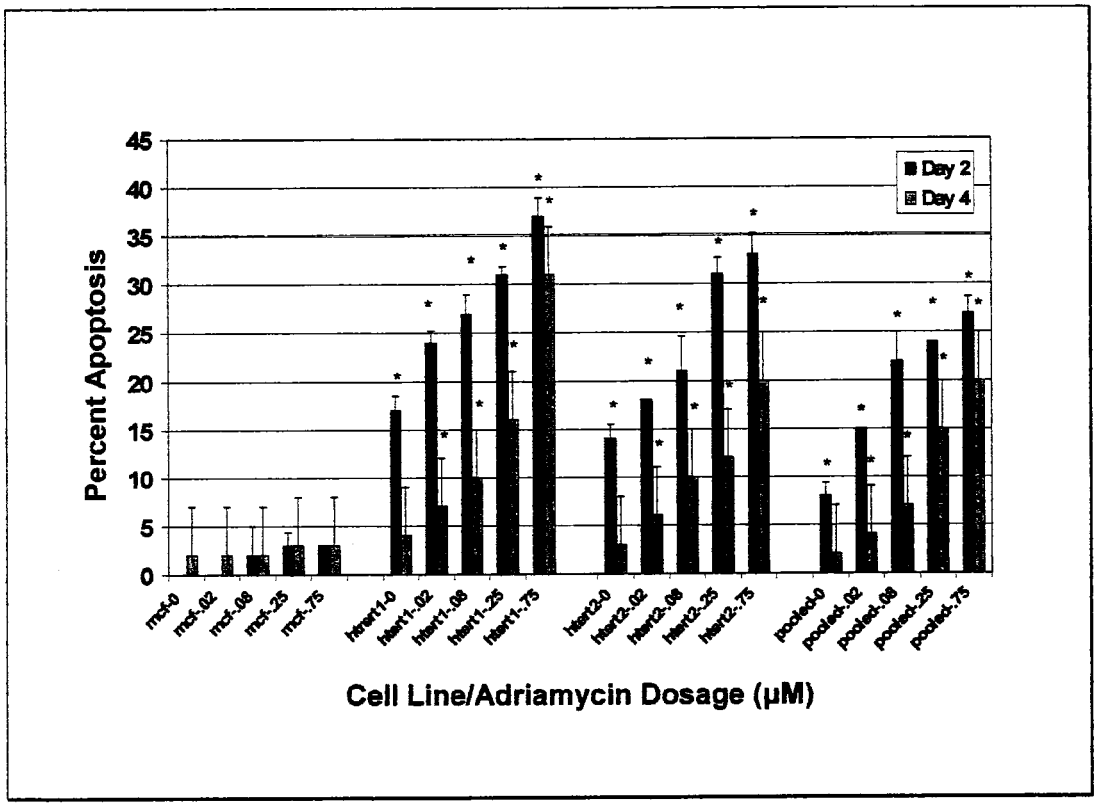


Figure 31. Apoptosis in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Same experimental procedure as Figure 30 except that the cells are stained for TUNEL. All cell lines transfected with three sets of siRNA revealed significantly high levels of apoptosis after AdR treatment with the largest percent apoptosis found in the hTERT1 siRNA cell line followed by hTERT2 and Pooled siRNA cell lines. Columns represent the calculated mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

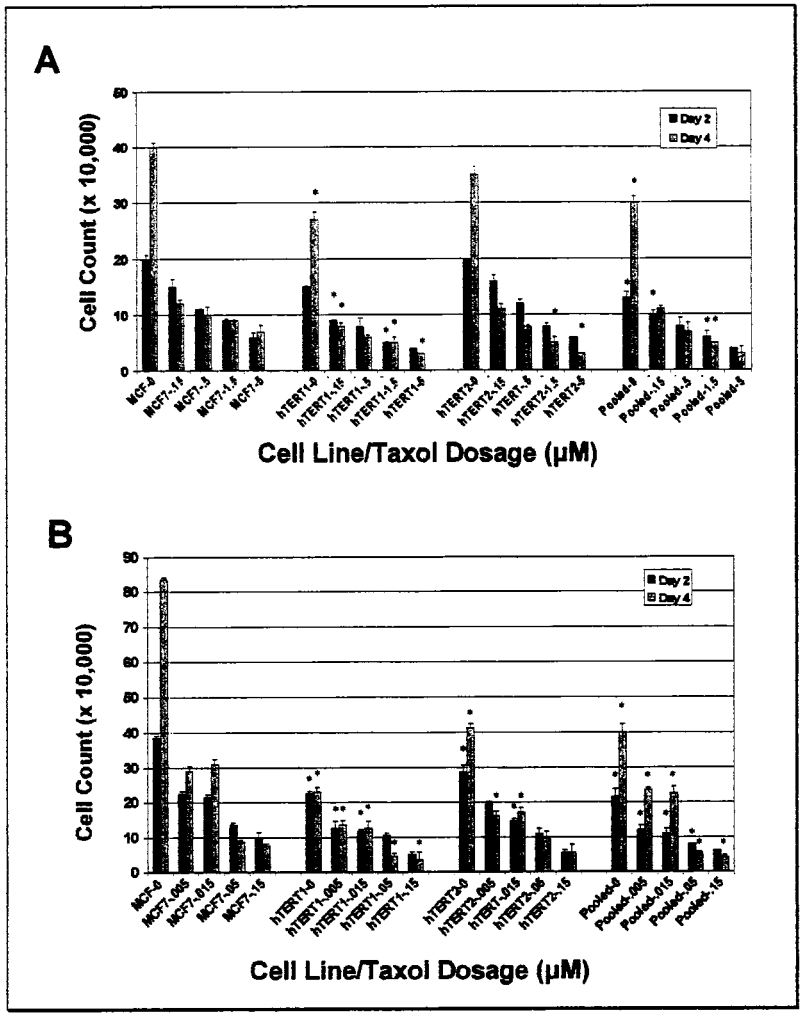


Figure 32. Growth of MCF-7 Cells Post-Treatment with Synthetic siRNAs and Taxol. Cells were transfected with siRNA constructs as indicated. Three days later, cells were treated with various concentrations of taxol. Samples were then taken 2 and 4 days post-treatment and counted in duplicate. **A.** With the higher concentrations, the taxol concentrations were too toxic for both the control and siRNA cell lines. **B.** Significantly slowed growth was observed all siRNA transfected cell lines after treatment with the lower concentrations of taxol. Significance for both graphs was determined by a two-tailed t-test and displayed on the graphs. *, $P < 0.05$.

repeated with a lower scale of taxol dosages (0.0-0.15μM). As compared to the control MCF-7, cells transfected with the hTERT-1 siRNA displayed signficantly slowed growth by day 2 in the range of 0.0-0.015μM taxol, but by day 4 all samples showed significantly slower growth. As for those cells transfected with the hTERT-2 siRNA, considerably reduced levels of growth were observed by day 4 in the range of 0.0-0.015μM taxol and on day 2, growth was considerably slower for the cells treated with taxol at 0.0 and 0.015μM. Finally, for those cells treated with the Pooled siRNA, differences in growth when compared to MCF-7 cells occurred on both days at all concentrations except for day 2 at concentrations of 0.15μM taxol.

We also observed cellular senescence occurred within the cells post-treatment in order to determine if senescence could be induced regardless of telomerase activity or telomere length. We found high levels of senescence in the cells transfected with hTERT-1 for both days and at every concentration of treatment (Figure 33). The hTERT-2 transfected cell lines only showed significant levels of senscence at day 2 with taxol concentrations of 0.0, 0.005, 0.015 and 0.15μM as well as at day 4 at 0.015-0.15μM. Similarly, the MCF-7 cells containing Pooled siRNA revealed elevated levels of senescence at day 2 with taxol concentrations of 0.005 and 0.015μM but by day 4 at cells treated with 0.0 and 0.005μM taxol also showed significant levels of senescence.

Sensitization of the cells was also tested by measuring levels of apoptosis (Figure 33). Cells transfected with hTERT-1 and Pooled siRNAs showed elevated levels of cell death with all concentrations as well as for both samples. As for cell lines infected with hTERT-2, at day 2 cell lines treated with concentrations of 0.015 and 0.15μM showed

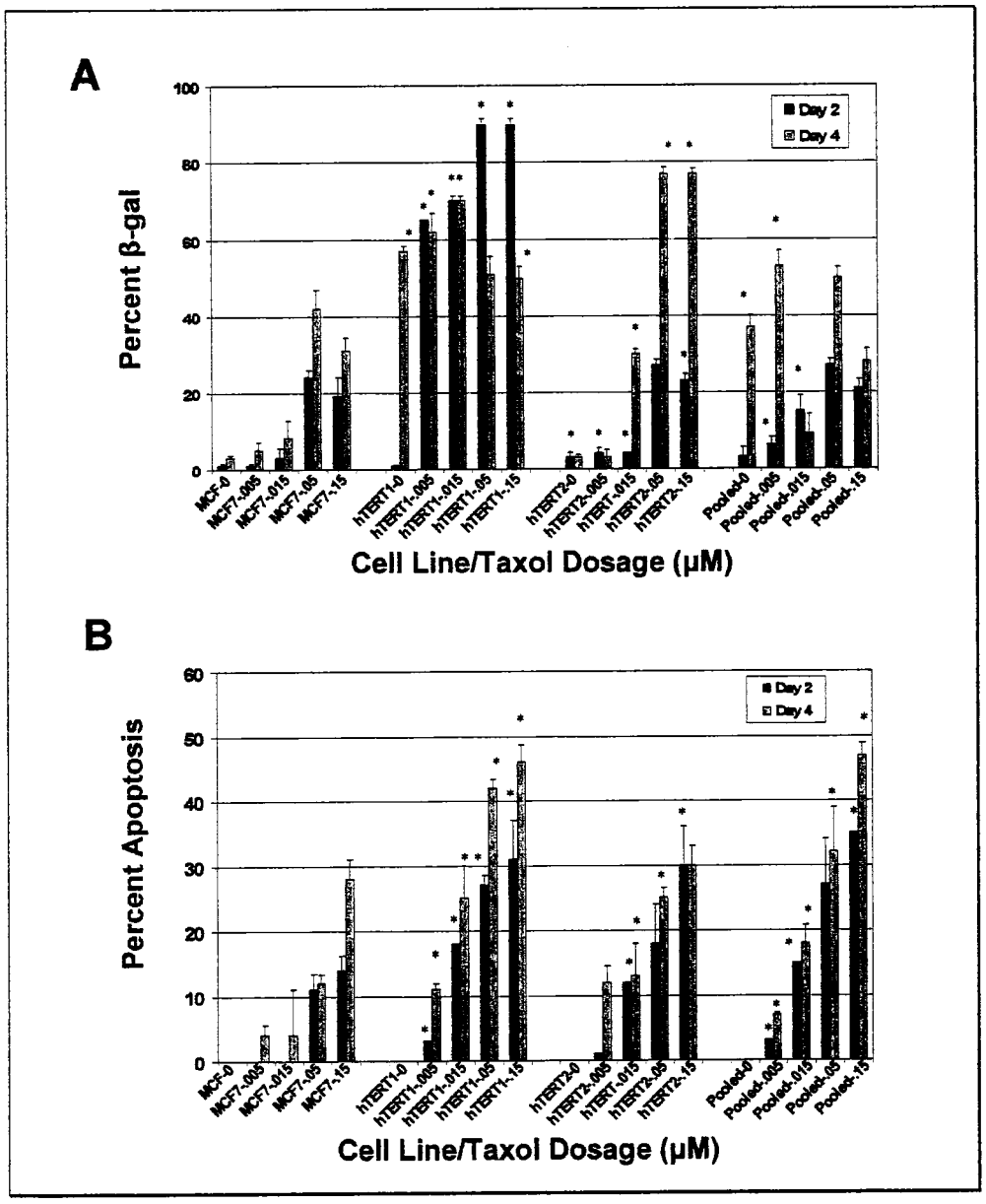


Figure 33. Cells Treated with Synthetic siRNAs and Taxol Showed Increased Sensitivity. Experimental methods are the same as in Figure 33. Cells were in fixed and incubated overnight with a β-gal staining solution. **A.** In the hTERT1 and hTERT2 siRNA transfected cell lines, noteworthy differences in senescence were observed at all taxol concentrations on both days samples were taken after taxol treatment. **B.** TUNEL assay showing percent apoptosis with hTERT1 revealing the greatest induction of cell death at all concentrations of taxol tested. Columns are the mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

significantly high levels of apoptosis but interestingly on day 4 only those cells administered dosages of 0.005 and 0.05μM taxol showed appreciable percent cell death.

DN-hTERT Inhibits Telomerase Activity and Induces Telomere Shortening

The rate-limiting component of the telomerase complex is the hTERT protein; therefore, expression of the hTERT gene within the cells is critical for telomerase activation. Normal human somatic cells contain only hTR and are without telomerase activity, whereas the majority of tumorigenic cells have telomerase activity, partially because they express hTERT. Previous reports have shown that dominant negative forms of hTERT are able to induce telomerase inhibition, telomere shortening and apoptosis of tumor cells (Hahn et al. 1999; Zhang et al. 1999), but other studies have demonstrated the effect was either apoptosis or cellular senescence depending on the cell line (Colgin et al. 2000). Therefore, we wanted to further examine the biological effects of telomerase inhibition specifically in breast tumor cells in order to ascertain the ultimate cellular consequence of blocking telomere maintenance via DN-hTERT and to compare to siRNA experiments in terms of sensitization.

We obtained the catalytically inactive, dominant negative form of hTERT from Dr. William Hahn (Dana Farber Cancer Center, Boston, MA) (Hahn et al. 1999) with two substitutions in the third RT motif of hTERT (Figure 34). At positions 710 and 711, the aspartic acid and valine residues were switched to alanine and isoleucine respectively. The DN-hTERT and pBABE, used as the empty vector control, retroviral vectors were infected into MCF-7 cells followed by selection with puromycin (800ng/mL) for 5 days

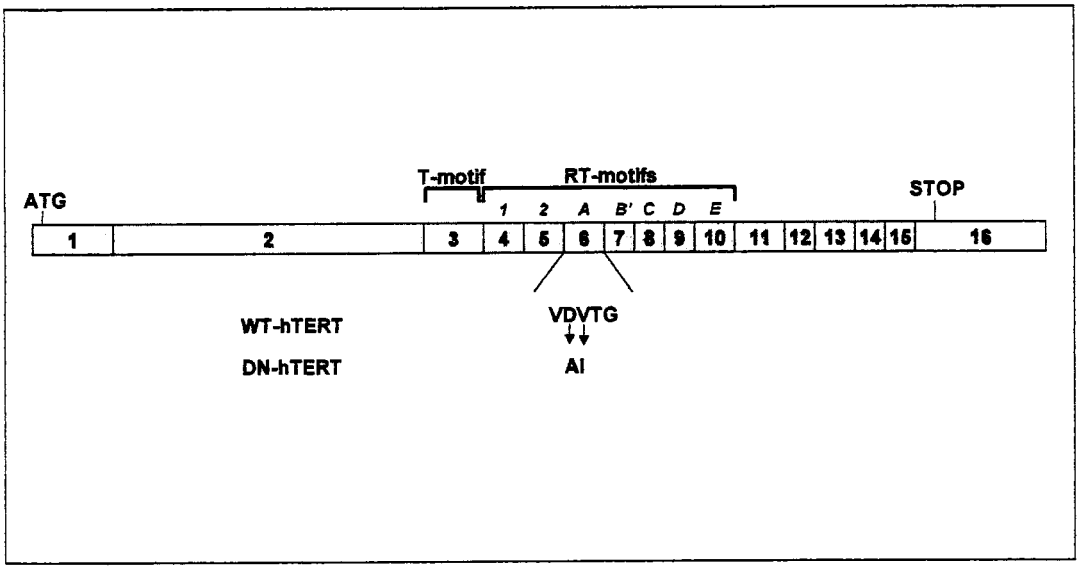


Figure 34. Schematic of Dominant Negative hTERT (DN-hTERT).
The DN-hTERT was created by substituting the aspartic acid and valine residues at positions 710-711 in the third RT motif (motif A)of hTERT with alanine and isoleucine, respectively.

and maintained in low levels of puromycin (50ng/ML). Telomerase activity was analyzed in clonal isolates using the TRAP assay. We found that introduction of DN-hTERT resulted in significant reduction of telomerase activity in all of the MCF-7 clonal populations. However, there was variability among the clones, but with the greatest knockdown of activity was found in clones 2, 6, and 10 (Figure 35).

Using RT-PCR, RNA expression levels of the hTERT for each clonal cell line were determined in order to establish the differences between endogenously and exogenously expressed RNA (Figure 36). Considerable disparities in the quantitative amount of DN-hTERT RNA between the clones were observed as compared to the same clones in which both sets of hTERT RNAs were amplified. As predicted, these differences correlated with the telomerase activity variability observed. Therefore, those clones with greater expression of DN-hTERT RNA also had lower levels of telomerase activity, which was expected.

To determine if increased expression of DN-hTERT could be detected at the protein level, western blot analysis was utilized to identify differences between the clonal populations (Figure 36). Three clones (DN2, DN6, and DN9) were analyzed as those showed inter-clonal variation of telomerase activity and DN-hTERT RNA levels. Compared to the β-actin control, the quantity of the hTERT protein was different between each clone. DN-hTERT clone 2 (DN2) had the lowest level of hTERT followed by DN6 and DN9 respectively. This finding was unexpected because DN2 and DN6 had higher levels of exogenous DN-hTERT RNA, indicating hTERT protein levels should be equally elevated. Furthermore, DN9 had large levels of hTERT protein as seen in the

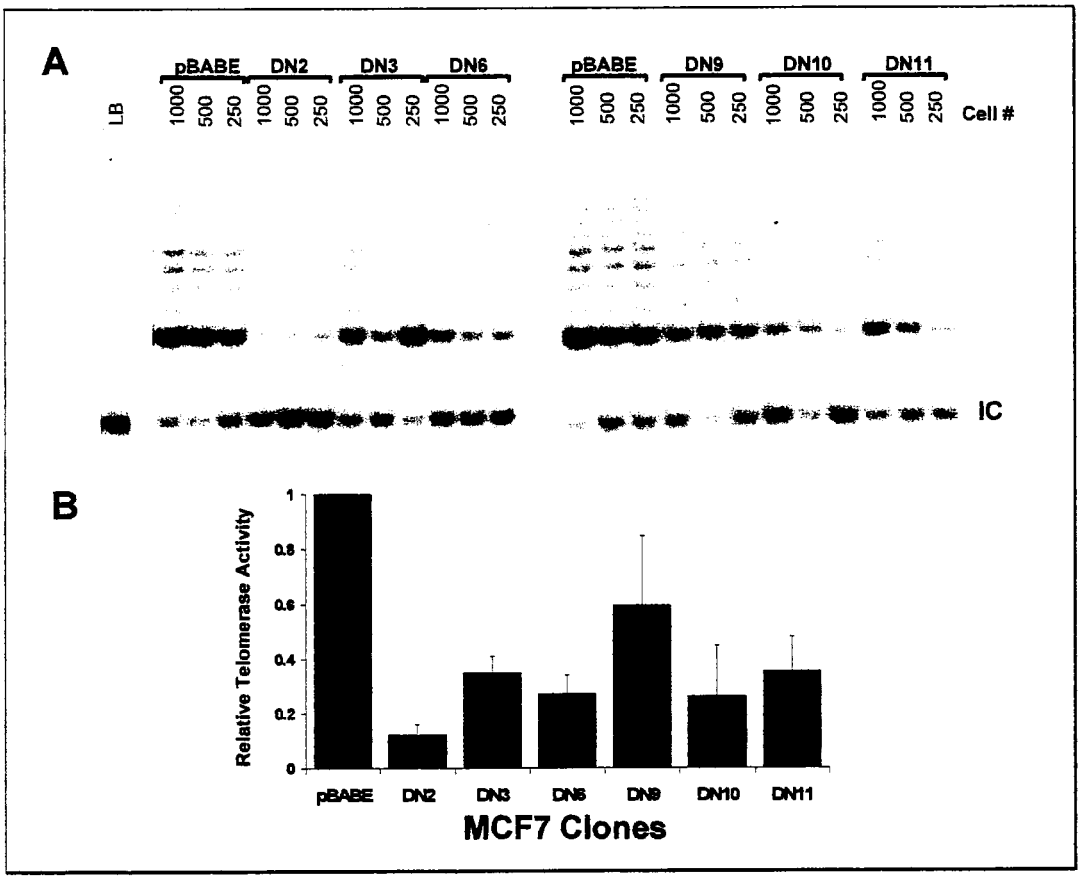


Figure 35. DN-hTERT Causes Decreased Telomerase Activity in Clones of MCF-7 Cells. The MCF-7 breast cancer cells were stably infected with dominant negative hTERT and clonal populations were obtained. **A.** Representative TRAP assay showing 250, 500, and 1000 cell equivalents. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC). Each sample was also normalized to the pBABE/MCF-7 cell line and shown as a percent of activity in relation to the pBABE/MCF-7 cell line. In several of the clonal populations (2,6,10), knockdown of telomerase was 75 % or greater.

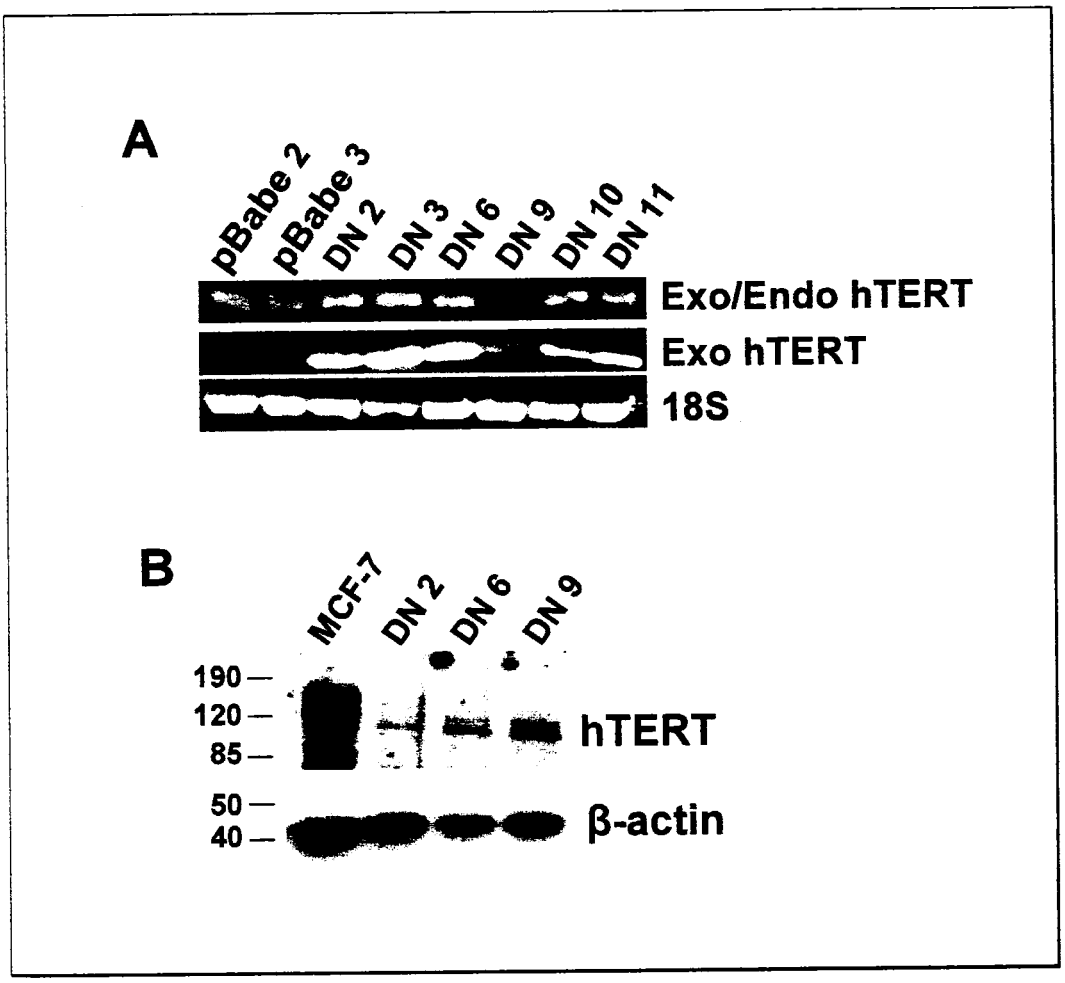


Figure 36. Levels of DN-hTERT mRNA and hTERT Protein Expression are Varied in MCF-7 Clones. A. RT-PCR was conducted to determine the expression of both the constitutive levels of hTERT as well as the introduced DN-hTERT levels in the various clonal populations. Exogenous hTERT expression levels showed great variability amongst the clones, which correlated with telomerase activity. **B.** Cell pellets were collected and 15μg of protein lysate was subjected to Western analysis. Immunoblots were probed with anti-hTERT and anti-actin as a loading control. Similarly, hTERT protein levels were reduced in the clones with exogenous DN-hTERT.

Western blot, but low levels of DN-hTERT RNA. Both instances point to the possibility that the presence of DN-hTERT within the cell induces the degradation of endogenous hTERT protein quantities, ultimately leading to a decline in overall hTERT levels.

Approximately, one month post-infection cell growth slowed and morphological changes occurred within different clonal populations. Because the cells became large and flattened in appearance, we examined the various clones for senescence using both morphology and β-galactasidase (β-gal) staining. Depending on the clone, the majority of the cells stained positive for senescence, indicating that even though the cells are metabolically active and are incapable of dividing, they are not dead. Shown in Figure 37 is only the DN2 clone because these clones had the highest levels of senescence as compared with the pBABE control, but note also that even at the highest levels of senescence not all of the cells are positive and some cells within the population continue to proliferate. However, it does appear that the levels of senescence observed within each clone can be correlated with the expression levels of DN-hTERT determined previously. We also examined the incidence of cell death through the TUNEL assay (Figure 37). We show clones DN2 and DN11 in order to display the differences in the quantities of cells staining positive for apoptosis. However, the extent of apoptosis within the clonal populations was not significant, implying that at this stage the majority of the cells were undergoing senescence rather than cell death.

Telomere length was assessed over time to determine the effects of telomerase inhibition by DN-hTERT. Samples from both pBABE and DN-hTERT clones were taken at various population doublings and telomere lengths were analyzed by TALA.

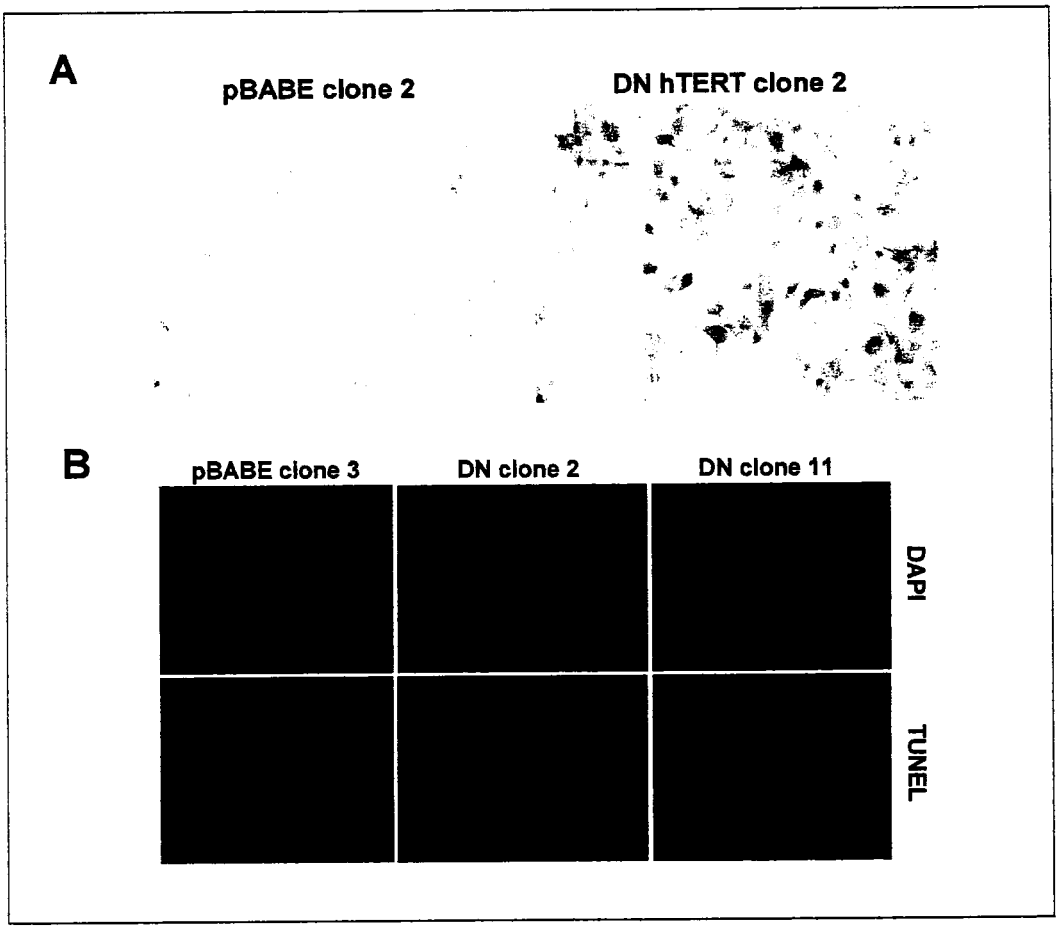


Figure 37. Increased Senescence and Apoptosis is Observed in DN-hTERT/MCF-7 Clones. **A.** Some MCF7 clones displayed a senescent phenotype and high levels of staining for β-gal about one month post-infection with DN hTERT. **B.** Using the TUNEL assay, elevated levels of apoptosis were detected in the dominant negative hTERT/MCF7 clones, although most cells undergo senescence.

Importantly, within every cell, the population of telomere lengths can be very heterogeneous differing from one chromosome to another, as well as between cells due to the various factors affecting telomeres including telomeric maintenance and damage or even expression of telomere binding proteins. Therefore, as seen in the TALA, the telomeres are represented by a smear due to the assorted lengths within and between cells. We found an ongoing rate of telomere loss over time for all of the clones (Figure 38 and 39). As compared to normal MCF7, MCF-7 over-expressing hTERT and pBABE control cell lines, the various clones displayed a marked decrease in telomere length of 1Kb or more with the DN2 clone being the most significantly shortened followed by DN10. The other clones were variable in shortening with DN9 observed to have elongated telomeres. Furthermore, the amount of shortening as cells aged appears to correlate with level of DN-hTERT expression determined earlier (Figures 35 and 36).

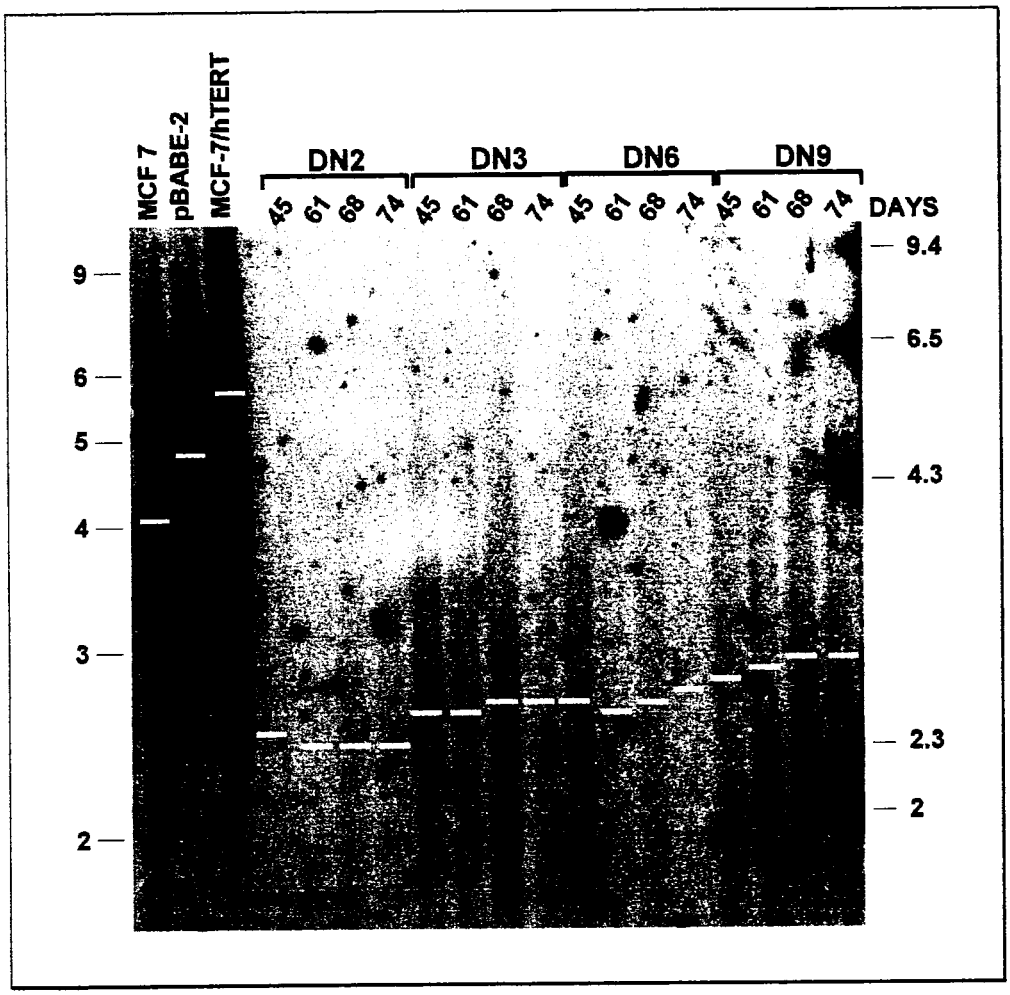


Figure 38. DN-hTERT causes Telomere Shortening. 5μg of DNA was isolated from DN-hTERT expressing cells and digested with three restriction enzymes. The digested DNA was incubated with a ^{32}P-labeled telomere probe overnight and separated on a 0.8% agarose gel. Samples were sequentially collected over a month's time for four different clonal populations. The average telomere lengths determined by this telomere amount and length (TALA) assay, as marked on each lane with a white bar, showed considerable shortening in several clones of DN-hTERT/MCF-7 cell lines (2, 3, and 6). The average telomere length was quantitated using ImageQuant software and telo-quant excel program.

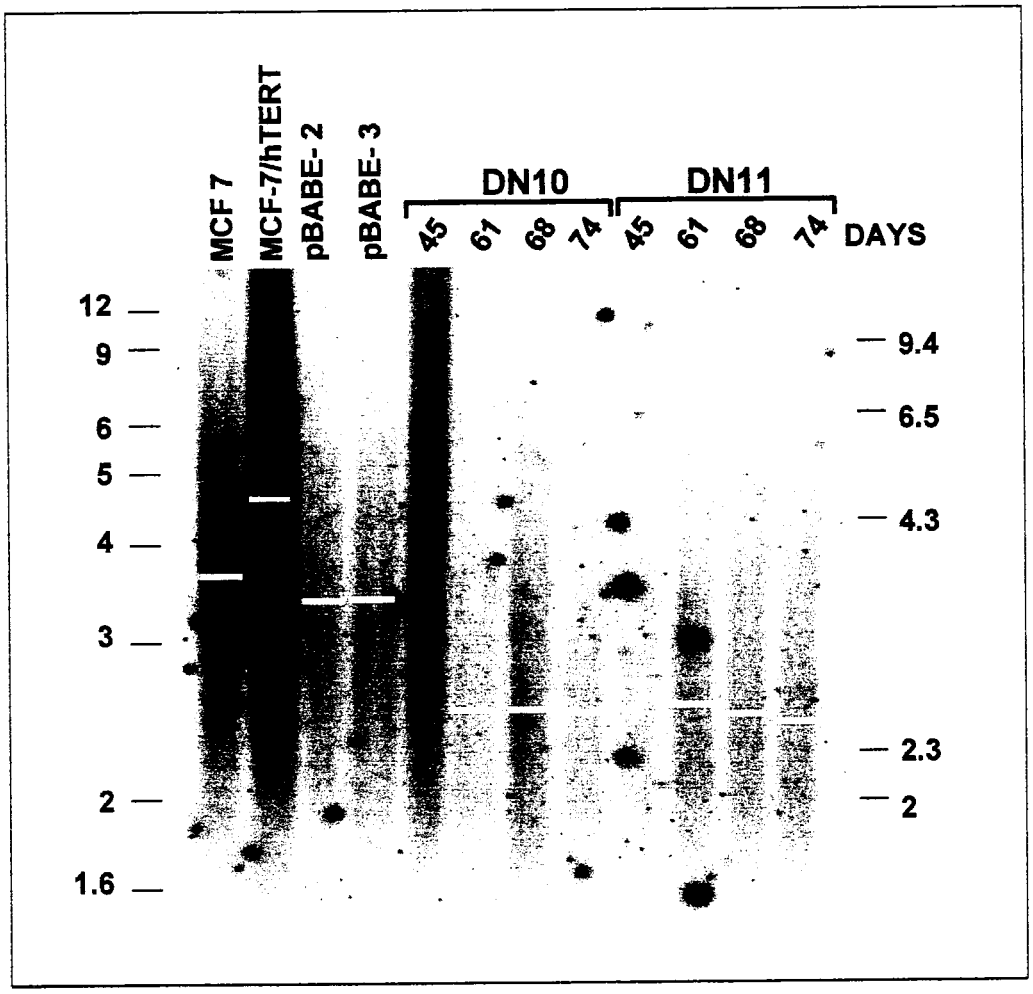


Figure 39. DN-hTERT causes Gradual Telomere Shortening. Same experiment as in Figure 38. Samples were sequentially collected over a month's time and the telomere lengths determined by the TALA assay. The average lengths of clones 10 and 11 of the DN-hTERT/MCF-7 cell lines were significantly shorter than the control cell lines (MCF-7, MCF-7/hTERT, and pBABE/MCF-7). The average telomere length was quantitated using ImageQuant software and telo-quant excel program.

DN-hTERT Causes Sensitization of Breast Tumor Cells to Adriamycin

Previous studies have established increased sensitivity to chemotherapeutic drugs after telomerase inhibition in a variety of tumor cell lines including lung cancer, cervical cancer and leukemia (Misawa et al. 2002; Nakamura et al. 2005; Ward and Autexier, 2005). Therefore, we wanted to assess if expression of DN-hTERT causes increased sensitization in breast cancer cells, specifically MCF-7 cells. Using colony forming assays, we examined the cells' ability to survive and proliferate after treatment with the DNA-damaging agent AdR. Three of the DN-hTERT clones with differing amounts of telomerase activity and telomere lengths were selected roughly 2 months post-infection for sensitization by AdR and tested for colony forming efficiency. Approximately, 2000 cells were seeded in triplicate and were administered an acute AdR treatment for 2 hours with varying concentrations (0.0-0.75μM). Ten days later, plates were stained with crystal violet and colonies counted. In comparison to the MCF-7 and pBABE cell lines, all of the DN-hTERT clones exhibited a greater sensitivity to AdR as seen by the considerable reduction in the number of colonies (Figure 40). Clonal growth rates were ascertained as critically lower than controls in all of the concentrations except at 0.0625μM in the DN9 cell line, which are significantly smaller than clinically relevant dosages at 0.75μM. Furthermore, clone DN9, which has longer telomeres and less DN-hTERT expression than the other clones (hence, only slightly inhibited telomerase activity), showed sensitization as low as 0.125μM AdR.

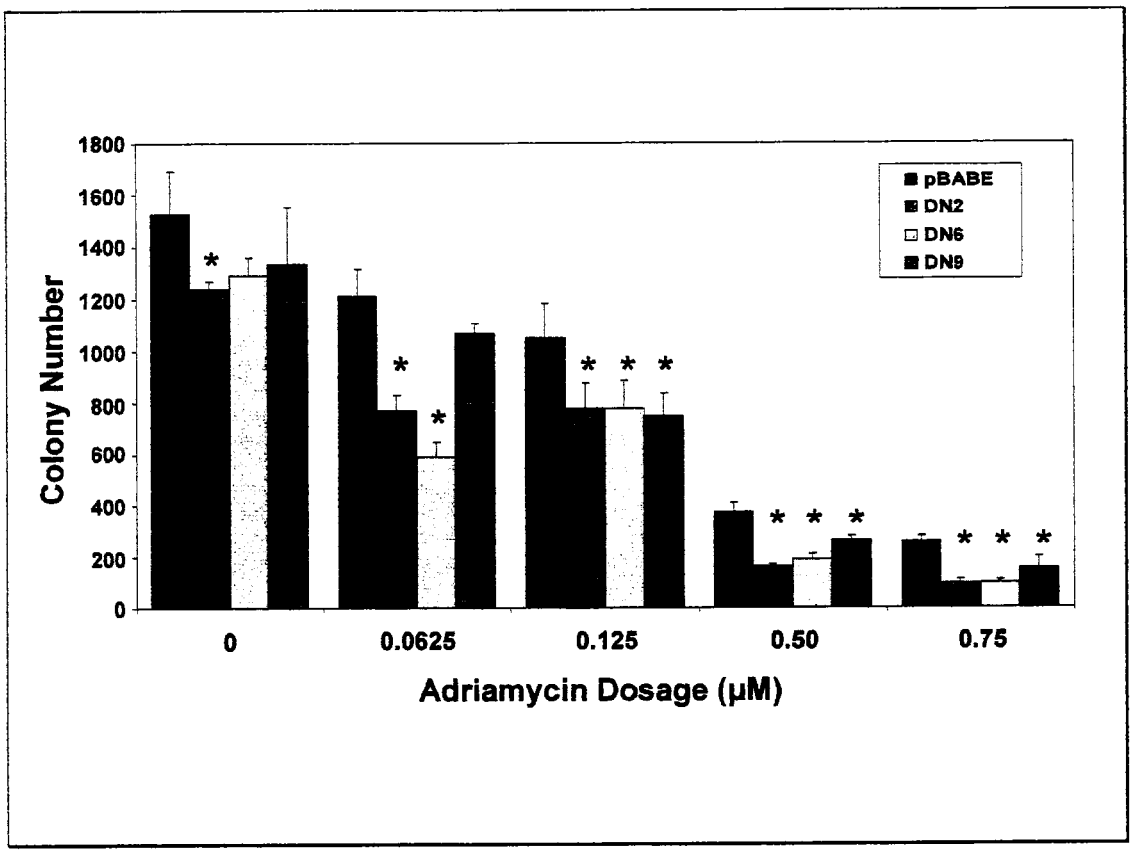


Figure 40. Increased Sensitivity after AdR Treatment. DN-hTERT/ MCF-7 cells were treated with a 2 hour acute treatment with various amounts of AdR. Colonies were stained and counted after 10 days of growth. The three DN-hTERT clones displayed significantly increased sensitivity as seen by decreased colony growth after treatment at lower dosages. Clonal differences in sensitivity correlated to levels of DN-hTERT expression and telomerase activity within the population.

This increased sensitivity of the clonal populations with shorter telomeres implies that there is increased susceptibility of shortened telomeres to AdR and thereby DNA damage, which will be significant in the induction of apoptosis or senescence. Therefore, we wanted to determine protein levels of p53 and p21 post-treatment with AdR. Since we saw higher levels of senescence in clones with elevated telomerase inhibition, we expect to see induction of p53 because it is required for senescence to occur. We utilized the same clones (DN2, DN6 and DN9) and administered a 2 hour acute AdR treatment with varying concentrations (0.0-0.75μM) (Figure 41). We assessed up-regulation of the DNA damage repair proteins at 6 and 24 hours after AdR treatment.

Basal levels of p53 (i.e. untreated cells) were elevated in all DN-hTERT clones with especially high quantities in the DN2 clone. However, p21 was only up-regulated in clones DN2 and DN6, implying a constant level of stress within the clonal populations. After exposure to AdR, the only difference, as compared to the control pBABE cells, was at the 6 hour time point at 0.75μM with up-regulation of p53 and significantly higher levels of p21. Also observed at 6 hours was the induction of high levels of p21 in clones DN2 and DN6 after treatment with 0.25μM AdR.

DN-hTERT Clones Recover by Telomerase Reactivation

Instead of the projected lag time of growth and telomere shortening followed by death or senescence, we observed from these long-term cultures the emergence of surviving cells that reverted back to normal phenotype and growth rate. Furthermore,

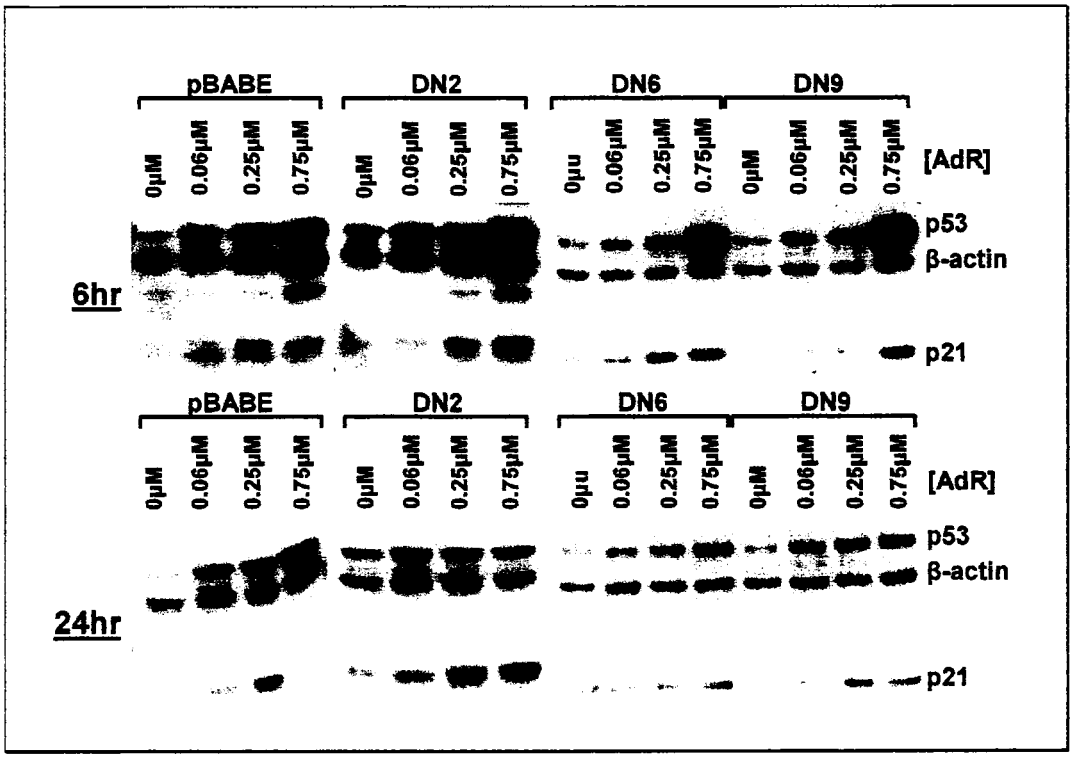


Figure 41. Induction of DNA Damaging Proteins after Treatment with AdR. DN-hTERT/ MCF-7 cells were treated with a 2 hour acute treatment with various amounts of AdR. Samples were taken 6 and 24 hours post-treatment. Cell pellets were collected and 15μg of protein lysate was subject to Western Blot analysis. Immunoblots were probed with anti-p53, anti-p21, and anti-actin as a loading control. The basal levels of p53 in all DN-hTERT clones were elevated as compared to controls with especially high levels found in the DN2 cell line.

these cells regained higher levels of telomerase activity than those found in normal MCF-7 and pBABE control cell lines (Figure 42).

Previous studies have also shown the reactivation of telomerase in human leukemia cell lines (AML and PML) and a murine kidney tumor cell line after telomerase inhibition with a dominant negative TERT (Delhommeau et al. 2002; Klapper et al. 2003; Sachsinger et al. 2002). To uncover the reason for this recovery, we examined the RNA levels of endogenous and exogenous hTERT using RT-PCR (Figure 43). We wanted to compare expression levels before and after recovery as well as detect differences in RNA levels between the clones. Similar levels of exogenous DN-hTERT were detected as before when telomerase activity was decreased. However, clonal variability was not found after recovery, and high levels of endogenous hTERT and DN-hTERT were observed (Figure 43). Therefore, after the clones had recovered, the levels of telomerase activity did not correlate with RNA expression levels of DN-hTERT, possibly suggesting a mechanism independent of transcriptional upregulation of hTERT.

Consequently, we wanted ascertain how the hTERT protein levels were affected within the clones by the reactivation of telomerase activity using a western blot analysis (Figure 43). We looked at protein expression in five of the DN-hTERT clones and found considerably higher levels of hTERT compared to those seen previously (see Figure 36), not to mention the fact that hTERT levels appeared equivalent in intensity as compared to the pBABE/MCF-7 control cell line. Thus, even in the presence of the same levels of DN-hTERT RNA, endogenous hTERT protein was maintained at a constant level,

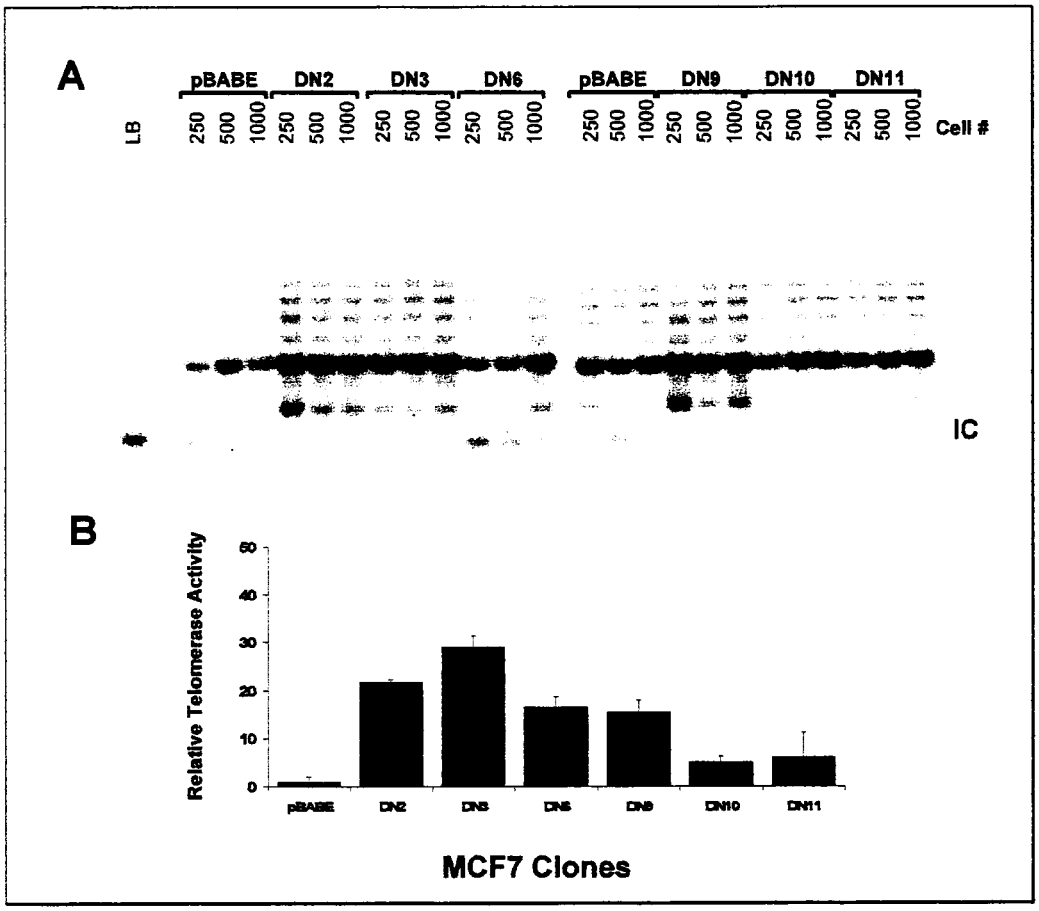


Figure 42. Loss of Telomerase Repression and Recovery. Approximately 4 months post-infection, the DN-hTERT cells went through "crisis" and telomerase activity was detected as a measure of recovery. **A.** Representative TRAP assay showing 250, 500, and 1000 cell equivalents of the DN-hTERT/MCF7 breast cancer cells. **B.** Quantitation of the relative telomerase activity was accomplished as stated in Figure 36. Telomerase activity was found to be highly up-regulated in all clones with a minimum of a 5 fold increase than in the pBABE/MCF-7 controls.

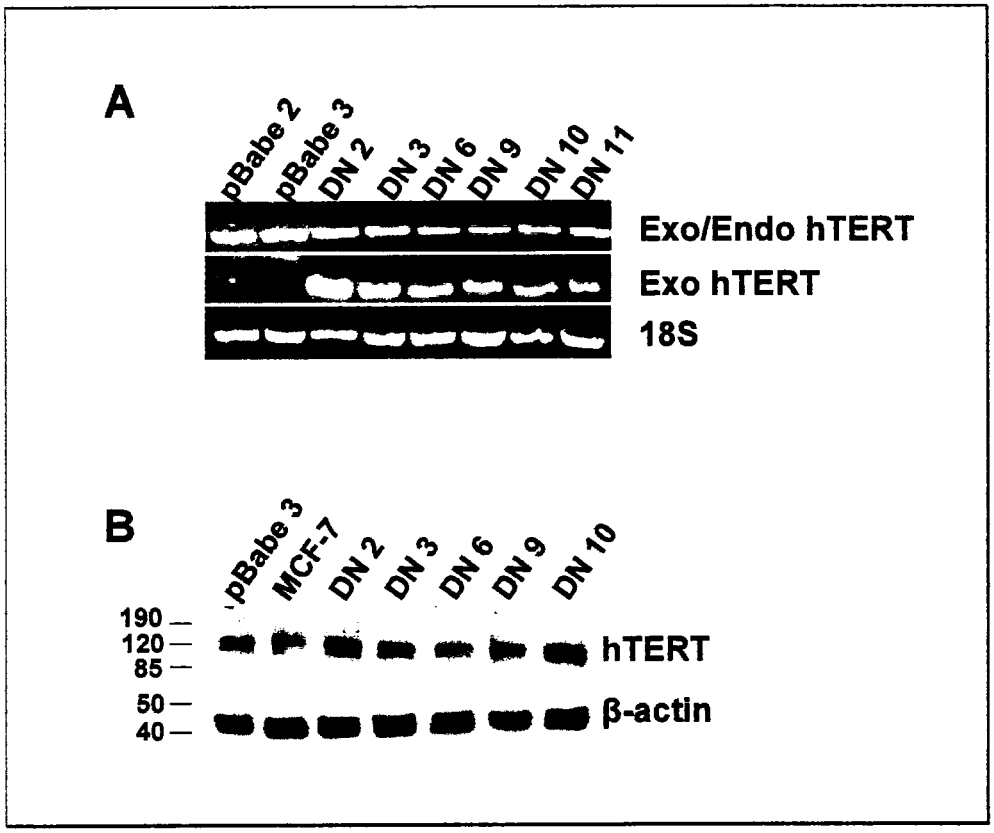


Figure 43. hTERT Expression Levels are Elevated Post-Recovery. A. RT-PCR was conducted to determine the expression of both the overall levels of hTERT (exo/endo) as well as the exogenous DN-hTERT (exo) levels in the various clonal populations. **B.** Cell pellets were collected and 20μg of protein lysate was subject to Western analysis. Immunoblots were probed with anti-hTERT with anti-actin serving as a loading control. Similar high levels of exogenous DN-hTERT were detected as 3 months previously when telomerase activity was knocked-down.

essentially equivalent to that of the MCF-7 cells, indicating that the DN-hTERT is being inactivated or overtaken by the higher levels of hTERT protein.

With this recovery or reactivation of telomerase activity we wanted to ascertain if this also resulted in elongation of the telomeres. Four clones were analyzed approximately three to four months post-infection with DN-hTERT using the TALA assay (Figure 44). All of the clones appeared to have undergone a slight lengthening in overall telomere size, but telomere elongation was especially apparent in clones DN2 and DN10. Recent studies have demonstrated that telomerase will elongate the telomeres that are most likely to be involved in chromosomal abnormalities that cause genomic instability (der-Sarkissian et al. 2004; Zou et al. 2004), so even though the average size of the telomeres were not vastly different when compared to earlier clones during crisis, this could be due to the preferential elongation of the shortest telomeres within the cell and perhaps overtime the other telomeres will continue to lengthen.

Comparison of Senitization of Clone DN2 During Crisis and Post-Recovery

First, we tested if DN-hTERT expression resulted in sensitization of clonal populations to AdR during crisis and after re-activation of telomerase post-recovery. Crisis is defined by the characterizations of the cell line observed earlier including telomerase inhibition, shortened telomeres, slowed growth, decreased levels of hTERT and a senescent-like state. Cells were exposed to an acute 2 hour treatment with various concentrations of AdR (0.0-0.75μM). Samples were taken 2 and 4 days post- treatment. Initially, we measured growth of the cell lines, in duplicate, in order to determine if the

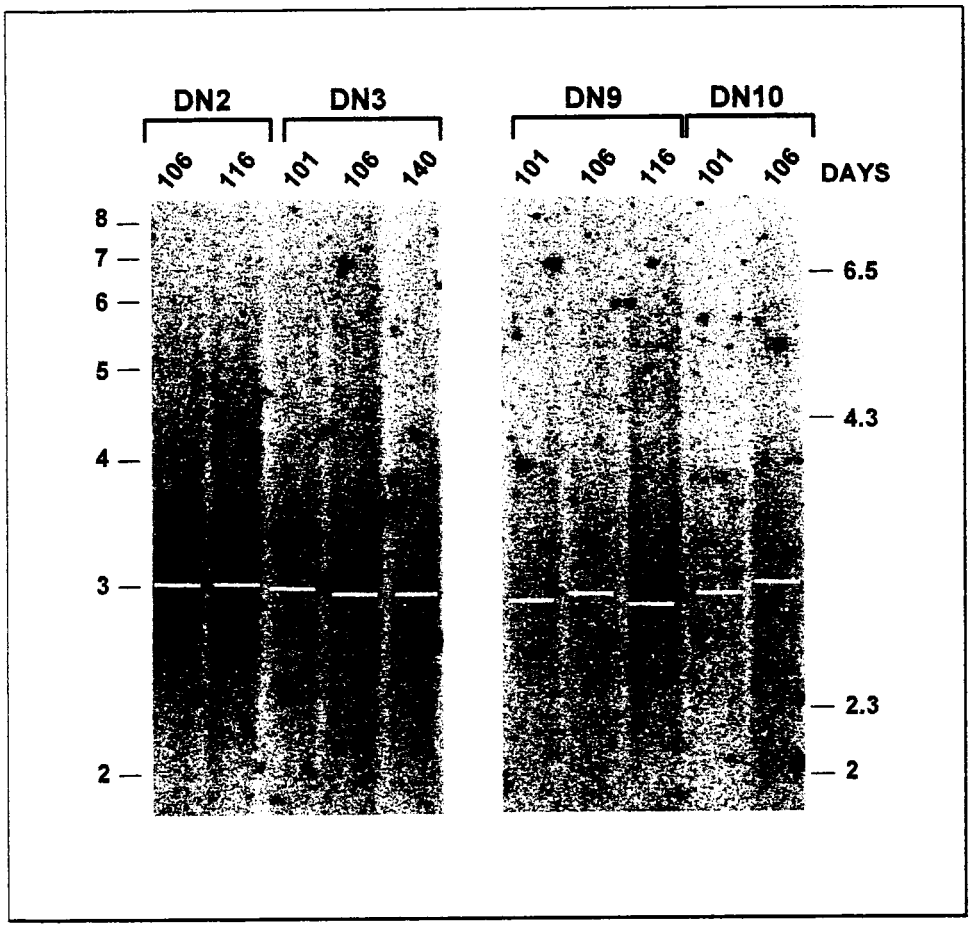


Figure 44. Telomere Lengths were Assessed Post-Recovery. Samples were sequentially collected between month 3 and 4 in four different clonal populations and telomere lengths were determined by the TALA assay. DN-hTERT/MCF-7 clones 2 and 10 exhibited elongated telomeres as time progressed and as the cell lines reactivated telomerase.

chemotherapeutic agent slowed the growth of cells regardless of telomerase activity (Figure 45). In comparison with the pBABE control cell line, only the DN2 clones in crisis (i.e. with inhibited telomerase activity) showed significantly less growth for both days and every concentration. The AdR treatments appeared to have no effect on the recovered DN2 clonal cell line, which had almost equivalent growth as the pBABE cell line. Perhaps, the reactivation of telomerase provided a protective function for the telomeres because the most critically short telomeres had been elongated. Following growth, we assessed the induction of senescence with β-galactosidase expression. We attained the same results as with growth. Only the DN2 cells in crisis displayed significantly higher levels of senescence for every concentration and for both days (Figure 45). Other studies have shown induction of senescence in MCF-7 cells 4 days after exposure to 1μM AdR (Elmore et al. 2002). Therefore, generation of such high levels of senescence 2 days post-treatment and at concentrations of AdR as low as 0.02μM is extremely significant revealing definite sensitization of DN-hTERT cells to AdR. Lastly, we examined the percent apoptotic cells after treatment with AdR using the TUNEL assay (Figure 46). Neither population of cells revealed considerably high levels of cell death at day 2 or day 4. In fact, there were overall low levels of apoptosis at every concentration regardless of the cell line. However, this is not totally unexpected because other studies using MCF-7 cells have shown that only senescence occurs post-treatment with AdR due to the presence of p53 because once p53 is knocked-out using E6, increased apoptosis occurs (Elmore et al. 2002).

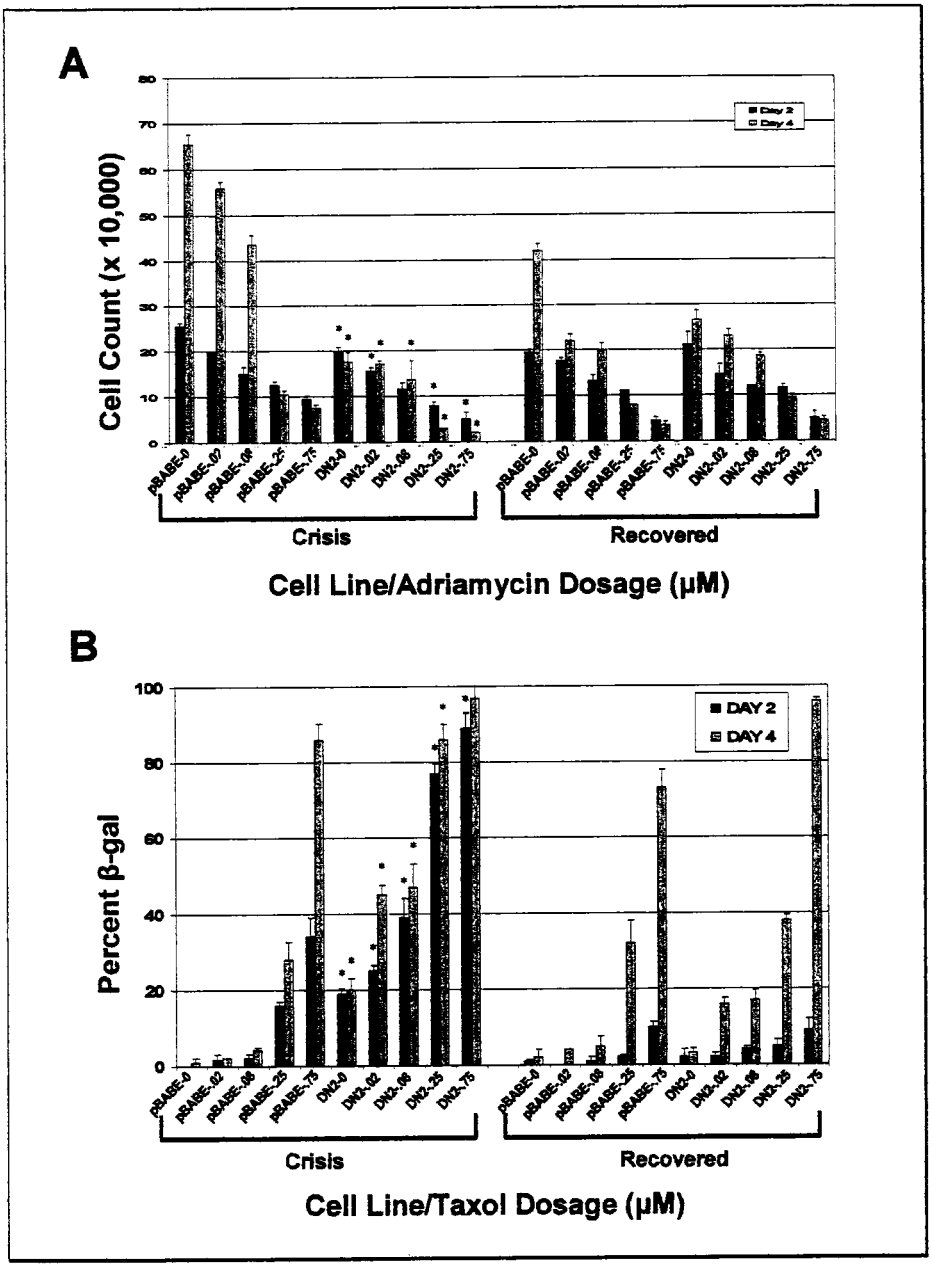


Figure 45. Decreased Growth and Increased Senescence in the DN2 Clone during Crisis but not in Recovered cells after Treatment Adriamycin (AdR). The DN2/MCF-7 clone at different stages, crisis and recovered, was exposed to various concentrations of AdR and samples were then taken 2 and 4 days post-treatment. **A.** Cell numbers were counted in duplicate. Only cells in crisis had significantly slower cellular growth compared to controls at dosages lower than clinically relevant. **B.** β-galactosidase staining for senescence, calculated for 3 independent fields of 100 cells. Similarly, only the DN2 cells in crisis displayed elevated levels of senescence at all concentrations of AdR. *, $P < 0.05$.

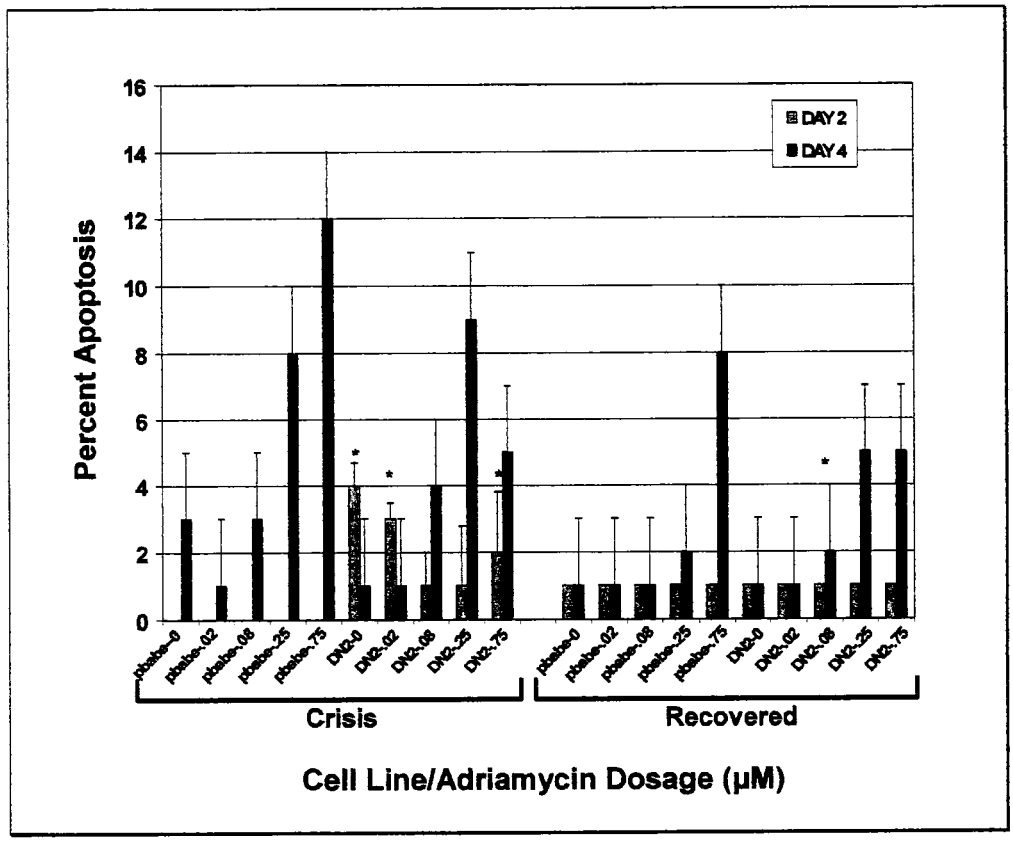


Figure 46. Sensitization of DN2 Cell Lines to Apoptosis after Treatment with AdR does not occur. Quantitation of a TUNEL assay 2 and 4 days post-treatment with AdR as was calculated in Figure 31. Neither the DN2 cells during crisis or after recovery revealed any substantial divergence in percent apoptosis as compared with the pBABE controls. *, $P < 0.05$.

In order to provide another means of sensitization in MCF-7 breast cancer cells, we utilized taxol, which has a distinctive effect compared to AdR as explained above. The same sensitization experiment was used as with AdR. Briefly, cells were exposed to various concentrations of AdR (0.0-0.75μM) for 2 hours with samples taken 2 and 4 days post-treatment. Again, we analyzed growth and found only the DN2 clones in crisis showed significantly slowed growth at every concentration as compared to controls (Figure 47). Treatment with taxol had minimal effects on the growth of DN2 post-recovery as compared to pBABE controls. We also assessed senescence post-treatment with taxol and found only the DN2 clones in crisis displayed considerably higher levels within the cell as compared to controls (Figure 47). Finally, using the TUNEL assay we looked apoptosis after treatment with taxol (Figure 48). Significantly higher levels of apoptosis were observed in the DN2 crisis cell line but not so in the DN2 cell line post-recovery, suggesting that the DN2 cells behave more like parental MCF-7 cells than those with decreased telomerase.

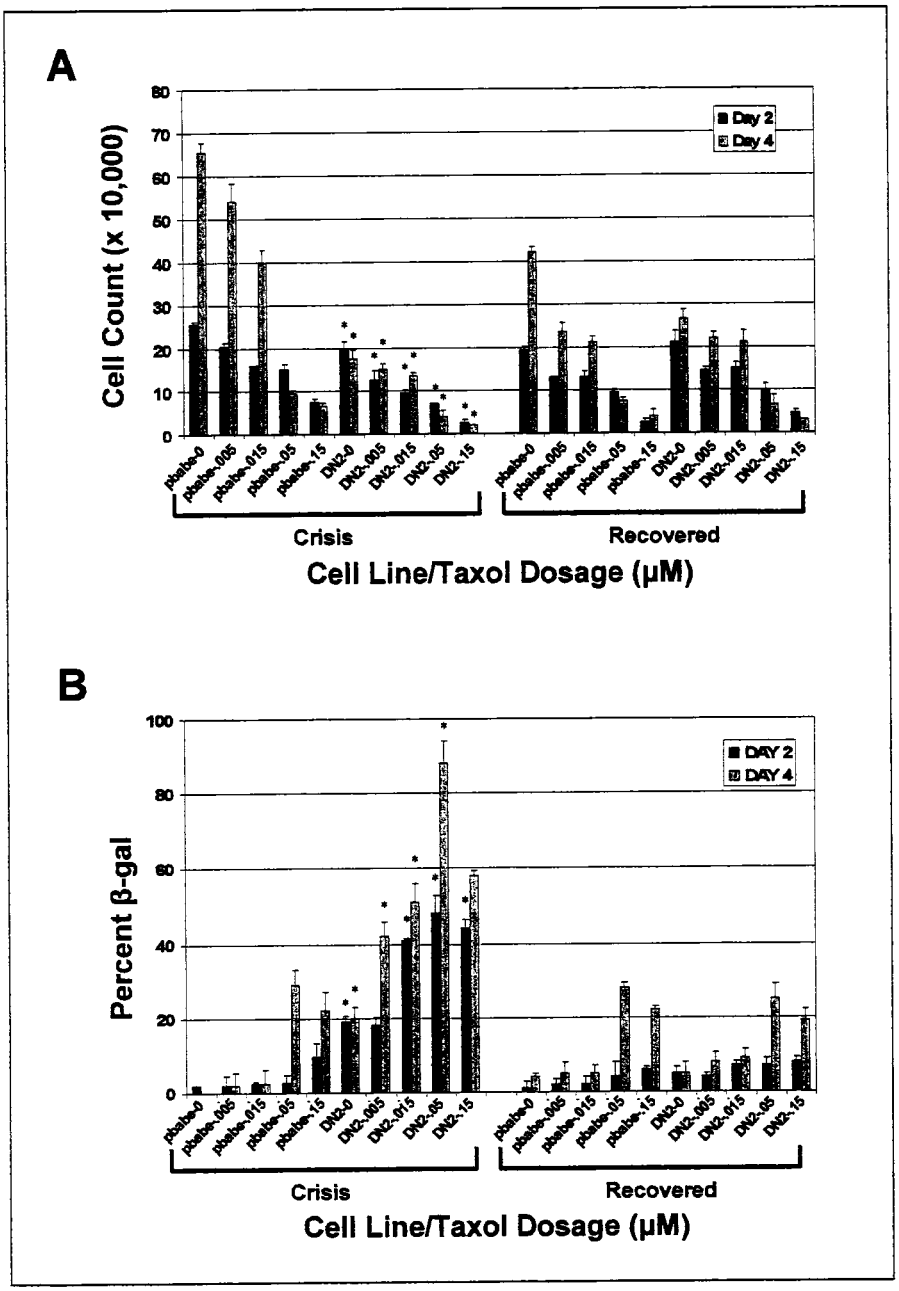


Figure 47. Taxol Causes Decreased Growth and Increased Senescence in the DN2 Clone during Crisis but not after Recovery. The DN2/MCF-7 clone at different stages, crisis and recovered, was exposed to various concentrations of taxol and samples were then taken 2 and 4 days post-treatment. **A.** Cell numbers were counted in duplicate. Only cells in crisis had significantly slower cellular growth, as compared with controls, at dosages lower than clinically relevant. **B.** β-galactosidase staining was done and calculated for 3 independent fields of 100 cells. Similarly, only the DN2 cells in crisis displayed elevated levels of senescence at almost all concentrations of taxol. *, $P < 0.05$

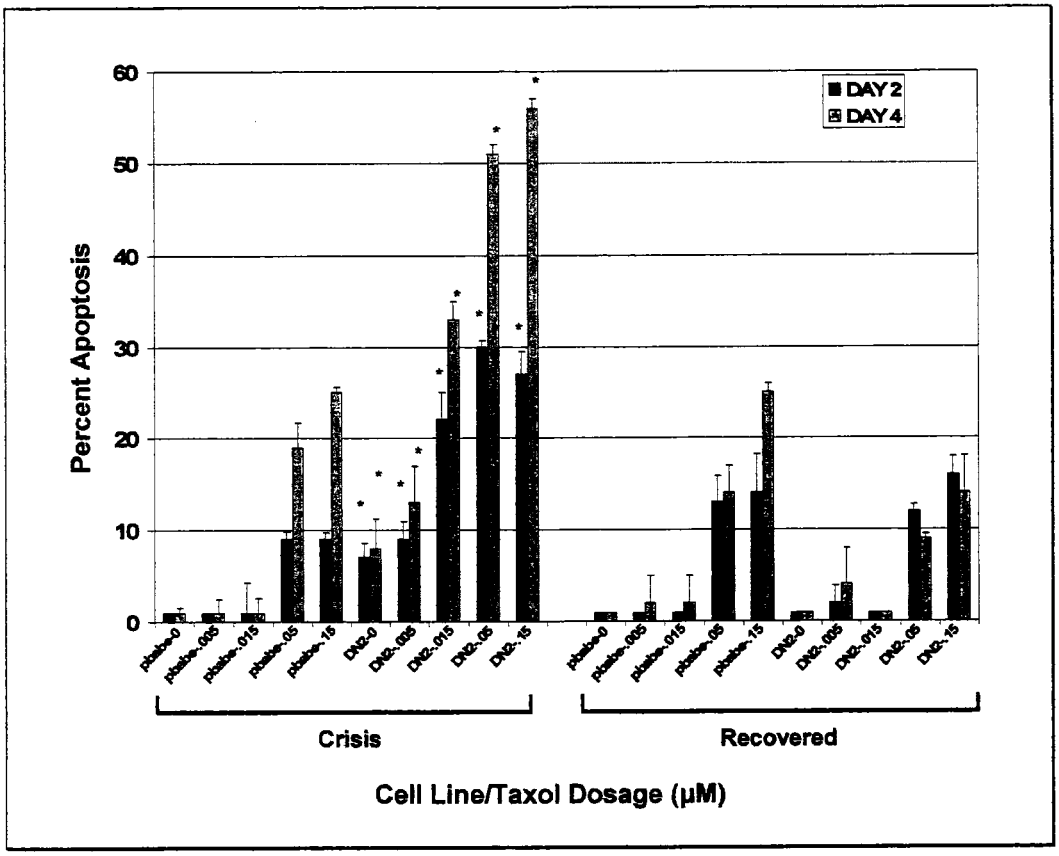


Figure 48. Sensitization of DN2 Cell Lines to Apoptosis after Treatment with Taxol. Quantitation of a TUNEL assay 2 and 4 days post-treatment with AdR. Only the DN2 cells during crisis revealed any substantial divergence in percent apoptosis as compared with the pBABE controls. *, $P < 0.05$.

Chapter 5

Discussion

Telomerase Inhibition Techniques in Combination with Chemotherapeutic Drugs

Telomerase is expressed in nearly 90% of human tumors and up-regulated in 95% of breast carcinomas, making its detection extremely promising for aiding in cancer diagnosis and prognosis, as well as treatment. Most often treated with conventional cytotoxic chemotherapies, breast cancer still remains resistant to apoptosis post-treatment (Rasbridge et al. 1994). Our goal was to provide a comparison of the different telomerase inhibition strategies on sensitizing breast cancer cells to standard chemotherapeutic agents. We demonstrate high levels of senescence and/or apoptosis of MCF-7 breast cancer cells by pre-treating with the different strategies of telomerase inhibition including siRNA for both hTR and hTERT, shRNA for hTR, and DN-hTERT followed by exposure to two different types of chemotherapies, Adriamycin and taxol.

Since the telomerase holoenzyme is composed of multiple components, there are many potential targets for achieving telomerase inhibition. Many studies have displayed telomerase inhibition targeting the core components of telomerase, hTR or hTERT, using a variety of methods including ribozymes, antisense, N3'→P5'-*thio*-phosphoramidate and mutant template hTR (Hao et al. 2005; Yeo et al, 2005; Nakamura et al. 2005; Dikmen et al. 2005; Blackburn, 2005). Our data demonstrate the use of synthetic siRNAs as an effective tool to inhibit telomerase activity in MCF-7 breast tumor cells by targeting both hTR and hTERT. This methodology is successful because the synthetic siRNAs are

incorporated into a mechanism that naturally occurs within the cell, RNA interference. However, in this case, DICER would not have to process the oligonucleotides in that they are transfected into the cell in the appropriate length, ~21 bp long, along with the two-nucleotide 3'-UU overhangs on both ends.

We demonstrate inhibition of hTR and hTERT RNA levels, using RT-PCR, and this knock down of expression was accomplished quite easily using a transient transfection with almost 100% efficiency in some cases. An effect, telomerase inhibition measured by the TRAP assay, was observed within 24 hours after the synthetic siRNAs were administered to the cells. Furthermore, one dosage of synthetic siRNAs caused a rapid response of slowed proliferation. Sensitization of MCF-7 cells was observed approximately 2 days post-treatment with the traditional chemotherapies, AdR and taxol. We utilized two chemotherapeutic agents because they attack the cell in very distinct methods. AdR is a topoisomerase II inhibitor and DNA-damaging agent that produces DNA strand breaks, while taxol stabilizes the microtubules during mitosis thereby preventing sister chromatid separation.

Similar to our observations, other studies conducted in human cervical carcinoma cell lines found treatment with the shRNA hTERT-1 resulted in slowed proliferation (Nakamura et al. 2005). We determined that using the synthetic hTERT-1 siRNA not only caused slowed growth within MCF-7 breast cancer cells, but also resulted in a highly effective knockdown of RNA levels and decreased telomerase activity. Within 48 hours of treatment with chemotherapy we found sensitization of cells as seen by further inhibited proliferation, as well as increased senescence and apoptosis. Comparable

findings were observed after knocking down levels of hTR in MCF-7 cells utilizing the siRNA hTR-2. Unexpectedly, using siRNAs targeting only hTR or hTERT singularly produced an almost equivalent response in terms of growth and sensitization as pooling these siRNAs together. The expectation being that the more siRNAs targeting the two core components, the more effective the knockdown of telomerase, because the RNA and protein are simultaneously being inhibited resulting in a more drastic response within the cells and therefore enhanced sensitization after drug treatment. However, we found no evidence that more is better, perhaps because the RNAi mechanism became saturated (Barik, 2004). RNAi is a naturally occurring mechanism, so there are other pathways in the cell that are dependent on this mechanism. The possibility exists that the use of multiple synthetic siRNAs within each cell became too overwhelming and the RNAi pathway could not accommodate all of the synthetic siRNAs along with its other functions, such as the generation and function of miRNAs (Hutvagner et al. 2004).

Generally, we found that AdR caused significantly elevated levels of senescence while taxol results in significantly higher levels of apoptosis just as other studies have shown previously (Saunders et al. 1997, Elmore et al. 2002). The uniqueness of our study revolves around the dosage and time period. We determine both chemotherapies caused these effects within a much quicker timeframe and at lower dosages than clinically relevant after pre-treatment with the telomerase inhibiting siRNAs. We observed sensitization within 48hrs of the drug treatment at acute doses of AdR as low as 0.02μM and Taxol of 0.005μM without delay in response to the drug treatments. Interestingly, we also observed high levels of senescence with taxol and high levels of

apoptosis with AdR, which is atypical. We observed significantly elevated levels of senescence and apoptosis after drug treatment, when the cells normally choose one over the other, suggesting that senescence and apoptosis may not be mutually exclusive events. The data presented here does not directly address this mutual exclusivity, but it is clear that the cells appear to undergo senescence earlier when exposed to AdR and then undergo apoptosis later.

We also attempted to reduce telomerase activity permanently through the use of retroviral and lentiviral systems. With these particular systems, the designed oligonucleotides are permanently inserted into the genome. These oligonucleotides also functioned in the RNAi pathway, similar to the synthetic siRNAs, but after synthesis within the cell, the oligonucleotides form short-hairpin RNA that have to be processed and sliced by DICER in order to activate the RNAi mechanism. Both of the vectors utilized contained puromycin resistant markers, and therefore, post-infection, we drug selected the cells with either the retroviral or lentiviral cassettes for 5 days to isolate only those cells containing the appropriate transgene. With the retroviral infected cells, which only infects dividing cells, clonal populations had to be established taking an additional 3 to 4 weeks for them to grow enough to be tested and manipulated, which is a major drawback of this system. Furthermore, we found highly variable results in terms of telomerase knockdown over time post-retroviral infection. All of the clonal populations displayed a decreased level of telomerase but not nearly as severe as with the synthetic siRNAs, so as a result sensitization studies were not conducted in these cell lines.

However, the hTR-T and hTR-1 sequences used in the pSuper.retro vector also produced variable results when transfected synthetically.

On the other hand, with the lentiviral cassettes, because they infect dividing and non-dividing cells, clonal populations were not isolated, and cells were tested immediately after puromycin selection. However, even when using the lentiviral method of infection with the same siRNA sequence, hTR-2, that had proven successful when synthetically administered to MCF-7 cells, telomerase activity was decreased but not as significantly nor was this inhibition maintained past two weeks. Using the stably infected shRNAs produced some sensitization of the cells, but not as dramatically as with the synthetic siRNAs in terms of dosage and time period. After treatment with AdR, we observed slowed growth as well as high levels of senescent cells at the lower dosages (0.02-0.25μM) by day 4 as compared to the empty vector control cells. As for the cells treated with taxol, we determined considerably slowed growth in the lower dosages (0.0-0.015μM) along with elevated levels of apoptosis in the higher drug concentrations by day 4 (0.05-0.15μM). The difference observed in terms of sensitization observed between the transient and stably expressed siRNA could be attributed to the possibility that the RNAi pathway becomes saturated with prolonged expression of the shRNAs as mentioned earlier with the pooled siRNAs or that the cells become partially resistant to the shRNA expression.

Another method we utilized to stably decrease telomerase activity was the use of a dominant negative form of hTERT. MCF-7 cells were infected with the retroviral vector containing the DN-hTERT sequence, followed by puromycin selection for 5 days

and the growth of clonal populations, approximately 3 to 4 weeks. We confirmed the presence of the exogenous hTERT using RT-PCR, showing high RNA levels with distinct expression patterns per clone. In addition, decreased hTERT protein levels using the western blot and reduction of telomerase activity were ascertained, but in both instances, we observed great variability among the clonal populations, which correlated to the amount of DN-hTERT RNA expression observed by RT-PCR. From these experiments, our assumption is the presence of DN-hTERT within the cell may cause degradation of endogenous hTERT protein, resulting in reduction of telomerase activity within the cell. Unlike with the use of the synthetic siRNAs or shRNAs, this telomerase inhibition was maintained over months and not simply days. As other studies have shown previously, we found telomerase inhibition followed by telomere shortening and sensitization after expression of DN-hTERT and treatment with various anticancer drugs (Cerone et al. 2006). Our results are unique in that we utilized lower drug concentrations than those utilized in the other studies and still concluded sensitization (i.e. the colony forming assays). Furthermore our study measured levels of senescence and apoptosis after treatment with a range of 5 different drug concentrations for each chemotherapeutic drug, AdR and Taxol; whereas, previous studies only looked at one drug concentration per chemotherapeutic agent.

We tested sensitization of DN-hTERT clones prior to significant telomere shortening, almost directly after the clonal populations were established. In the first study, using a colony forming assay, we showed decreased proliferation and colony formation of clones DN2, 6 and 9. Only for clones DN2 and DN6 did we find

sensitization at AdR dosages as low as 0.0625μM. Even though clone DN9 had longer telomeres than the other clones, some shortening had occurred within this cell line, suggesting that perhaps there is a threshold of telomere length that has to be reached before sensitization can occur.

In the second set of sensitization experiments, we chose to examine the clone with the greatest amount of telomerase knockdown, DN-2, by treating this clone with an even lower dose to determine if smaller AdR concentrations could produce similar sensitization results. This clone showed equal levels of sensitization as seen with the use of the hTERT-1 synthetic siRNA. Post-treatment with both of the chemotherapeutic drugs showed that senescence and cell death occurred more rapidly in DN2 as compared with the controls at lower dosages than clinically relevant. As previously shown by other studies, we found induction of senescence with AdR and apoptosis with taxol. Specifically, with AdR we observed slowed growth and senescence within 48hrs of drug treatment at dosages 0.02μM. For taxol, we detected slowed growth and high senescence and apoptosis at concentrations of 0.005μM by day 2 post-treatment, again suggesting that cells may be capable of undergoing both senescence and apoptosis. Interestingly, we also saw high levels of senescence with taxol, which is usually not the case to observe significantly elevated and equal levels of senescence and apoptosis after drug treatment, usually the cells choose one over the other. These results also differ from another study that found sensitization of HeLa cells only after treatment with DNA-damaging agents such as AdR but not to microtubule-targeting reagents such as taxol, implying a specific interaction between DNA repair and telomerase (Nakamura et al. 2005). These

observations demonstrate that the disruption of telomere maintenance limits cellular lifespan in human cancer cells, validating hTERT as an important target for the development of anti-neoplastic therapies.

Most Effective Means of Sensitization

Overall, we have found high levels of sensitization with RNAi and with DN-hTERT knockdown of telomerase activity. The most effective synthetic siRNA tested was hTERT-1. We observed significant levels of slowed growth, senescence and apoptosis as compared to the controls before chemotherapeutic treatment and even higher levels were observed post-treatment, regardless of chemotherapy administered indicating perhaps, hTERT is a better target for future development of anti-telomerase therapies than hTR or targeting both components simultaneously using RNAi. Other studies have also demonstrated increased sensitivity to topoisomerase inhibitors in MCF-7 cells after treatment with a hammerhead ribozyme directed against the T motif of hTERT (Ludwig et al. 2001). Here, we demonstrate sensitization at a wide range of concentrations of chemotherapies using synthetic siRNA and transient inhibition of telomerase, which has not been done previously and was rapid and occurred at much lower dosages as had been shown before.

Even though studies have revealed that siRNAs are more stable in mammalian cells than antisense and ribozymes, there still remains the problem of delivery of chemically synthesized siRNA into the cells (Bertrand et al. 2002). Thus, we utilized the retroviral and lentiviral plasmid delivery system of siRNA into the cells. However, these

methods did not generate the same quality of sensitization in the breast tumor cells as the hTERT-1 siRNA but with the lentiviral system we were targeting the template region of hTR with the hTR-2 siRNA sequence.

The use of a DN-hTERT transgene within the MCF-7 breast tumor cells appears to be the most successful, stable means of telomerase inhibition observed in comparison to RNAi. We determined that telomerase activity was stably inhibited for an extended period of time, approximately 4 months, and showed that the cells exhibited the significant telomere shortening, slowed proliferation, elevated senescence and decreased levels of hTERT protein within the cells. As for testing sensitization of the cells, we selected the clone with the most significant knockdown of telomerase and shortest telomeres, DN2. We compared the clonal cell line before and after recovery of telomerase activity to the MCF-7 parental cell line. Again both chemotherapies were utilized with a range of concentrations per drug. After treatment with AdR in the DN2 clone during crisis, we found significantly slowed grow and high levels of senescence at all drug concentrations, while treatment with taxol caused substantially slowed growth, high levels of senescence and apoptosis regardless of drug concentration administered. However, this sensitization did not occur in the recovered DN2 cells in which telomerase had been reactivated or in the MCF-7 parental cell line, revealing a possible protective function of telomerase. Perhaps, once reactivated telomerase functions by elongating the shortest telomeres in the cells thereby reducing genomic instability and preventing sensitization

We show the combination of DN-hTERT and standard chemotherapies to be a highly effective means to induce apoptosis and/or senescence in breast tumor cells because the lag period is shorter, in that the patient would not have to wait until all telomeres became critically short as proven by our study that some telomere shortening clearly enabled sensitization within the cells. In addition, the concentrations that produced sensitization with the DN2 cells were at doses much lower than clinically relevant and are insufficient to trigger cytostasis, which would be beneficial to the patient in terms of a reduction in toxicity and side effects from the chemotherapeutic drugs.

Mechanism for how Decreasing Telomerase Activity Sensitizes Cells

We have shown that pre-treatment with the synthetic siRNAs in MCF-7 breast tumor cells was a very effective means of inhibiting telomerase activity in a transient fashion. We then tested sensitization of these cell lines by treating them with two chemotherapies at various concentrations, observing increased levels of senescence and apoptosis. This sensitization occurred within 5 days of initial siRNA transfection, meaning that there was not sufficient time for the telomeres to shorten to critical lengths. Instead perhaps, the chemotherapies caused telomere dysfunction, which could not be repaired due to the lack of active telomerase at the telomeres. It has been suggested that telomerase, specifically its function in telomere maintenance, has a protective role for cells attempting to repair DNA damage (Chan and Blackburn, 2002). Without net global telomere shortening, the telomere dysfunction, which was caused by the lack of telomerase and the damage induced by the drug treatments, may result in loss of

chromosome end protection such as the capping function of hTERT, thereby inducing senescence or apoptosis. Another study has suggested that the cancer cells may have adapted to the high levels of telomerase activity within the cells and become "addicted to this upregulation of telomerase (Li et al. 2005). Therefore, when the telomerase levels are abruptly decreased the cancer cells are unable to cope which renders them susceptible to a change in telomerase level resulting in a rapid growth-inhibitory response. Another possibility is the revolves around the idea that MCF-7 cells may be on the threshold of short telomeres, so that blocking telomerase would cause an immediate shortening of telomeres, within one cycle. This insult would then cause the instability and sensitization observed in our breast cancer cells.

As seen by the Western analysis of cells transfected with hTR-2 siRNA after treatment with AdR, basal levels of p53 were higher compared to the scrambled siRNAs, suggesting a potential increase in DNA damage and repair in the absence of telomerase. In addition, we observed a transient increase in the p53 levels at 6 hours after treatment, which had disappeared by 24 hour post-treatment. Studies have suggested that after AdR treatment of MCF-7 cells, p53 function induces senescence, but that inhibiting p53 in the same cell line results in increased incidence of apoptosis after treatment with AdR (Elmore et al. 2002). Here, we demonstrate significantly elevated levels of apoptosis in MCF-7 cells transfected hTR-2 after treatment with AdR as compared to the control MCF-7 cells, even though in the presence of wild-type, functional p53. This suggests that the levels of DNA damage at the telomere are significant, which could normally be compensated for by higher levels of telomerase.

Breast cancer cells infected with DN-hTERT displayed sensitization to chemotherapeutic drugs, but only after telomere shortening had occurred (Cerone et al. 2006). Using the DN-hTERT transgene in MCF-7 cells, we inhibited telomerase, which resulting in telomere loss after growth over time, and then treated cells with various chemotherapies at a range of concentrations. Similar to other results, we found that telomerase inhibition sensitizes cells to chemotherapeutic drugs, contingent upon telomere shortening but not complete loss of telomeric sequence or function. However, we show sensitization at a wide range of drug concentrations, while the other study only examines one concentration per chemotherapy. One possible reason for this sensitization with AdR is related to the study that suggested that AdR preferentially targets the telomeres (Elmore et al. 2002). Therefore, in our case where telomere shortening has occurred in some telomeres, this subset of shorter telomeres are more susceptible to DNA-damaging reagents such as AdR.

However, in certain clones where telomeres are longer at earlier time points, studies have shown that telomerase preferentially elongates the shortest telomere (Zou et al. 2004; der-Sarkissian et al. 2004). So, in the absence of this regulation within the cancer cells due to the DN-hTERT, perhaps those telomeres that are not continually being elongated and may be more affected by the repression of telomerase (compare DN2 with shorter average telomeres with DN6 or DN9 with longer telomeres), sensitization still occurs.

Another possibility for the explanation of how decreasing hTERT levels causes increased sensitization of breast tumor cells is the contribution of functional hTERT to

genomic instability within the cell, in that hTERT cannot perform its additional protective functions or telomere maintenance mechanisms. Other studies have indicated that hTERT not only guards the chromosome by elongating telomeres but also "caps" the chromosomes, providing karyotypic stability (Chan and Blackburn, 2002). Furthermore, overexpression of hTERT has been demonstrated to aid the process of malignant transformation, regardless of telomere length, making the cells more anti-apoptotic (Kang et al. 2004).

In the breast cancer cells, PMC42, hTERT down-regulation was shown to decrease apoptosis resistance, causing induction of cell death (Cao et al. 2002). They also established some sort of interplay between hTERT and p53; specifically, hTERT and p53 knockdown elevates the levels of death in these cells, whereas increasing p53 while hTERT is inhibited results in the opposite, rescue from apoptosis. MCF-7 cell lines are also p53 positive and in the DN-hTERT clones with greatest transgene expression, DN 2, we find higher basal levels of p53, suggesting a DNA damage response within the cells without treatment. However, after AdR treatment, the only significant upregulation of p53 as compared to the MCF-7 controls is early after treatment with the high, clinically utilized concentration of 0.75μM AdR.

Recovery of the DN-hTERT Clonal Populations

Approximately 4 months post-infection of the DN-hTERT gene, we found recovery of telomerase activity and telomere elongation for all of the clones over time.

While the molecular basis of this recovery is yet unknown, our RT-PCR data suggests that it is not due to the loss of the transgene or transcriptional silencing. However, this phenomenon of recovery of cells has been documented in several other studies in which a catalytically inactive form of hTERT was used to inhibit telomerase activity with each theory on the mechanism of recovery being distinctive. One study proposes that even though they attained a clonal population, perhaps some of the cells within the population silenced the DN-hTERT transgene. This action relieved the inhibition pressure within the cells thereby producing a growth advantage over those cells containing active DN-hTERT. Therefore, after a significant period of time growing in culture, the cells with silenced mutant hTERT would be selected for and thus take over the population of cells (Rahman et al. 2006). Unlike our results, that particular study revealed decreased DN-hTERT expression levels in late passage cells with reactivation of telomerase, while we showed to change in DN-hTERT mRNA levels before and after recovery.

In a study conducted in a murine kidney tumor cell line, RenCA, after a period of time post-infection with a murine dominant-negative TERT, the cells reactivated telomerase, which was correlated with considerable increases in endogenous mTERT mRNA levels in the presence of DN-mTERT expression (Sachsinger et al. 2002). Similarly, another study, utilizing human AML cell lines, after infection with DN-hTERT, also demonstrated telomerase reactivation and recovered proliferation rates similar to that of control cell lines (Delhommeau et al. 2002). They found the rescue of the cells after telomere shortening resulted from one of two possibilities, including loss of mutant hTERT expression or upregulation of endogenous hTERT mRNA while the DN-

hTERT was still expressed. Our results demonstrate neither option. We found the reversion of telomerase inhibition without the up-regulation of endogenous hTERT mRNA transcription and with continued high expression of the DN-hTERT transgene. Interestingly, no reversion to an ALT (alternative lengthening of telomeres) phenotype was observed in our system (Jevapalan et al., 2005).

The mechanism for the recovery of telomerase activity observed in the DN-hTERT/MCF-7 breast cancer cells is as of yet unknown as we found high levels of exogenous transgene expression without a significant increase in hTERT mRNA levels, suggesting a mechanism independent of transcriptional upregulation of hTERT. We also determined the hTERT protein levels to be almost equivalent in intensity as compared to the control cell line. One possible explanation for this recovery of telomerase activity has to do with telomerase assembly within the cell. Possibly due to the increase in stress imposed by telomerase inhibition and shortening telomeres, there is an upregulation of chaperone proteins resulting in an increased quantity of assembled telomerase within each cell (Akalin et al., 2001). Because there are few molecules of telomerase within every cell, less than 100 and of those complexes approximately only 10-20% assemble and function properly, elevated levels of chaperones may enable the formation of a greater number of functional telomerase complexes resulting in higher quantities of functional telomerase activity.

Clinical Implications of Telomerase Inhibitors

As mentioned previously, telomerase inhibition is a slow method utilized to cause apoptosis in cancer cells in that there is a lag period, dependent on the length of the telomeres, between administration and observable phenotype (Corey, 2002). Despite advances in diagnosis, staging and management, metastatic disease is still essentially incurable resulting from its evasion of traditional chemotherapy and potentially acquired drug resistance. Thus, anti-telomerase therapies would be beneficial as an adjuvant therapy to traditional chemotherapeutics, perhaps to sensitize cells, but likely not as a primary treatment.

In vivo delivery of siRNAs has been applied quite readily with great success in postnatal and adult mice, which involves the siRNAs or plasmid encoding shRNA being injected hydrodynamically into the tail vein (McCaffrey et al. 2002; Layzer et al. 2004; Lewis et al. 2002; Sorensen et al. 2003). Significant reductions of target sequences were observed mostly in the liver but also in the pancreas, lung, kidney and spleen. However, this method can not be translated into treatment of human disease but remains a good proof of principal technique.

The uses of retroviral or lentiviral plasmids to deliver siRNA or shRNA in the therapy of human disease is limited due to a few critical problems, including inefficient *in vivo* delivery, insufficient delivery to the desired tissue, and unknown toxicity for the patient (Dorsett and Tuschl, 2004; Hannon and Rossi, 2004). In terms of delivery, the virus-based expression vectors are not cell or tissue specific, therefore, there is no guarantee that the appropriate quantities of the siRNA will reach the targeted tumor cell

or that problems will not arise from the localization of the therapies in the wrong tissues (Sioud, 2004). Another possible hazard is the disruption or dysregulation of important genes via the chromosomal integration of the viral genome (Barquinero et al. 2004; Bartosch et al., 2004).

Some researchers have developed methods to overcome these pitfalls using the selective expression of hTERT in cancer cells. A tumor-specific antigen for therapeutic vaccines was created and has shown preliminary signs of efficacy, including a lack of toxicity to normal and stem cells in the Phase I/II trials (Vonderheide et al, 2004). Realistically, our synthetic siRNAs would not be of much use in the human body unless topically applied, which is not likely, as seen for treatment of ocular diseases such as age-related macular degeneration. Fomivirsen, induced by cytomegalovirus, is placed on the retina and was approved in 1998 by the FDA to treat retinitis (Marwick, 1998). In addition, phase I trials of shRNA-based therapy against HIV began in 2006. Therefore if the transgenes of the shRNA or DN-hTERT could be targeted to cancer cells somehow through the use of hTERT or being expressed by the hTERT promoter then they would have significant benefits as adjuvant cancer therapy treatments not only in breast cancer but all telomerase-expressing cancers.

Applicability of Telomerase Inhibitors in Future Studies

Experimental systems have taught us that ectopic telomerase can protect against genomic instability, a driving force in both aging and cancer. Here we have shown with

multiple telomerase inhibition strategies that MCF-7 breast cancer cell growth was halted *in* vitro, partially resulting in the induction of apoptosis. However, with those techniques that were stably maintained over a significant period of time, specifically with the DN-hTERT, high levels of cell death occurred only post-treatment with the chemotherapeutic agents AdR and taxol. Therefore, combining telomerase inhibition with chemotherapy is a more effective strategy for cancer treatment than either one alone. With most telomerase inhibitors, there is a lag period between inhibition and meaningful biological effects. We have demonstrated a more realistic approach, which is using telomerase inhibition as a means to sensitize breast cancer cells to more standard therapeutic modalities, hence, as an adjuvant treatment that produces the desired effect (e.g. apoptosis and/or senescence) in a quicker timeframe and at lower dosages than traditionally utilized. This idea of combination treatment of telomerase inhibitors and chemotherapy resulting in sensitization of cancer cells is not a MCF-7-specific phenomenon. Other studies have also shown telomerase inhibition can sensitize leukemia, cervical carcinomas as well as other types of breast cancer to various chemotherapies (Ward and Autexier, 2005; Nakamura et al. 2005).

We concluded the most effective means to inhibit telomerase is by targeting hTERT using RNAi. Therefore, it seems logical to ascertain the effects of permanent insertion into the genome using the lentiviral system. Even though this technique was unsuccessful using the hTR-2 target sequence, stable suppression of hTERT might be more efficient in the MCF-7 cells in reducing telomerase activity as observed with the

DN-hTERT transgene infection. We could also utilize the lentivectors *in vivo*, injecting them into mice to determine inhibition of tumor growth.

In the DN-hTERT cell lines, we show that these cells failed to exhibit high levels of immediate death but displayed growth retardation, which could also be tested by their ability to grow in soft agar. This would demonstrate that telomerase inhibition, either by knocking down hTERT, alters the proliferative potential of the breast cancer cells thereby leading to decreased tumorigenic potential because even limited reduction in growth rates *in vitro* would imply some therapeutic value for patients.

In order to confirm or add onto the results of this study, we could conduct the same set of experiments in paired drug-resistant and drug-sensitive cell lines as well as to assess if growth inhibition and if enhanced chemotherapeutic sensitivity occurred. This would indicate if our strategy to sensitize cancer cells utilizing the combination of telomerase inhibition pre-treatment followed by chemotherapy would also prevent recurrence post-treatment.

In addition, our success *in vitro* would lead us to the next step, which is applying the same knowledge using an *in vivo* system. It is important to determine if our methodologies would reduce the tumorigenicity *in vivo* by generating xenograft mouse models. Clonal isolates expressing the DN-hTERT transgene as well as appropriate control vectors including pBABE and WT-hTERT would be injected subcutaneously into immunodeficient nude mice, followed by treatment with chemotherapies at various concentrations and assessing the differences in tumor growth. Ideally, we expect that the

lag period between inhibition and biological effect was not required and a significantly decreased tumor growth at lower drug concentrations.

Summary

This work has provided insights into the consequences of different genetic mechanisms of telomerase inhibition in breast cancer cells as pre-treatments to chemotherapies in order to sensitize cells. While we demonstrate that telomerase can be inhibited in MCF-7 cells transiently using RNAi, we also provide evidence that telomerase inhibition can be maintained long-term utilizing a DN-hTERT transgene. Furthermore, using both tools, sensitization was shown by slowed cellular growth, senescence and apoptosis post-treatment with AdR and taxol. We measured levels of senescence and apoptosis after treatment with a range of drug concentrations for each chemotherapy, while previous studies only examined one drug concentration per chemotherapeutic agent.

After transfection with the synthetic siRNAs and chemotherapies, we show a rapid sensitization response of the cell whether targeting hTR or hTERT. We found the rate of senescence or apoptosis occurs quicker than previously shown, within 48 hours of treatment and at much lower doses of the chemotherapeutic drugs than clinically relevant. This would clearly be highly desirable to patients with respect to treatment and reduced toxicity/side effects from the chemotherapies once a suitable delivery system has been developed.

As expected, after treatment with AdR we observed senescence as had previously been shown in breast cancer cells, but we also discovered elevated levels of apoptosis (Saunders et al. 1997; Elmore et al. 2002). In a similar manner, we predicted the induction of cell death in the MCF-7 cells with taxol treatment (Saunders et al. 1997), but not the considerably high levels of senescence simultaneously. However, we have found that MCF-7 cells appear to undergo senescence earlier when exposed to AdR and then undergo apoptosis later. Although this study does not directly address mutual exclusivity of cellular fate, we observed significantly elevated levels of senescence and apoptosis after drug treatment thereby suggesting that senescence and apoptosis may not be mutually exclusive events.

Importantly, we find that stable suppression of telomerase using the DN-hTERT results in telomere shortening, elevated senescence and/or apoptosis as well as increased sensitization to chemotherapeutic agents, which may be dependent on telomere shortening. As seen from colony forming assay with the DN9 clone, the combination of telomerase inhibition and slight telomere shortening resulted in sensitization at low dosages of AdR, suggesting that perhaps there is a threshold of telomere length that has to be reached before sensitization can occur. Similar to the studies with the synthetic siRNAs, especially hTERT-1, we show post-treatment with AdR and taxol caused a more rapid occurrence of senescence and cell death in the DN2 clone as compared with the controls at lower dosages than clinically relevant. We also found that the DN-hTERT clones post-recovery were not sensitized to AdR and taxol implying a protective role for telomerase. However, it remains to be determined the mechanism of telomerase

reactivation in the MCF-7 breast cancer cells since other possibilities unrelated to upregulation of endogenous hTERT mRNA have not been explored, such as chaperone involvement in telomerase complex assembly.

Understanding the complicated nature of sensitization of breast cancer cells in terms of senescence vs. apoptosis after pre-treatment with anti-telomerase therapies followed by chemotherapeutic treatment would be beneficial in the development of telomerase inhibitors as neoadjuvant therapies. Specifically, therapeutic opportunities would be greatly improved if a synergism between telomerase inhibition and established anti-tumor strategies could be proven to kill the breast cancer cells and prevent recurrence.

List of References

References Cited

Barik, S. 2004. Control of nonsegmented negative-strand RNA virus replication by siRNA. Virus Res. **102**: 27-35.

Barquinero, J. et al. 2004. Retroviral vectors: new applications for an old tool. Gene Ther. **11**(suppl 1): S3-S9.

Bartosch, B. et al. 2004. Strategies for retargeted gene delivery using vectors derived from lentiviruses. Curr. Gene Ther. **4**: 427-443.

Baumann, P., and Cech, T.R. 2001. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science **292**: 1171-1175.

Berns, K., et al. 2004. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature **428**: 431-437.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409**: 363-366.

Bertrand, J.R., et al. 2002. Comparison of antisenese oligonucleotides and siRNAs in cell culture and *in vivo*. Biochem. Biophys. Res. Commun. **296**: 1000-1004.

Bilaud, T., Brun, C., Ancelin, K., et al. 1997. Telomeric localization of TRF2, a novel human telobox protein. Nat. Genet. **17**: 236-239.

Blackburn, E.H. 2005. Telomerase and Cancer. Mol. Cancer Res. **3(9)**: 477-482.

Blasco M.A., Lee H-W, et al. 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell **91**: 25-34.

Bodnar, A.G., Ouellette, M., Frolkis, M., et al. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science **279**: 349-352.

Broccoli, D., Smogorzewska, A., Chong, L., et al. 1997. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat. Genet. **17**: 231-235.

Brummelkamp, T.R., et al. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science Apr 19; **296**(5567): 550-553.

Bryan, T.M., Goodrich, K.J., and Cech, T.R. 2000. Telomerase RNA bound by protein motifs specific to telomerase reverse transcriptase. Mol. Cell **6:** 493-499

Cao, Y. et al. 2002. TERT regulates cell survival independent of telomerase enzymatic activity. Oncogene **21**: 3130-3138.

Chan, S.W. and Blackburn, E.H. 2002. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. Oncogene. 21: 553-563.

Chen, J-L., Blasco, M.A., and Greider, C.W. 2000. Secondary structure of vertebrate telomerase RNA. Cell **100**: 503-514.

Chiu, C.P., Dragowska, N.W., Kim, et al. 1996. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells **14**(2): 239-248.

Colgin, LM., Wilkinson, C. et al. 2000. The hTERTα splice variant is a dominant negative inhibitor of telomerase activity. Neoplasia **2**(5): 426-432.

Comolli, L.R., et al. 2002. A molecular switch underlies a human telomerase disease. Proc Natl Acad Sci U S A. **99**(26): 16998-17003.

Corey, D.R. 2002. Telomerase inhibition, oligonucleotides, and clinical trials. Oncogene **21**: 631-637.

de Lange, T. 2002 Protection of mammalian telomeres. Oncogene **21**: 532-540.

Delhommeau, F., et al. 2002. Telomere dysfunction and telomerase reactivation in human leukemia cell lines after telomerase inhibition by the expression of a dominant-negative hTERT mutant. Oncogene **21:** 8262-8271.

der-Sarkissian, H. et al. 2004. The shortest telomeres drive karyotype evolution in transformed cells. Oncogene **23**: 1221-1228.

Dez, C., Henras, A., et al. 2001. Stable expression in yeast of the mature form of human telomerase RNA depends on its association with the box H/ACA small mucleolar RNP proteins Cbf5p, Nhp2p and Nop10p. Nucleic Acids Res. **29**(3): 598-603.

Dikmen, Z.G., et al. 2005. *In vivo* inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. Cancer Res. **65(17)**: 7866-7873.

Dorsett, Y. and Tuschl, T. 2004. siRNAs: applications in functional genomics and potential as therapeutics. Nat. Rev. Drug Discov. **3**: 318-329.

Dragon F., Pogacic, V., et al., 2000. In vitro assembly of human HA/ACA small nucleolar RNPs reveals unique features U17 and telomerase RNAs. Mol. Cell Biol. **20**(9): 3037-3048.

Dykxhoorn, D.M., Novina, C.D., and Sharp, P.A. 2003. Killing the messenger: Short RNAs that silence gene expression. Nat. Rev. Mol. Cell Biol. **4:** 457-467.

Elbashir, S.M., Lendeckel, W. & Tuschi, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15**: 188-200.

Elmore, L.W., Rehder, C.W., Di, X., McChesney, P.A., Jackson-Cook, C.K., Gewirtz, D.A., and Holt, S.E. 2002. Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J. Biol. Chem. **277**: 35509-35515.

Elmore, L.W., Turner, K.C., Gollahon, L.S., Landon, M.R., Jackson-Cook, C.K., and Holt, S.E. 2002. Telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization. Cancer Biol. and Therapy **1**(4): 391-397.

Elmore,L.W., X.Di, Y-M.Di, S.E.Holt, and D.A.Gewirtz. 2005. Evasion of chemotherapy-induced senescence in breast cancer cell: implications for treatment response. Clin. Cancer Res. **11**:2637-43.

Gil, J., and Esteban, M. 2000. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. Apoptosis **5:** 107-114.

Farwell, D.G., et al. 2000. Genetic and epigenetic changes in human epithelial cells immortalized by telomerase. Am J Pathol.**156**(5): 1537-47.

Feng, J., Funk, W.D., et al. 1995. The RNA component of human telomerase. Science **269:** 1236-1241.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391**: 806-811.

Ford, L.P., Suh, J.M., et al., 2000. Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. Mol. Cell Biol. **20**(23): 9084-9091.

Forsythe, H.L., Jarvis, J.L., Turner, J.W., et al., 2001. Stable association of hsp90 and p23, but not hsp70, with active human telomerase. J.Biol. Chem. **276:** 15571-15574.

Gan, Y., et al. 2001. Telomere amount and length assay. Pharm. Res. **18**(12): 1655-1659.

Greider, C.W. 1996. Telomere length regulation. Annu. Rev. Biochem. **65:** 337-365.

Greenberg, R.A., O'Hagan, R.C., Deng, H., et al. 1999. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene **18**: 1219-1226.

Griffith, J.D., Comeau, L., Rosenfield, S., et al. 1999. Mammalian telomeres end in a large duplex loop. Cell **97**: 503-514.

Gu, J., et al. 2005 Roles of tumor suppressor and telomere maintenance genes in cancer and aging-an epidemiological study. Carcinogenesis **26(10)**: 1741-1747.

Hahn, W.C., Stewart, S.A., et al. 1999. Inhibition of telomerase limits the growth of human cancer cells. Nat Med. Oct **5**(10): 1164-70.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293**: 1146-1150.

Hande, K.R. 1998. Clinical applications of anticancer drugs targeted to topoisomerase II. Biochim. Biophys. Acta **1400**: 173-184.

Hannon, G.J., and Rossi, J.J. 2004. Unlocking the potential of the human genome with RNA interference. Nature **431**: 371-378.

Hao, Z-M. et al. 2005. Intensive inhibition of hTERT expression by a ribozyme induces rapid apoptosis of cancer cells through a telomere length-independent pathway. Cancer Biology and Therapy **4**(10): 1098-1103.

Haque, S.J., and Wiliams, B.R.G. 1998. Signal transduction in the interferon system. Semin. Oncol. **25**: 14-22.

Harley, C., Futcher, A.B., and Greider, C.W. 1990. Telomeres shorten during ageing of human fibroblasts. Nature **346**: 866-868.

Hastie, N.D., Dempster, M., Dunlop, M.G., et al. 1990. Telomere reduction in human colorectal carcinoma and with aging. Nature **346**: 866-868.

Hendersin, IC, Harris, JR, Kinne, DW and Hellman, S. 1998. Cancer of the breast. In Cancers: Principles and Practice of Oncology, 3rd Ed. V.T. DeVita, S. Hellman, and S.A. Rosenberg, eds. (Lippincott, Philadelphia, PA) pp. 1197-1268.

Herbert B-S, Wright, A.C., et al. 2001. Effects of chemopreventive and antitelomerase agents on the spontaneous immortalization of breast epithelial cells. J. Natl. Cancer Inst. **93**: 39-45.

Holt, S.E., Shay, J.W., and Wright, W.E. 1996. Refining the telomere-telomerase hypothesis of aging and cancer. Nat. Biotech. **14**: 836-839.

Holt, S.E., et al., 1999. Functional requirement of p23 and Hsp90 in telomerase complexes. Genes Dev. **13:** 817-826.

Hoos, A., Hepp, H.H., Kaul, S., et al. 1998. Telomerase activity correlates with tumor aggressiveness and reflects therapy effect in breast cancer. Nat. Genet. **36:** 984-988.

Horwitz, S.B., et al. 1993. Taxol: mechanisms of action and resistance. J. Natl. Cancer Inst. Monogr. **15**: 55-61.

Huard, S., Moriarty, T.F., and Autexier, C. 2003. The C terminus of the human telomerase reverse tgranscriptasse is a determinant of enzyme processivity. Nuclei Acids Res. **31:** 4059-4070.

Hutvagner, G., et al. 2004. Sequence-specific inhibition of small RNA function. PLoS Biol. **2**: E98.

Jaffrezou, J-P., et al. 1996. Daunorubicin-induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis. EMBO J. **15**(10): 2417-2424.

Jemal A., et al. 2005. Cancer statistics. CA. Cancer J. Clin. **55**:10-30.

Jevapalan, J.N. 2005. Activation of the ALT pathway for telomere maintenance can affect other sequences in the human genome. Hum. Mol. Genet. **14**(13):1785-94.

Kang, H.J., et al. 2004. Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. J Neurosci. **24**(6): 1280-1287.

Kim,N.W. et al. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science. **266**(5193): 2011-2015.

Klapper, W., et al. 2003. DNA damage transiently increases TRF2 mRNA expression and telomerase activity. Leukemia **17**: 2007-2015.

Kraemer, K. et al. 2004. Chemosensitzation of bladder cancer cell lines by human telomerase reverse transcriptase antisense treatment. J. Urol. **172**: 2023-2028.

Kyo, S., Takakura, M. Kanaya, T., et al. 1999. Estrogen activates telomerase. Cancer Res. **59:** 5917-5921.

Layzer, J.M., et al. 2004. *In vivo* activity of nuclease-resistant siRNAs. RNA **10**: 766-771.

Lee, B-Y. et at. 2006. Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell **5**: 187-195.

Lee, S.R., Wong, J.M., Collins, K. 2003. Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. J. Biol. Chem. **278**: 52531-52536.

Lewis, D.L., et al. 2002. Efficient delivery of siRNA for inhibition of gene expression of postnatal mice. Nat. Genet. **32**: 107-108.

Li, S., Crothers, J., et al. 2005. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. J. of Biol. Chem. June 24; **280**(25): 23709-23717.

Ling, Y-H. et al. 1993. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. Cancer Res. Apr 15; **53**(8): 1845-52.

Liu, Y., Snow, B.E., et al. 2000. The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo. Curr. Biol. **10**: 1459-1462.

Ludwig, A., et al. 2001. Ribozyme cleavage of telomerse mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. Cancer Res. 61: 3053-3061.

Misawa, M. et at. 2002. Inhibiton of human telomerase enhances the effect of chemotherapeutic agents in lung cancer cells. Internatl. J. Oncology **21:** 1087-1092.

Masutomi, K., et al. 2003. Telomerase maintains telomere structure in normal human cells. Cell **114:** 241-253.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. and Tuschi, T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110: 563-574.

Marwick, C. 1998. First "antisense" drug will treat CMV retinitis. JAMA. **280**: 871.

Meyerson, M. et al. 1997. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell. **90**: 785-795.

McCaffrey, A.P., et al. 2002. RNA interference in adult mice. Nature 418:38-39.

McManus, M.T., and Sharp, PA. 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. **3**: 737-747.

Milross, C.G., et al. Relationship of mitotic arrest and apoptosis to antitumor effect of paclitaxil. J. Natl. Cancer Inst. **88**: 1308-1314.

Mokbel, K. 2000. The role of telomerase in breast cancer. Eur. J. Surg Oncol. **26:** 509-14.

Mourelatos, Z., Dostie, J., et al. 2002. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. **16**: 720-728.

Moyzis, R.K., J.M. Buckingham, L.S. cram, et al. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A. **85**: 6622-6626.

Muller, I., Niethammer, D., and Bruchelt, G. 1998. Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity. Int. J. Mol. Med. **1**: 491-494.

Nakamura, et al. 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science **277:** 955-959.

Nakamura, T.M, & Cech, T.R. 1998. Reversing time: origin of telomerase. Cell **92**: 587-590.

Nakamura, T.M., Moser, B.A., and Russell, P. 2002. Telomere binding of checkpoint sensor and DNA repair proteins contributes to maintenance of functional fission yeast telomeres. Genetics **161**: 1437-52.

Nakamura, M. et al. 2005. Efficient inhibition of human telomerase reverse transcriptase expression by RNA interference sensitizes cancer cells to ionizing radiation and chemotherapy. Hum. Gene Ther. **16**(7): 859-868.

Naldini, L., et al. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272**: 263-267.

Newbold, R.F. 2002. The significance of telomerase activation and cellular immortalization in human cancer. Mutagenesis **17**: 539-550.

Olovnikov, A.M. 1971. Principle of marginotomy in template synthesis of polynucleotides. Dokl Akad Nauk SSSR **201**: 1496-1499.

Rahman,R., Mo, L., Cui, W. 2006.Telomerase with mutated catalytic motifs has dominant negative effects on telomerase activity and inhibits cell growth. Biochem. and Biophys. Res. Com. **350:** 796-802.

Ramirez, R.D, Sheridan, S., et al. 2004. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Canc. Res. **64**: 9027-9034.

Rao, S., et al. 1995. Characterization of the taxol binding site on the microtubule. 2-(m-Azidobenzoyl)taxol photolabels a peptide (amino acids 217-231) of beta-tubulin. J Biol Chem. Sep 1; **270**(35): 20235-20238.

Rasbridge, S.A., et al. 1994. The effects of chemotherapy on morphology, cellular proliferation, apoptosis and oncoprotein expression in primary breast carcinoma. Brit. J. Cancer. **70**: 335-341.

Sachsinger, J. et al. 2001. Telomerase inhibition in RenCa, a murine tumor cell line with short telomeres, by overexpression of a dominant negative mTERT mutant, reveals fundamental differences in telomerase regulation between human and murine cells. Cancer Res. **61**(14): 5580-5586.

Seimiya, H., Sawada, H., et al. 2000. Involvement of 14-3-3 protein in nuclear localization of telomerase. Embo J. **19**(11): 2652-2661.

Shay, J.W. and Bacchetti, S. 1997. A survey of telomerase activity in human cancer. Eur. J. Cancer **33**(5): 787-791.

Silverman, R.H. 1997. In Ribonucleases: structure and function (eds G. D'Alessio and J.F. Riordan) Ch **16**, 515-551 (Academic Press, St Louis).

Sioud, M. 2004. Therapeutic siRNAs. Trends Pharmacol. Sci. **25**: 22-28.

Somlo, G., et al. 2001. High-dose paclitaxel in combination with doxorubicin, cyclophosphamide and peripheral blood progenitor cell rescue in patients with high-risk primary and responding metastatic breast carcinoma: toxicity profile, relationship to paclitaxel pharmacokinetics and short-term outcome. Br. J. Cancer **84**(12): 1591-8

Sontheimer, E.J. 2005. Assembly and function of RNA silencing complexes. Nat. Rev. Mol. Cell Biol. **6:** 127-138.

Sorensen, D.R., et al. 2003. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J. Mol. Biol. **327**: 761-766.

Tabara, H., et al. 1999. The rde-1 gene, RNA interference, and transposon silencing in C. *elegans*. Cell **99:** 123-132.

Tesmer, V.M., Ford, L.P., Holt, S.E., et al. 1999. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. Mol. Cell. Biol. **19**: 6207-6216.

Vaziri, H. and Benchimol, S. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr. Biol. **8**(5): 279-282.

Vickers, T.A., et al. 2003. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J. Biol. Chem. **278:** 7108-7118.

Vonderheide, R.H., et al. 2004. Vaccination of cancer patients against telomerase induces functional antitumor CD8+ T lymphocytes.Clin. Cancer Res. 10: 828-839.

Vulliamy, T. et al. 2001. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature. Sep 27; **413**(6854): 432-435.

Ward, R.J., and Autexier,C. 2005. Pharmacological telomerase inhibition can sensitize drug-resistant and drug-sensitive cells to chemotherapeutic treatment. Mol. Pharm. **68**(3): 779-786.

Wilda, M., Fuchs, U., Wossmann, W., and Borkhardt, A. 2002 Killing of leukemic cells with BCR/ABL fusion gene by RNA interference (RNAi). Oncogene **21:** 5716-5724.

Wenz,C. Enenkel, B., Amacker, M., et al. 2001. Human telomerase contains two cooperating telomerase RNA molecules. EMBO J. **20**: 3526-3534.

Weinrich, S.L., Pruzan, R., Ma, L., Ouellette, M., et al. 1997. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat. Genet. **17**: 498-502.

Williams, B.R.G. 1999. PKR; a sentinel kinase for cellular stress. Oncogene **18**: 6112-6120.

Wright, W.E., and Shay, J.W. 1992. The two-stage mechanism controlling cellular senescence and immortalization. Experimental Gerontology **27**: 383-389.

Wright, W.E., Piatyszek, M.A., Rainey, W.E., et al. 1996. Telomerase activity in human germline and embryonic tissues and cells. Dev. Genet. **18**(2): 173-179.

Wu, Y. et al. 2007. Taxol induced apoptosis regulates amino acid transport in breast cancer cells. Apoptosis **12**(3): 593-612

Yasumoto, S., Kunimura, C., Kikuchi, K., et al. 1996. Telomerase activity in normal human epithelial cells. Oncogene **13**(2): 433-9.

Yi, X., Tesmer, V.M., Savre-Train, I., Shay, J.W., and Wright, W.E. 1999. Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol. Cell Biol. Jun; **19**(6): 3989-97.

Yvon, A.M., et al. 1999. Taxol suppresses dynamics of individual microtubules in living human tumor cells. Mol. Biol. Cell **10**: 947-959.

Zaleski, G., et al. 1994. Doxorubicin-induced DNA degradation in murine thymocytes. Mol. Pharmacology Nov; **46**(5): 901-8.

Zaug, A.J., Podell, E.R., Cech, T.R. 2005. human POT1 disrupts telomeric G-quadruplexes allowing telomerase extension in vitro. Proc. Natl. Acad. Sci. U.S.A. **102**: 10864-10869.

Zhang, E., et al. 2005. Silencing $p21^{Waf1/Cip1/Sdi1}$ expressionincreases gene transduction efficiency in primitive human hematopoietic cells. Gene Therapy **12**: 1444-1452.

Zhang, X., Mar V. Zhou, W., et al. 1999. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev. **13**: 2388-2399.

Zhou C., Liu, F. 2003. Inhibition of human telomerase reverse transcriptase gene expression by BRCA1 in human ovarian cancer cells. Biochem Biophys Res Commun. **303:**130-136.

Zou, Y. etal. 2004. Does a sentinel or a subset of short telomeres determine replicative senescence? Mol. Biol. Cell **15**: 3709-3718.

Appendix A

p21 Inhibition using RNAi and Chemosensitization of Breast Tumor Cells

Knockdown of p21 using a shRNA in MCF-7 Cells

Our lab has previously shown the molecular and cellular consequences of Adriamycin treatment in breast tumor cells (Elmore et al. 2002; Elmore et al. 2005). After acute exposure to Adriamycin, MCF-7 cells senescence approximately three days later and down-regulate telomerase. Telomere length has been proven to be an important trigger for senescence. However, we have shown that senescence can be induced without net shortening of the telomeres (Elmore et al. 2002). That the most critical event is the preservation of telomere structure/integrity and p53 levels only when this occurs do we have AdR-induced senescence in MCF-7 cells. Adriamycin and ROS have been shown to preferentially attack the telomeres (Elmore et al. 2002). Senescence is also characterized by transient p53 activation, high levels of reactive oxygen species as well as sustained $p21^{waf\text{-}1}$ expression. From these results I wanted to further examine and understand the relationship between p21 and senescence. Perhaps there is a threshold of p21 needed to maintain the senescence phenotype, and if I can knock down the p21 protein levels enough then the cell should undergo apoptosis instead of senescence.

We obtained the siRNA p21 sequence from Berns et al. (Berns et al. 2004), which targets the second exon and stably expressed the siRNA in the MCF-7 cells (Table 2)(Figure 49). The pSUPER.retro.gfp vector was utilized for infection, which

functions in the same manner as the pSUPER.retro vector, except this retroviral vector has a green flouresent protein (GFP) marker so that positive clones can be selected both visually and via antibiotic resistance. Once infected into the cell lines, selected with puromycin and clonal populations grown, clones were chosen visually and then screened for constitutive levels of p21 by western blotting (Figure 49). We found clonal variability in the levels of knockdown with the highest percentage ranging around 80% as seen in clones 7b and 11b.

TABLE 2. siRNA sequences for p21

Name	Target Sequence (5'-3')	Target Region
p21-b	408-***GACCATGTGGACCTGTCAC*** -426	Exon 2
p21-c	233-***CTTCGACTTTGTCACCGAG***-251	Exon 2

Sensitization of Breast Tumor Cell using siRNA Targeting p21 as a Pretreatment

We picked out three clones (7b, 11b, and 16) with various constitutive amounts of p21 and administered acute Adriamycin (AdR) treatment (1μM) to the MCF-7/p21 cell lines for 2 hours. Protein levels of p21 were determined at 0, 4, 24, and 48 hours post-treatment (Figure 50). Induction of the p21 occurs as soon as 4hrs after AdR and continues to be elevated up to 48 hrs. To further confirm these results we ascertained protein levels 24 hours after AdR treatment using immunohistochemistry. Again we observed decreased constitutive amounts of p21 post-infection with siRNA and then generation of high p21 levels after chemotherapy (Figure 50).

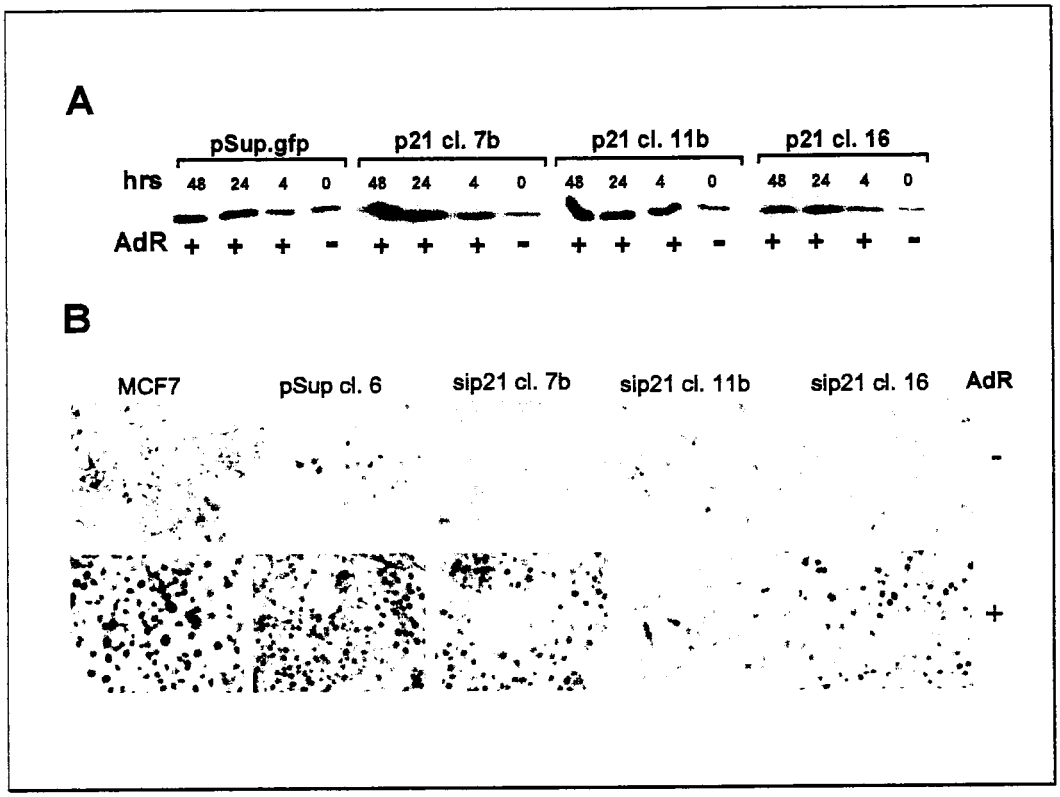


Figure 50. p21 induction after AdR treatment in MCF7/p21 knockdown clones. Using the Western Blot **(A)** and immunohistochemistry **(B),** p21 levels were measured at various time points before and after treatment with AdR. In both experiments the siRNAs were shown incapable of continued suppression of p21 post treatment with the chemotherapeutic agent.

In addition, after AdR treatment we found induction of senescence at levels higher as compared to the empty vector pSuper.retro.gfp/MCF-7 levels (Figure 51). The same three clones with decreased levels of p21 were treated for 2 hours with Adriamycin (1μM). Untreated MCF-7 cells undergo senescence three days after treatment (Elmore et al. 2002); whereas, in the MCF/p21 cell lines with the greatest amount of knockdown, senescence was apparent 24 hours after treatment and continued for at least five days afterwards. However, clone 16, with the most normal amount of p21, and the empty vector control displayed similar amounts of SA-βgal staining by day 3. This experiment needs to be repeated and quantitated before more conclusions can be made.

The cell lines were also tested for apoptosis (TUNEL) after acute Adriamycin treatment. We found increases in the amount of cell death as compared with the empty vector control cell lines (Figure 52). However, this portion of the project is not complete, and the data presented is quite preliminary and needs to be repeated due to varying results collected in the analysis, as well as increasing the number of time points analyzed.

We have clearly shown that blocking p21 function results in a transient decrease after treatment with Adriamycin followed by a large amplification in protein quantities. This constitutive 80% knockdown of p21 levels does not appear to be significant enough to be maintained post treatment with AdR. We observed the majority of the cells senescencing while others appear to undergo apoptosis. The reason for this result is still unknown and is under investigation. Because we see p21 knockdown with siRNA before

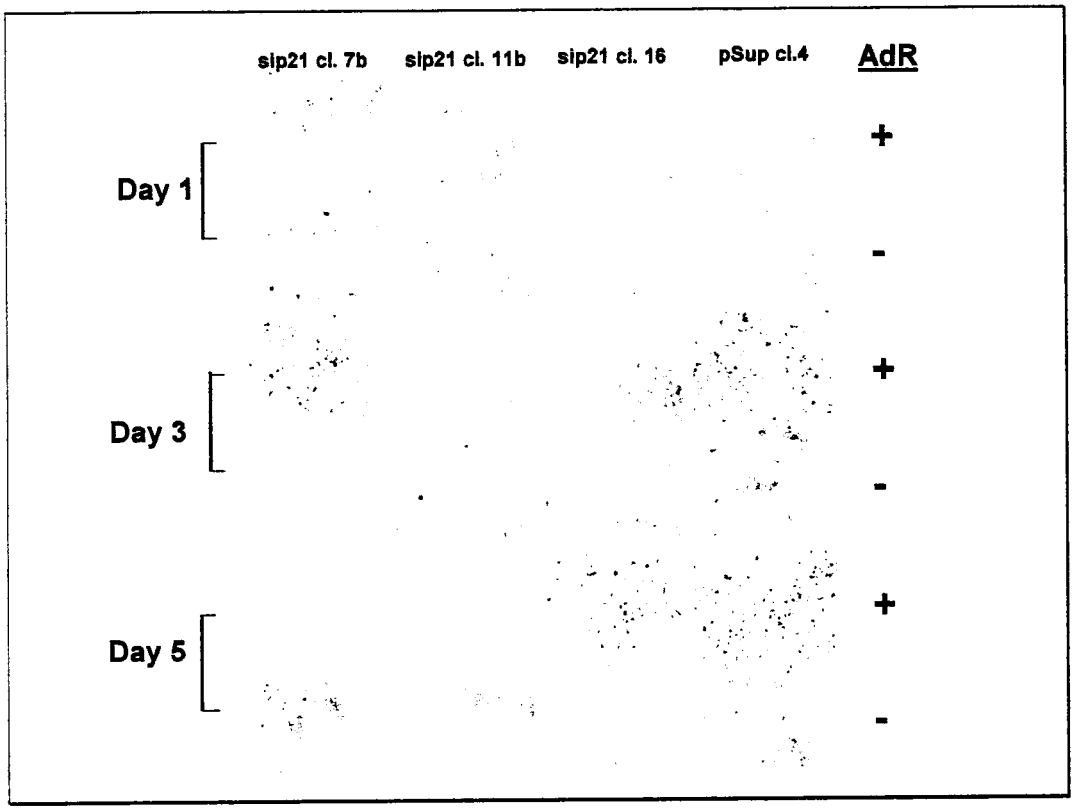
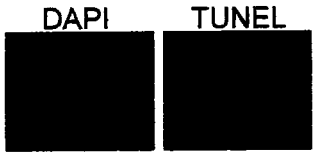


Figure 51. Senescence initiation in MCF7/p21 knockdown clones. By comparing the quantities of SA-βgal staining in the three p21 knockdown clones to the empty vector (pSup) clone, we determined the senescent phenotype occurs at a faster rate in the clones with lower levels of p21 after AdR treatment.

A



Clone 11b-DAY 9 (26%)

B

Sample	Clone 7b	Clone 11b	Clone 16	pSup cl. 6
UNTREATED				
Day 5	0	.2%	.65%	0
Day 7	0	0	.45%	0
TREATED				
Day 7	9.7 % (n=2)	9.3 % (n=3)	5.9 % (n=3)	1.7 % (n=3)
Day 9	20.7 % (n=6)	22.1 % (n=6)	21.8 % (n=6)	

Figure 52. Apoptosis in MCF7/p21 knockdown clones after acute AdR treatment. **A.** The TUNEL assay enables a comparison of the DAPI staining of the nuclei of each cell and the fluorescently labeled dead cells but TUNEL. **B.** From the samples taken, there appears to be significantly greater amounts of apoptosis occurring in the MCF-7 cells lines with p21 knockdown regardless of the level of decrease in protein.

but not after DNA damage, we will explore the option of utilizing additional siRNA to provide a more complete knockout of p21 expression in the MCF-7 cell line.

Multiple siRNA induced Inhibiton of p21 and Sensitization of Breast Tumor Cells

We have already established two cell lines that have significant p21 knock down with siRNA targeting exon 2 (408-426), MCF/p21 clone 7b and MCF/p21 clone 11b. As stated previously, the inhibition of p21 was not sufficient enough to be maintained post-AdR treatment. So we obtained another p21 siRNA sequence, which targets upstream of p21-b, but also in exon 2 (233-251) (Zhang et al. 2005), and conducted an infection (Figure 53). The new siRNA sequence was introduced into the old cell lines. The pSUPER-retro vector was again utilized for the second infection, except this one only has a puromycin selectable marker, no GFP. Once infected into the cell lines, selected with puromycin and clonal populations grown, the clones were screened for constituitive levels of p21 by western blotting. There were varying levels of knockdown with the greatest ranging around 100% which is greater than the original knockdown seen in the first p21 cell lines created indicating the use of multiple siRNA targeting p21 to be a more effective method of inhibition.

We then tested sensitization of these cell lines after pre-treatment with p21 knockdown. The MCF-7 cell lines, containing both siRNA constructs, were to an acute treatment with AdR (2 hours). Levels of p21 increased immediately with detectable levels seen at zero hours after dosage and peaking at fours hours post-treatment (Figure 53). We have clearly shown that blocking p21 function results in a transient decrease

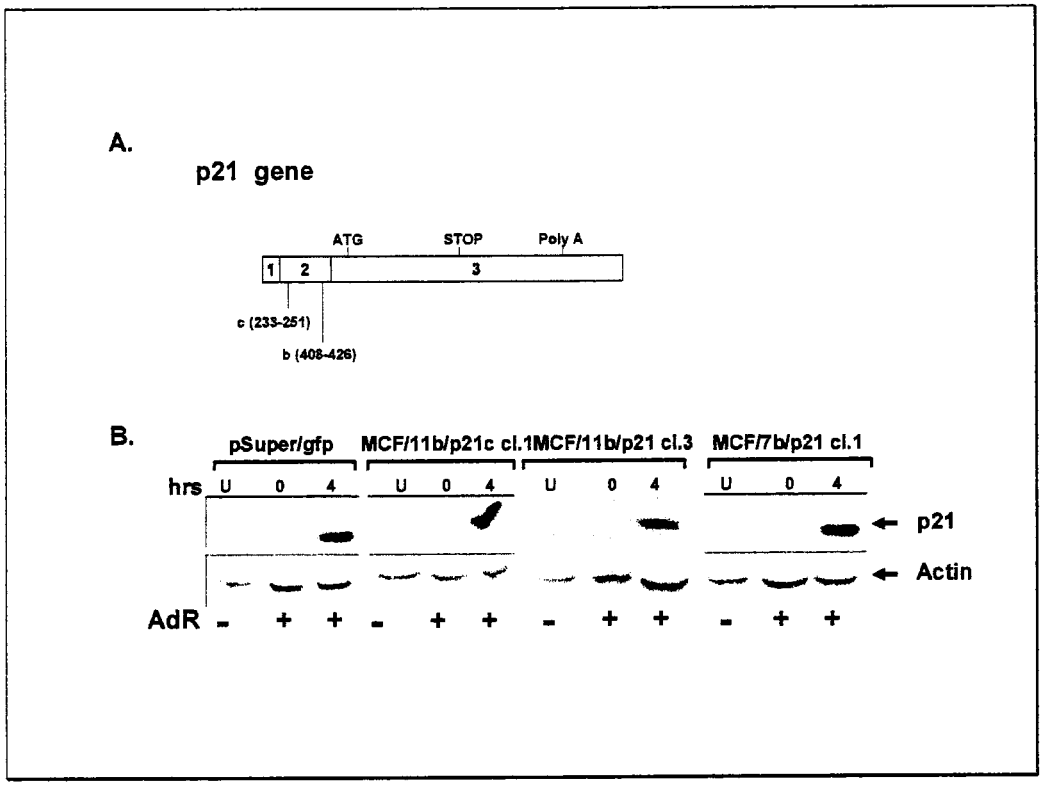


Figure 53. Induction of p21 after AdR treatment. A. Previously established cell lines MCF7/3b and MCF7/11b with p21 knockdown were infected with another siRNA sequence targeting p21. **B.** Constituitive levels of p21 were decreased over 90% in most of the clones. Elevated levels of p21 were detected post-AdR treatment (0 & 4 hours) even with the presence of two siRNAs per cell line.

after treatment with Adriamycin followed by a large amplification in protein quantities. This constitutive 80% knockdown of p21 levels does not appear to be significant enough to be maintained post treatment with AdR. We observed the majority of the cells senescencing while others appear to undergo apoptosis. The reason for this result is still unknown and is under investigation. Because we see p21 knockdown with siRNA before but not after DNA damage, we will explore the option of homologously knocking out p21 in the MCF-7 cell line as a cleaner system for p21 knockout.

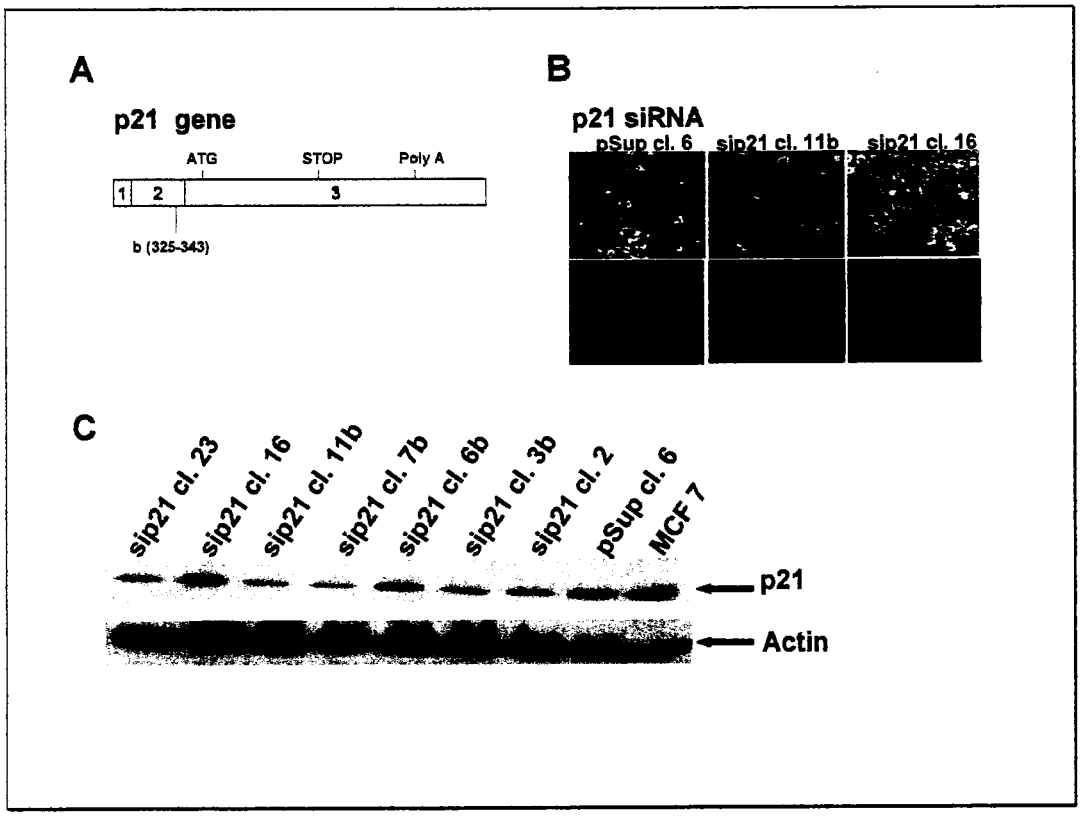


Figure 49. Knockdown of functional p21 in MCF7 cells by siRNA. A. MCF7 breast cancer cells were stably infected with siRNA targeting exon 2 in p21 protein. **B.** The clones were visualized and selected upon expression of GFP. C. Using a western blot, protein levels of p21 were assessed and found to be decreased ~80% in several of the clones. However, there was a range of reduction showing differences in infection efficiencies.

Vita

Ann Kennon Rigby Poynter was born August 24, 1978 in Richmond, VA. She graduated as valedictorian of Mary Baldwin College in 2000 receiving her Bachelor of Science in Biology with a minor in Chemistry. Following one year of experience as a lab specialist in the department of chemical engineering at the University of Virginia, she joined the department of human genetics at the Medical College of Virginia at VCU in 2001.

Grants

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Publications

Giassi, L.J.. **Poynter, A.K.**, and Gainer, John L. 2002. Trans Sodium Croscetinate for Hemorrhagic Shock: Effect of Time Delay in Initiating Therapy, Shock **18**:585-588.

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

Poynter, K.R., Elmore, L.W., and Holt, S.E. 2006. A Comparison of Telomerase Inhibition and Sensitization of Breast Tumor Cells. Manuscript in Preparation.

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Poynter,K.R. VCU: Massey Cancer Center Retreat, Richmond, VA. June 2006.

Poynter,K.R. VCU: Daniel T. Watts Research Poster Symposium, Richmond, VA. October 2006.