T-Oligo and its Associated Proteins in Apoptosis and Senescence

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THESIS

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This thesis is dedicated in memory of my father who was a great example in my life, and my mother who has been a constant support as I work to achieve my goals.

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TABLE OF CONTENTS

CHAPTER **TABLE OF CONTENTS** (continued) PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BEBM	Bronchial Epithelial Cell Basal Medium
BEGM	Bronchial Epithelial Growth Medium
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle's Media
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
HBSS	Hank's Balance Salt Solution
hnRNP	Heterogeneous nuclear ribonucleoprotein
IgG	Immunoglobulin G
KCl	Potassium Chloride
MEM	Minimal Essential Media
M.O.M.	Mouse on Mouse
Msi-1	Musashi Homolog 1
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NSCLC	Non-Small Cell Lung Cancer
OCT	Optimum Cutting Temperature
PBS	Phosphate-Buffered Saline
pRb	Retinoblastoma Protein
Pur-β	Purine-rich Element Binding Protein β

LIST OF ABBREVIATIONS (continued)

RNA	Ribonucleic Acid
RNAse	Ribonuclease
SDS Page	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering Ribonucleic Acid
ssDNA	Single Stranded Deoxyribonucleic Acid
TBST	Tris-Buffered Saline Tween 20
TEMED	Tetramethylethylenediamine
T-oligo	Oligonucleotide Homologous to the Telomere Overhang
Tris-HCl	Tris (Hydroxymethyl) Aminomethane-Hydrochloric Acid
βME	Beta-Mercaptoethanol
WT	Wild Type

SUMMARY

Telomeres are tandem repeats of the DNA sequence TTAGGG. The 3' end of each telomere consists of a single-stranded overhang that is normally concealed in a loop structure at the chromosome ends. An 11-base oligonucleotide homologous to the 3' end of the telomere sequence known as T-oligo has been shown to induce DNA damage responses such as apoptosis and senescence, and has also been shown to reduce tumorigenicity. This study presents T-oligo's ability to induce anti-cancer responses in melanoma and lung cancer cell lines, and investigates the effect T-oligo has on senescence and apoptosis inducing proteins along with proteins that are known to bind to T-oligo.

Published literature shows that when telomeres become critically short or have damaged DNA, the telomere overhang is exposed inducing apoptosis or senescence in cancer cells. T-oligo treatment which mimics the exposure of the telomere overhang sequence is able to induce senescence and apoptosis in cancer cells since they have altered cell cycle checkpoints. T-oligo may also induce DNA damage responses through binding to telomere-associated proteins or other T-oligo binding proteins.

We hypothesize that T-oligo will not induce DNA damage responses in normal bronchial epithelial cells (NBEC), however it will induce DNA damage responses in lung cancer and melanoma cell lines.

This study demonstrates that NBEC treated with T-oligo did not show apoptosis, senescence, or inhibition of proliferation, nor was there upregulation of p53 and p21. However, T-oligo did induce several DNA damage responses in cancer cells. T-oligo induced apoptosis in lung cancer cells. T-oligo reduced clonogenic capacity in both lung cancer and melanoma cell lines, and induced senescence in MU melanoma cells and H358 and SW1573 lung cancer cells. T-oligo induced proteins mediating senescence such as p21, p27, and p33 *in vitro* and induced proteins such as p53 and p73 which induce apoptosis. It was found that T-oligo associated proteins hnRNP C, Pur-β, and Msi-1 were modulated by T-oligo. T-oligo upregulated hnRNP C and Pur-β, and downregulated Msi-1. T-oligo was further able to reduce lung cancer tumors in a mouse model, and found to upregulate senescence associated proteins p21, p27, and p33 *in vivo*. These results verify our hypothesis that T-oligo is able to induce anti-cancer responses, and demonstrate the proteins and pathways that might be involved in mediating T-oligo induced DNA damage responses.

1. Introduction

1.1 Background

It is estimated that in 2012, there will be 303,410 new cases of lung cancer and melanoma, and lung cancer and melanoma may account for 169,520 deaths (1). Lung cancer is the leading cause of death over any other cancer in the United States (1). There are two major types of lung cancer: small cell and non-small cell lung cancer (NSCLC), which accounts for 85% of lung cancers (2). Metastatic melanoma kills nearly 80% of patients and the incidence of metastatic melanoma has risen during the past decade (3). Frequent molecular genetic changes such as deletions/mutations of p53, pRb, $p16^{INK4\alpha,}$ and overexpression of EGFR, HER2/neu and c-myc have been found in NSCLC (4). At the molecular level, lesions in B-RAF and $p16^{INK4\alpha}$/ARF genes have been linked to human melanoma (5-7).

Telomeres are tandem repeats of a specific nucleotide sequence found at the end of chromosomes. Telomeres act like biological clocks and shorten with each round of DNA replication, and when critically short, signal for senescence or cell death by apoptosis (8). In stem cells, telomeres are maintained by the enzyme telomerase, whose main component hTERT (Human Telomere Reverse Transcriptase) adds TTAGGG residues to the 3′ terminus of chromosome ends. hTERT is generally not expressed in normal somatic cells, but is expressed in the majority of primary tumors and cancer derived cell lines (9-11). The 3′ end of each telomere consists of a single stranded overhang of TTAGGG tandem repeats that has been proposed to stabilize a loop structure at the chromosome ends (8, 12), and plays an important role in signaling for DNA damage responses (8, 12). The telomere overhang is protected by various telomere-associated proteins such as TRF2 (Telomere repeat factor 2) and POT1

(Protection of Telomeres 1 homolog) (8, 13, 14). When these proteins disassociate from the telomere, the telomere overhang is exposed, causing apoptosis, senescence, and differentiation (8, 12, 15). These effects of telomere overhang exposure can be artificially induced by exogenous addition of an 11-base oligonucleotide identical in sequence to the telomere overhang (T-oligo) (9, 16-22). Treatment with T-oligo induces DNA damage responses (19) similar to responses that occur under physiological conditions.

1.2 Statement of Problem

Lung cancer and melanoma still have very few effective treatment options available to patients (23, 24). T-oligo, which is homologous to the 3′ overhang of the telomere has been shown to induce DNA damage responses like apoptosis and senescence in melanoma and lung cancer, and has minimal effects on normal cells. However, the mechanism of how T-oligo induces these responses is still relatively unknown and needs to be further investigated. The effect of T-oligo on several proteins that bind to T-oligo also needs to be further investigated. Additionally, the efficacy of T-oligo as a cancer therapeutic has not been determined.

1.3 Purpose of Study

The aim of this study is to investigate the mechanism of action of T-oligo as a cancer therapeutic in two NSCLC cell lines and two melanoma lines that are currently available. The mechanism of T-oligo-induced senescence and apoptosis, which are the major anti-cancer responses induced by T-oligo, will be investigated in this study. We will also study if T-oligo associated proteins are modulated upon treatment with T-oligo; specifically, the effects of T-oligo on hnRNP C (heterogeneous nuclear ribonucleoproteins) and Pur-β (Purine rich binding

element β). We will also explore the potential of T-oligo as a therapeutic agent for treatment of lung cancer in a xenograft model.

1.4 Significance of the Study

Currently, several studies are targeting telomeres as options for cancer therapy (8, 25, 26). This study will help demonstrate how telomere overhang exposure mediates DNA damage responses and whether this mechanism could be targeted for cancer therapy. This study validates that T-oligo may be an effective therapeutic in the treatment of lung cancer and melanoma, and the *in vivo* study on a mouse xenograft model helps determine its potential efficacy and role in inducing senescence *in vivo* in cancer.

2. REVIEW OF RELATED LITERATURE

2.1 Human Telomeres

Telomeres are highly specialized structures located at the ends of chromosomes and are structurally and functionally distinct from the rest of the chromosome. They vary in length depending on the species, individual, and even among cells of different origins within the same individual organism. In humans, telomeres average in length between 3 to 20 kb (27). Telomeres consist of double-stranded DNA (dsDNA) followed by a region of single-stranded DNA (ssDNA) which creates a G′ overhang at the 3′ end of the telomere (28-31). The structural integrity of the telomere is maintained by a protein complex, known as shelterin, through the formation of a lariat structure, or t-loop, which masks telomere ends from DNA repair mechanisms (28, 32).

The main purpose of telomeres is to protect the genomic material of the chromosome and thereby sustain cell viability. The t-loop structure is protected from exposure to extracellular DNA damage or repair mechanisms by multiple copies of POT1 (protection of telomerase) protein, an important ssDNA binding protein in humans (28, 33-35). A G-quadruplex is another higher order structure, formed by stacking of guanosine (G) tetrads by incorporating a 16-nucleotide d(GGGTTAGGGTTAGGGT) and a 6-nucleotide d(TAGGGT) sequence of telomeric 3′ overhang, folded via hydrogen-bonding (28, 33). The G-quadruplex protects the telomeric 3′ overhang from being accessed by telomerase, thereby regulating its catalytic activity (28, 33). The t-loop, aided by G-quadruplex structures, prevents the natural ends of the chromosome from being recognized as double-strand breaks and the resulting activation of DNA damage responses (28, 36-43). Telomeres also guard against inappropriate exonuclease degradation as well as end-

to-end fusions (28, 36-41, 44). For these reasons, proper telomeric function is essential for genomic stability. In normal human cells, telomeres shorten with each successive cell division, and upon reaching critical lengths they elicit DNA damage responses resulting in cell senescence, apoptosis, or further chromosomal instability (32). Increase of telomerase activity can result in cellular immortality and tumorigenesis (26, 45).

2.1.1 Normal Structure and Function of Human Telomeres

With the aid of the shelterin complex, telomeric DNA loops back on itself, forming a lariat structure known as a t-loop. The G rich strand invades the duplex telomeric repeat and where the single-stranded overhang is incorporated between two other strands, forming a 3′ single-stranded region, is called a displacement loop, or d-loop, which aids in elongation of telomeres by telomerase (28). Shelterin, a protein complex composed of the proteins TRF1, TRF2, POT1, TPP1, TIN2, and Rap1 bind along the length of the telomere repeats. The shelterin complex protects chromosome ends from DNA degradation, DNA repair mechanisms, and end-to-end fusion (28, 44, 46-52). Normal somatic cells shorten with each cellular division and after extensive cell divisions, telomeres become critically short and uncapped, resulting in DNA damage responses and chromosomal instability (53). Because of this “end-replication problem” associated with telomeres, they are often referred to as “biological clocks.”

2.1.1.1 Shelterin Complex

Six proteins located on the chromosomal terminus of telomeres, form a shelterin complex and are required for capping of telomeric ends and regulation of telomere length (8, 54). The shelterin complex is responsible for maintaining the lariat structure of the telomere and plays a

significant role in controlling telomere length homeostasis through suppression of nuclease activity at the telomere and through regulation of the telomere-lengthening enzyme, telomerase. Interestingly, the process by which telomere ends are protected resembles the initial steps of homologous recombination (13).

The proteins that make up the shelterin complex are tankyrase, telomere repeat binding factor 1 and 2 (TRF1, TRF2), protection of telomeres 1 (POT1), and TRF1-interacting protein (Tin2), TPP1 and repressor-activator protein (Rap1) (13, 54). TRF1 and TRF2 both bind to the dsDNA region of the telomere and are necessary for the recruitment and stabilization of the other shelterin factors. TIN2 interacts with the TRF1/TRF2 scaffold and attracts TPP1 and its binding partner POT1 (55-59). In this way, TIN2 is responsible for linking POT1 to the ssDNA of the telomere. RAP1 is known to bind with TRF2, but its exact function is yet to be determined (13).

It appears that TRF2 plays an important role in the t-loop structure of the telomere. TRF2 is found to bind double-stranded DNA on the telomere and protects the 3′ single strand overhang from being recognized as a DNA break. It has been found that inhibiting TRF2, or using a dominant-negative form causing over expression, leads to a rapid induction of apoptosis, mediated primarily through stabilizing p53 and upregulation of p53-dependent apoptotic genes as well as cyclin-dependent kinase inhibitors (CDKIs) (13). However, it is unknown how the altered telomere structure is recognized as damaged DNA (16). It has been suggested that the shortening of the telomere is not what necessarily leads to senescence, but rather the “uncapped” telomere resulting in a dysfunctional structure does (46). Uncapping, or exposure of the 3′ overhang, may occur due to critically short telomeres causing the loop structure to become

unstable (16). Studies have shown that cells expressing TIN2 mutations lead to telomere uncapping and growth arrest, with telomere dysfunction and cell death in a p53-deficient background (54, 60).

It has been discovered that expression of TRF1 and POT1 in *trans* results in extensive telomere elongation, while fusing TRF1 to POT1 abolishes this effect, inducing mild telomere shortening (54). This finding implies that a protein bridge between dsDNA and ssDNA may be responsible for the inhibition of telomerase access and promotion of telomere shortening (61). Further findings concerning shelterin proteins have shown that mutants in tankyrase1 with inactive ARC (ANK repeat cluster) IV, which is required for the TRF1 PARsylation, decreases binding of POT1 (54). POT1 is a downstream effector of TRF1-mediated telomere length control. The mutant tankyrase1 loosens the closed structure of the telomeric heterochromatin and elongates the telomeric 3′ overhang similar to that seen in wild-type tankyrase1 (62).

2.1.1.2 <u>Telomere Length and Cell Senescence, Apoptosis, and DNA Damage Foci</u>

Because the telomere-lengthening enzyme telomerase is not active in most human somatic cells, telomere length homeostasis is mostly dependent upon the rate of normal telomere erosion, known as the end-replication problem (63). During normal DNA replication, a primer initiates 5′-3′ replication, and since the primer is removed, the 5′ end of the synthesized strand lacks the first 10-15 base pairs (63). In addition, the ssDNA 3′ overhang essential for protection of chromosome ends is created by telomere-shortening nuclease activity. The rate at which normal human telomeres shorten appears to average about 100 base pairs per cell replication (63, 64).

Telomere dysfunction is related to the deregulation or displacement of telomere-binding proteins. First, normal telomere erosion reduces the number of binding sites for telomere-binding proteins until the Hayflick limit is reached and the structural integrity of the telomere is lost, activating DNA repair systems (28, 65). In addition to normal erosion, inactive shelterin proteins can lead to the deregulation of telomere length, eventually leaving the telomere ends unprotected and susceptible to DNA damage responses (66). Finally, alterations to telomeric repeats also cause telomere dysfunction since the telomere can no longer recruit protective telomere-binding proteins (66). It seems that telomeres are designed to eventually attract DNA damage responses in order to protect the fidelity of genomic material.

Cells normally respond to telomeric dysfunction by undergoing replicative senescence or apoptosis, but in some cases cells continue to proliferate. This can result in increased genomic instability caused by nucleolytic or enzymatic degradation, oxidative metabolism, as well as non-homologous end joining (NHEJ) and homologous recombination (HR) (67). For this reason, many cancers, neurodegenerative diseases, genome instability syndromes, premature aging, and normal aging correlate with deficiencies in telomere-binding proteins and DNA damage proteins (29).

2.1.2 Telomerase

The primary function of telomerase is to lengthen telomeres in order to increase cellular proliferation. It is normally active in germ line cells and inappropriately active in most cancers (31). Human telomerase is a ribonucleoprotein enzyme that contains a catalytic component

called TERT (telomerase reverse-transcriptase) (68, 69), a RNA template molecule called TERC (or hTR) (70), and a telomerase associated protein (71). The two primary subunits of telomerase are TERT and TERC. TERT uses TERC to add TTAGGG repeats to the 3' end of the telomeric overhang (54). Telomerase is regulated by a negative feedback loop involving shelterin; longer telomeres recruit more shelterin, which in turn inhibits telomerase activity (13).

TERC is constitutively expressed in most somatic cells, while higher expression of the catalytic subunit is restricted to cancer cells (54). Studies conducted on cells both positive and negative for telomerase have shown that the level of TERT messenger RNA (mRNA) is directly proportional to telomerase activity in some particular cell types (72-75). It has also been demonstrated that c-Myc-activated telomerase, through interaction with the TERT gene promoter, has caused transformation by facilitating immortalization, thus becoming a hallmark of human cancer (76). Low-level expression of telomerase is present in some normal fibroblasts and most somatic cells (77); this amount of telomerase cannot maintain telomere length but may play a role in maintaining chromosomal structure during each S phase (78).

2.1.3 Alternative Lengthening of Telomeres

Not all immortal cells with unlimited proliferative potential maintain telomere length with telomerase; some cells continue to elongate and stabilize their telomeres by inducing alternative lengthening of telomeres (ALT) pathway, resulting in acquired resistance to telomerase inhibiting compounds (79). Approximately 10-15% of human tumor cells, especially tumors of mesenchymal origin such as sarcomas and astrocytomas, have been shown to utilize ALT (80). Most ALT-positive human cells show highly heterogeneous telomere lengths when compared to

much more homogeneous telomere lengths of telomerase-positive immortal cells (81, 82). In addition, ALT-positive cells tend to display a significant number of telomeric DNA circles (t-circles), perhaps derived from t-loops, when compared to telomerase-positive cells (83). Normal cells and telomerase-positive cells appear to contain one or more factors that repress the ALT mechanism. It is unknown whether a single or multiple ALT mechanisms exist in human cells, or whether ALT is required for certain types of cells (84).

Promyelocytic leukemia protein (PML) nuclear bodies (NB) are present in most mammalian cell nuclei and a typical nucleus contains 5-30 bodies that appear as discrete foci varying in size from 0.2-1µm in diameter. However, the structural organization and the precise functions of these nuclear bodies remain unclear (85). These nuclear foci display frequent telomeric exchange events as well as the extrachromosomal t-circles (86). Many proteins necessary for DNA recombination, repair, and replication associate with ALT-associated promyelocytic leukemia bodies (APBs), and since many proteins involved in homologous recombination are required for ALT, this suggests that ALT mechanisms involve recombination (87). In one study, APBs were found in 17/17 ALT cell lines, 0/20 telomerase-positive cell lines, and 0/5 mortal cell strains, with a few exceptions noted (87). Another study showed a strong correlation between the presence of APBs in tumors and heterogeneous telomere length phenotypes characteristic of ALT (80). Results of fluorescence microscopy shows that telomeric DNA in ALT-positive cells associates dynamically with other telomeric DNA as well as PML-nuclear bodies, suggesting that perhaps telomeric DNA becomes located in APBs as part of the ALT mechanism (88). APB suppression has been shown to result in loss of rapidly changing

telomere lengths associated with ALT as well as progressive telomere shortening observed in telomerase-negative somatic cells (89).

2.2 Telomerase and Human Cancers

The DNA replication process is susceptible to errors, and without proper damage responses, mutagenesis may occur, potentially resulting in cellular death, senescence, or malignant transformation (90). As described above, it is believed that critically shortened telomeres attract DNA damage responses in order to protect genetic fidelity. In addition, nearly all cells have telomere maintenance mechanisms with potential for unlimited proliferation, such as telomerase and ALT(91). For this reason, the inappropriate activation of telomerase has become the target of many studies related to tumorigenesis and carcinogenesis, as well as the treatment of existing cancers.

Malignant cells are characterized by their lack of regulation of cell cycle checkpoints and unlimited proliferation. Transformed cells as a rule have invasive capabilities, and exhibit activation of telomerase (or ALT) (92). In fact, abnormal telomerase expression is found in most human tumors, cancer cells, and cancer stem cells, and is shown to promote tumorigenesis and have more malignant potential such as metastasis (93). For many types of cancer cells, telomerase expression can be used for screening, early detection, and prognosis (94).

2.2.1 Carcinogenesis and Immortalization

Carcinogenesis and cellular immortalization are related but independent events involving telomeres and often telomerase or ALT. Normal somatic cells are designed to stop dividing at

the “Hayflick limit”, also known as mortality stage 1 (M1), when perhaps a single uncapped telomere is recognized as broken or otherwise damaged DNA (95). However, if a mutation has caused inactivation of tumor suppressors’ p16/Rb (retinoblastoma protein) and p53 and/or activation of oncogenes and mitogenic signaling pathways, the cell can continue prolific replication beyond M1 (96). Cancer cells replicate more often than normal cells, and thus their telomeres shorten at a faster rate (97). Interestingly, telomeres in cancer cells have been found to be much shorter (5 kb) compared to normal cells (10-20 kb) (98, 99). Transformed cells that have bypassed M1 will usually stop dividing at mortality stage 2 (M2), the “crisis” at which telomeres have become critically shortened and end-end fusions occur (96).

In order for cancer cells to overcome M2, which is extremely rare in human cells, the upregulation or reactivation of telomerase is almost always required, but other telomere maintenance mechanisms such as ALT can also cause immortalization of cancer cells and subsequent progression to advanced malignancies (96). Some early stage clinical cancers may have bypassed M1 but not M2 and so remain untransformed, while cancer cell lines and “cancer stem cells” have overcome both M1 and M2 (100). In contrast, normal lymphocytes and stem/progenitor cells have highly regulated telomerase activity and experience extended lifespans but are still not transformed since they have not overcome M1 and M2 (101). Some benign tumors may also express low levels of telomerase activity (102).

2.2.2 Activation of Telomerase

Under ordinary circumstances, transformation of normal cells and the immortalization of cancer cells are prevented by cell senescence, which leads to permanent cell cycle arrest (101).

Telomerase has been implicated in immortalization as described above, as well as carcinogenesis and tumorigenesis. Telomerase can become activated in normal somatic cells making them susceptible to transformation and malignancy (103). One study found that while inducing telomerase activity in ALT cells does not alter telomere maintenance in these cells, the ectopic expression of TERT in these ALT cells was found to lead to expression of a tumorigenic phenotype (103).

Telomerase activity has been detected in more than 80% of cancer tissues, with a very strong correlation between the severity of the cancer and the incidence and level of telomerase expression. Higher levels of telomerase activity are found in advanced stages of cancer than early stages, which suggests that the progression of cancers may depend on telomerase. (104). Telomerase activation is often required for *in vitro* transformation of cells, but hTERT expression may occur in various stages of tumorigenesis or carcinogenesis *in vivo* (105).

2.2.3 Telomerase and Stem Cells

There are two known pathways by which cancer cells attain immortality: first, they can originate from a normal telomerase-negative cell which has bypassed both M1 and M2 as described above, or alternately, they may originate from a telomerase-positive cell such as a cancer stem cell (106, 107). A gradual increase in telomerase activity is found in the cancers caused by this first mechanism, while cancers resulting from the second mechanism show high telomerase activity from early stages (106, 107). Cancer stem cells originating from normal cells that innately express telomerase activity may not experience M1 and M2 because they inherently hold potential for immortalization (106, 107).

Cancer stem cells are broadly defined as “cancer-initiating cells” which have both self-renewal capacities throughout their lifetimes as well as the ability to differentiate into heterogeneous lines of cancer cells (100). Cancer stem cells are rare and are thought to exist among the heterogeneous cell mass that makes up a tumor (108). Telomerase is used to mark “stemness” since telomere length and telomerase activity indicate the developmental stage of the stem cell (109, 110).

Lymphocytes and most stem/progenitor cells in self-renewal tissues express telomerase upon mitogenic stimulation (111). Telomerase activity is suppressed upon differentiation or senescence so that normal somatic cells never attain immortality unless there is a loss of tumor suppressor genes or activation of oncogenes (104, 112). If aged human stem cells with dysfunctional telomeres enter into the cell cycle under conditions of stress or tissue regeneration, DNA damage responses may be activated and affect self-renewal, but stress-free conditions allow potential for malignant transformation of normal stem cells into cancer stem cells if DNA damage checkpoint controls are lost (13).

2.3 Therapeutic Targets and Drugs Using Telomeres and Telomerase

Various approaches of telomeres and telomerase targeting based on the direct or indirect method of telomerase inhibition have been studied, and a variety of compounds have been tested on many possible therapeutic targets in the treatment of tumors and cancers. Telomerase targeting strategies can be assigned to two general categories: one is directly targeting telomerase by inhibiting the activity of its catalytic subunit, hTERT, or targeting the RNA template of telomerase, hTERT, leading to telomere shortening and inhibition of telomerase activity and cell

proliferation. Another approach is indirectly targeting telomerase with G-quadruplex stabilizers or targeting telomere and telomerase associated proteins, such as tankyrases or HSP90, thus blocking telomerase access to telomeres or inhibiting binding of telomerase-associated proteins, which could consequently lead to telomere uncapping resulting in cell apoptosis (25, 45).

2.3.1 Telomerase Template Antagonist TERC Oligonucleotide GRN 163L

GRN163L, a 13-mer, lipid-conjugated NPs telomerase template antagonist, targets hTERC efficiently due in part to its lipophilic palmitoyl tail at the 5' terminal end of its sequence 5'-Palm-TAGGGTTAGACAA-3') (113). It forms stable duplexes with single-stranded RNA, resists degradation, demonstrates high affinity and specificity for targets, and behaves as a competitive enzyme inhibitor or template antagonist (114). It has been studied in many cancer types *in vitro* and in pre-clinical *in vivo* xenograft models, providing support for the use of telomerase template antagonists as targeted cancer therapeutics (115, 116). Telomerase template antagonists are the first telomerase inhibitors to have reached clinical trials in different cancer types (29). Currently, GRN163L is undergoing six stage I and stage I/II clinical trials targeting various cancers (114).

GRN163L is reported to inhibit cancer cell growth before the majority of telomeres in cells become critically short, and Jackson et al. reported that cells given GRN163L before they were attached to culture dishes were prevented from adhering to the dishes in cells with telomerase, and also in telomerase-deficient immortal cells utilizing the ALT mechanism (117).

2.3.2 G-Quadruplex Stabilizers Telomestin, BRACO19, and RHPS4

The G-rich telomeric DNA found in humans and other vertebrates is capable of forming G-quadruplexes, four-stranded DNA secondary structures (99). Compounds that stabilize these G-quadruplexes have been shown to inhibit telomerase activity and telomere maintenance, making the DNA G-quadruplex a target for cancer therapy (99). G-quadruplex-targeting compounds have been shown to prevent telomere capping and maintenance, and thereby induce rapid apoptosis in telomerase-active cells as well as inhibit ALT (34).

Synthetic G-quadruplex stabilizing ligands have been tested in several cancer types, showing rapid loss of hTERT expression, telomere shortening, inhibition of cell growth, and apoptosis thought to be caused by changes in telomere capping (118, 119). G-quadruplex ligands may also trigger telomere uncapping by causing dissociation of telomere-associated proteins (120, 121)(29). Three of the most commonly studied G-quadruplex binding ligands include acridines (trisubsituted BRACO19 and pentacyclic RHPS4), and a natural product (telomestatin), among many others (28).

2.3.2.1 Telomestatin

Telomestatin is the most potent small-molecule inhibitor of telomerase and one of the most promising G-quadruplex targeting drugs (122). It is a natural product isolated from *Streptomyces anulatus* 3533-SV4 and targets intermolecular G-quadruplex structures over duplex DNA (123, 124). Telomestatin is capable of inducing telomere dysfunction and activating DNA damage responses, and has been shown to safely inhibit telomerase activity, shorten telomeres, cause apoptosis, and increase chemosensitivity in cancer cells (118). A major

limitation of telomestatin is the difficulty of its synthesis, making it unfeasible for large-scale production (125). A recent study has reported a development of a new (S)-stereoisomer of telomestatin-(R), which has shown four-fold greater telomerase inhibiting activity than the (R)-stereoisomer (124).

2.3.2.2 BRACO19

BRACO19 is a telomere-targeting trisubstituted acridine derivative designed by computer modeling to act as G-quadruplex stabilizer (126, 127). It has very low cytotoxicity and very high quadruplex-binding and telomerase-inhibiting activity (29). BRACO19 produces anti-tumor effects soon after treatment and has been shown to suppress the catalytic function of telomerase in cancer cells, destabilize the telomere-capping complex, and induce end-to-end chromosomal fusions (128). It shows high *in vivo* activity against a variety of cancers, inhibiting growth, inhibiting nuclear hTERT expression, and increasing atypical mitosis (129). The major limitation of BRACO19 is its lack of membrane permeability (130).

2.3.2.3 RHPS4

RHPS4 is a five-ring acridine identified to be a potent submicromolar-level inhibitor of telomerase, but showing acute cytotoxicity only at significantly higher concentrations (131). Studies have shown that the promising results of short-term treatment of cancer cells with RHSP4 are caused by telomere dysfunction rather than shortening based on presence of telomeric fusions, polynucleated cells, and telophase bridges in treated cells (132). A combination therapy of RHPS4 with cisplatin in breast cancer cell lines has shown a synergistic

effect (127). RHPS4 has shown promising preclinical results and may enter into clinical studies in the near future (29).

2.3.3 Nucleoside Analog AZT

The nucleoside analog and reverse transcriptase inhibitor azidothymidine (AZT) was one of the first drugs tested for hTERT inhibition (118, 133-135). AZT has been shown to weakly decrease telomerase activity and cell proliferation, but these effects are not specific for telomerase over other polymerases (136).

2.3.4 Small Molecule Inhibitor BIBR1532

A widely studied small molecule inhibitor targeting hTERT is BIBR1532 (2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid), a non-competitive catalytic inhibitor with a high specificity for telomerase (137, 138). BIBR1532 is a non-nucleotidic small molecule synthetic compound that inhibits telomerase by non-competitive binding to the active site of hTERT (54, 137, 139). BIBR1532 has been shown to inhibit 50% of telomerase activity in several types of tumor cells and long-term treatment can significantly decrease tumor growth *in vivo* (140). While the long lag time expected for classical telomerase inhibitors may make them ineffective for clinical cancer treatment, BIBR1532 has been shown to sensitize drug-resistant cancer cells to other chemotherapeutics (141).

2.3.5 T-oligo

T-oligo is an oligonucleotide homologous to the telomere overhang sequence (GTTAGGGTTAG) known to accumulate in the nucleus (18, 22, 142), which has been shown to signal potent DNA damage responses (10). T-oligo activates multiple independent DNA damage pathways such as ATM, p95/Nbs1, E2F1, $p16^{INK4A}$, and p53 to modulate downstream effectors (16, 18). Studies targeting cancer cells with T-oligo have found upregulation of proapoptotic proteins E2F1, p53, p73, p21, as well as senescence proteins (19). These cellular responses occur without affecting the cells own telomeres (16, 17, 19, 143) and are independent of telomerase (17). Studies further show that T-oligo induces senescence through both p53 and pRb pathways (16, 17). T-oligo has been shown to induce apoptosis in melanoma cells (9)(19), lymphocytic leukemia (19)(22), breast carcinoma cells (22), human lymphoma lines (144), prostate cancer cells (21), and ovarian cancer cells (145). T-oligo has also shown the ability to inhibit angiogenesis in melanoma cells lines (20), and can increase cellular reactive oxygen species levels through a p53 dependent pathway, thus indicating that T-oligo acts to reduce oxidative damage to cells (143). T-oligo has efficacy against breast cancer, lung cancer, glioma, and melanoma xenograft mouse models, and has no harmful side effects on mice (8).

T-oligo has been found to reduce tumorigenicity and metastasis of melanoma as well as induce differentiation, senescence, and apoptosis of melanoma cells (9) while normal human melanocytes treated with T-oligo show only transient cell cycle arrest (16). MM-AN cells briefly treated with T-oligo and injected into flank or tail veins of SCID mice showed 85-95% reduction of tumor volume and number of metastases, and T-oligo administered intralesionally or systemically selectively inhibited growth of previously established MM-AN tumor nodules in

the flank and peritoneal cavity by 85-90% (9). T-oligo has been found to induce the p53 homologue p73 to activate downstream effectors and downregulate the inhibitor of apoptosis protein livin/ML-IAP (9).

The potency of T-oligo against cancer cell lines and relatively short treatment times suggests its potential as a cancer therapeutic, and its lack of harmful effects on normal cells suggests its combination with other compounds may increase anti-tumor activity without increasing cytotoxicity (8).

3. RESEARCH APPROACH

3.1 Materials and Methods

3.1.1 Culturing of Cells

H358 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 0.01 M HEPES, and 1 mM sodium pyruvate.

SW1573 cells (ATCC, Manassas, VA) were cultured in MEM supplemented with 10% Fetal Bovine Serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM Non-essential amino acids.

MU and AN melanoma cells (obtained from Dr. HR Byers from Boston University) were cultured in MEM with 10% Fetal Bovine Serum, antibiotic-antimycotic solution containing 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.25 μg amphotericin B/mL. Fetal Bovine Serum and all medium supplements were obtained from Invitrogen (Carlsbad, CA). Cells were maintained in a 25 cm^2 vented cap tissue culture treated flasks (Corning, Corning, NY).

H358 cells for experiments were detached from flasks using 0.25% trypsin/EDTA at a concentration of 2.5 g/L trypsin and .38 g/L EDTA (Invitrogen, Carlsbad, CA) by washing twice with 2 mL of 0.25% trypsin in 25 cm^2 flask. After cells detached they were collected in RPMI-1640 medium with 10% FBS spun down at 800 rpm and resuspended in the same medium. Similarly, SW1573, MU, and AN cells were detached using 2 mL of 0.05% trypsin/EDTA at a concentration of 0.5 g/L trypsin and 0.2 g/L EDTA (Invitrogen, Carlsbad, CA).

Normal Bronchial Epithelial Cells (generous gift from Dr. Y Zhao) were plated for experiments in 6 well plates. Some cells were saved and cultured in 25 cm^2 flasks. Cells were maintained in BEBM (bronchial epithelial cell basal medium) supplemented with gentamicin sulfate, transferrin, bovine pituitary extract, hydrocortisone, epidermal growth factor, insulin, epinephrine, triiodothyronine, and retinoic acid per manufactures instructions (#CC-3170 Lonza, MD), and maintained at 7% CO_2 and 37°C.

3.1.2 **Western Blot Procedure**

H358, SW1573, MU, MM-AN, and normal bronchial cell lysates were prepared by plating the respective cells in 35 x 10mm tissue culture dishes (BD Franklin Lakes, NJ) in 2 mL of their respective medium described above. Cells were incubated at 37° C and 7% CO_2 for 24 hours. Medium was then removed and washed twice with non-supplemented medium. Cells were then treated with diluents (20 µL distilled water/mL media), 40µM of an oligonucleotide homologous to the telomere overhang (T-oligo pGTTAGGGTTAG) (Midland Certified Reagent Company, Midland, TX), and 40uM of a complementary oligonucleotide (complement 40µM, pCTAACCCTAAC) (Midland Certified Reagent Company, Midland, TX). Medium with T-oligo or complementary oligonucleotide was prepared in medium for respective cell line with 10% FBS, and 40 µM T-oligo/complementary oligo. T-oligo or complementary oligo were added to the plated cell lines and incubated at 37° C and 7% CO_2 incubator for 6, 18, 24, 48, 72, and 96 hrs respectively. Cells were washed two times with1X, pH 7.4 cold PBS (Phosphate-Buffered Saline), and then treated with 100 µL of a protein extraction buffer containing complete mini protease inhibitor cocktail (Roche, Indianapolis, IN. 11836153001), phosphate-protein extraction buffer composed of 20 mM Tris-HCl pH 8, 150 mM NaCl, 100 mM NaF, 1% NP-40,

10% glycerol (#BP-116P Boston Bioproducts, Boston, MA) and 0.1mM sodium orthovanadate (Boston Bioproducts, Boston, MA), and kept on ice for 45 seconds after which cells were scraped using a cell scraper and were lysed using a 1 mL syringe. Cell lysate were further disrupted using a SONIFIER cell disruptor 350 (Branson Sonic Power, Danbury, CT) using 5 pulses of 1 second each with settings of 15 for percent duty and 3 for output control. Lysate was then centrifuged at 14,000 rpm for 20 min in the cold room. Lysate was then transferred to new centrifuge tubes kept on ice. Protein estimation was done using the Bradford method. A protein estimation curve was prepared with BSA (Fisher Scientific, Rockford, IL) and distilled water as shown below. Stock solution of BSA used was 1 mg/mL.

Table I Protein Standard Curve

Cuvette	**Distilled H_2O**	**BSA**
Blank	100 µL	0 µL
1 µg	99 µL	1 µL
2 µg	98 µL	2 µL
4 µg	96 µL	4 µL
6 µg	94 µL	6 µL
8 µg	92 µL	8 µL
10 µg	90 µL	10 µL
12 µg	88 µL	12 µL
14 µg	86 µL	14 µL
16 µg	84 µL	16 µL

To 3 μL of the lysate sample, 97 μL distilled water was added. 900 μL of the Bradford reagent (BioRad, Hercules, CA) was then added to make the total to 1ml and samples were mixed with a vortex mixer. Bradford reagent was prepared by using one part Bradford reagent and 4 parts distilled water.

Samples were then estimated on a Beckman du 650 spectrophotometer (Beckman Coulter, Brea, CA) by first using a blank and then preparing a protein standard curve. Protein concentrations in samples were analyzed in duplicate. 50 μg of protein samples were loaded in Laemelli's loading buffer composed of 250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 8% βME, and 0.02% bromophenol (Boston Bioproducts, Boston, MA). Lysates were heated at 100°C for 5 min and then centrifuged for 30 seconds at 14,000 rpm. Samples were run on an SDS PAGE gel for 2 hours at 90 volts. The gel was then removed from the electrophoresis apparatus and then transferred onto a nitrocellulose membrane using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad, Hercules, CA). Gels were transferred for 45 minutes at 18 volts. Blots were rinsed three times in TBST (Tris-buffered saline solution) containing 250 mM Tris, 27 mM KCl, 1.37 mM NaCl and a pH 7.4 (Boston Bioproducts, Boston, MA) with 0.05% Tween 20 (Fisher Scientific, Rockford, IL) and blocked for 1 hour using 5% milk. The blot was then rinsed three times in TBST and the primary antibody prepared by adding the appropriate dilution to TBST containing 2% bovine albumin. Primary antibody was added to the blot and kept overnight at 4 °C on a rocker in the cold room. The next day the primary antibody was removed and the blot was washed in TBST for 1 hour with three, 20 minute changes of fresh TBST. Appropriate secondary antibody diluted in 1% milk in TBST was then added to the blot for 1 hour with constant rocking. Blot was washed for 20 minutes three times in fresh TBST. To visualize

proteins, membranes were treated with prepared Pierce® ECL Western Blotting Substrate (Fisher Scientific, Rockford, IL. 32109) for 60 seconds, and exposed to autoradiography film (Scrip Inc., Boling Brook, IL. 688-0013) for various time points, after which the film was fixed and developed using the Konica SRX-101A developer (Konica Minolta, Philadelphia, PA).

3.1.3 **Preparation of Gel**

10% gels were prepared by adding 5 mL of distilled water, 3.15 mL 4X resolving buffer containing 1.5 M pH 8.8 Tris-HCl, and 0.4% SDS (Boston Bioproducts, Boston, MA), 4.15 mL 30% Bis/acrylamide (BioRad, Hercules, CA), 50 μL 10% APS, and 5 μL TEMED were added together and mixed using a pipette. Mixture was then added to gel plate and layered with water containing supersaturated butanol and was allowed to set for almost an hour. The stacking gel was then prepared by adding 3.05 mL distilled water, 1.25 ml 4X stacking buffer solution containing 500 mM pH 6.8 Tris-HCl and SDS 0.4% (Boston Bioproducts, Boston, MA), 700 μL 30% Bis/acrylamide, 25 μL 10% APS, and 5 μL TEMED was added to 50 mL tube and mixed together. Butanol was removed from top of gel and the gel was rinsed twice with distilled water. Stacking gel was then added to gel plate and comb was inserted. Gel was allowed to set for 30 min. When not using the gel the same day they were stored at 4°C in moist paper towels.

3.1.4 **Preparation of Antibodies**

β-actin antibody (Sigma, St. Louis, MO) was prepared by adding 2% bovine albumin (Sigma, St. Louis, MO) in TBST and a 1:5000 dilution of the primary antibody. hnRNP C1/C2 and E2F1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was prepared by adding 2% bovine albumin in TBST and a 1:250 dilution of the primary antibody. Pur-β antibody (generous

gift from Dr. Robert Kelm, University of Vermont) was prepared by adding 2% bovine albumin in TBST and a 1:2900 dilution of the primary antibody. Msi-1 antibody (Millipore, Billerica, MA) was prepared by adding 2% bovine albumin in TBST with a 1:1000 dilution. p21 and E2F1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was prepared using a 1:1000 dilution and adding it to TBST with 2% bovine albumin. p53-DO1 and p73 antibody (Oncogene, San Diego, CA) were prepared using a 1:1000 dilution and adding it to TBST with 2% bovine albumin.

3.1.5 **Densitometric Analysis of Western Blot**

Films were scanned into a .jpg format using a HP scanner, and densitometric analysis was performed using NIH ImageJ software following the method outlined at http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j.

3.2 **Normal Bronchial Epithelial Cells**

3.2.1 **Proliferation Experiment on Normal Bronchial Epithelial Cells**

50,000 NBEC were plated in triplicate in 6 well plates containing 2 mL BEGM (CC-3170, Lonza, Walkersville, MD) and incubated at 37°C in 7% CO_2 for 24 hours. Cells were treated with diluent, T-oligo, or complementary oligonucleotide prepared in BEBM (Bronchial Epithelial Basal Medium) which contains no supplements for 72 and 96 hours. Cells were then harvested by rinsing with 0.5 mL HEPES buffered saline solution (#CC-5604, Lonza, Waslkersville, MD) to each well and then adding 1 mL trypsin/EDTA (#CC-5604, Lonza, Walkersville, MD) to each well for 5 min. When cells detached, 2 mL trypsin neutralizing solution (#CC-5604, Lonza, Walkersville, MD) was added to the wells containing trypsin. Cells were subsequently collected and placed in labeled tubes containing MEM media. Tubes were

centrifuged 5 minutes at 1000 rpm. The supernatant was then removed from the tubes, pellet was dislodged and cells were re-suspended in 300 μL MEM. Cells were counted on a hemocytometer (Fisher Scientific, Rockford, IL). Four counts were made from each tube and the average was then taken of each sample. Viability of each sample was verified by adding 10 μL of the cell solution mixed with 10 μL of 0.2 μM trypan blue (Thermo Scientific, Waltham, MA).

3.2.2 **Apoptosis Experiment on Normal Bronchial Epithelial Cells and H358 Lung Cancer Cells**

50,000 NBEC were plated in triplicate in 6 well plates containing 2 mL BEGM and incubated at 37°C in 7% CO_2 for 24 hours. Cells were then treated with diluent, T-oligo, or complementary oligonucleotide prepared in BEGM containing no supplements for 48, 72, and 96 hours. Cells were harvested by rinsing twice with 0.5 mL trypsin (Lonza, Walkersville, MD) and then collected in a 15 mL tube and centrifuged at 1,000 rpm at room temperature. After centrifugation the supernatant was then removed and the cells were re-suspended in 6.5 mL plain DMEM, after which 3.5 ml of chilled 70% ethanol was added and tube was inverted to mix cell suspension together. Tubes were stored at 5°C until ready to be stained with propidium iodide (Sigma, St. Lous, MO). Before propidium iodide staining, tubes were centrifuged in an Eppendorf 5804R centrifuge (Eppendorf, Hauppauge, NY) at 7°C at 800 rpm for 5 minutes. The supernatant was removed and cells were re-suspended in 7 mL of PBS containing 1% FBS. Tubes were again centrifuged at 7°C at 800 rpm for 5 minutes. The cells were re-suspended in 300 μL propidium iodide which was prepared by adding 180 μL of 1 mg/mL propidium iodide and 80 μL of RNAse A solution 1 mg/mL to 9.81 mL PBS (Fisher Scientific, Rockford, IL), and transferred to flow cytometry tubes. Tubes were kept in the dark at room temperature for 2 hours

before running samples on the flow cytometer (Bectin-Dickinson, Franklin Lakes, NJ). Samples were run in 5 replicates and 10,000 events were recorded for analysis of apoptosis and cell cycle.

50,000 H358 cells were plated in triplicate in 6 well plates containing 2 mL RPMI-1640 and incubated at 37°C in 7% CO_2 for 24 hours. Cells were then treated with diluent, T-oligo, or complementary oligo prepared in RPMI-1640 containing no supplements for 96 hours. Cells were harvested and prepared for FACS analysis as mentioned above.

3.2.3 **Senescence Experiment on Normal Bronchial Epithelial Cells**

50,000 NBEC were plated in triplicate in 6 well plates containing 2 mL BEGM and incubated at 37°C in 7% CO_2 for 24 hours. Cells were then treated with diluent, T-oligo, or complementary oligo prepared in BEBM which contained no supplements for one week. After treatment for one week cells were washed two times with PBS. Cells were then fixed at room temperature using 3% formaldehyde for 5 minutes. 3% formaldehyde was then removed and cells were again washed two times with PBS. The β-galactosidase solution was prepared by adding 20 mg x-gal (Sigma, St. Louis, MO) to 1 mL of dimethylformamide (Sigma, St. Louis, MO). To prepare the staining solution 13.4 mL of distilled water added to the 1 mL x-gal solution along with 4 mL of 0.2 M citric acid/sodium phosphate buffer (pH 6.0), 1 mL of 100 mM potassium ferrocyanide, 1 ml of 100 mM potassium ferricyanide, 0.6 mL of 5 M sodium chloride, and 40 μL of 1 M magnesium chloride. The staining solution was then added to each well and kept in a 37°C incubator for 8 hours. Senescent cells which were stained blue were counted using 40x magnification on a Nikon Diaphot (Nikon, Mellville, NY) and 15 different microscopic fields were evaluated for each sample.

3.3 **Lung Cancer and Melanoma**

3.3.1 **Clonogenicity Assay**

100,000 cells of H358, SW1573, MU, and MM-AN cells were plated in triplicate in 60 mm x 15 mm dishes and incubated overnight in 37°C and 7% CO_2. Cells were treated with diluent, T-oligo, or complementary oligonucleotide for one week. Following a week long incubation period H358 cells were then harvested by washing cells two times with 0.25% trypsin at a concentration of 2.5 g/L trypsin and 0.38 g/L EDTA. Cells were detached and collected using the growth medium and pelleted by centrifugation for 5 minutes at 1,000 rpm. Cells were re-suspended in fresh growth medium and plated at 3,000 cells per dish. After 10 days cells were fixed in 100% methanol and stained with 1% methylene blue 10,000 mg/mL (Fisher Scientific, Rockford, IL) for 10 minutes. Methylene blue was removed and dishes washed repeatedly with water to remove excess methylene blue. Cells were counted using Kodak ROI analysis software (Kodak, Rochester, NY). The same procedure was performed for SW1573, MU, and MM-AN cells, however, they were detached with 0.5% trypsin/EDTA (0.5 g/L trypsin and 0.2 g/L EDTA). SW1573 cells were re-plated at 3,000 cells per dish and the same procedure was performed as was done for H358 cells. MU cells were re-plated at 500 cells per dish, and MM-AN cells were re-plated at 1,000 cells per dish and allowed to grow for one week. They were stained and analyzed using the same method.

3.3.2 **Senescence Experiment on MU Melanoma, H358, and SW1573 NSCLC**

Cells were plated at 25,000 cells in a 60 mm x 15 mm dish in triplicate. Cells were incubated at 7% CO_2 and 37°C for 24 hours. Cells were then treated with diluent, T-oligo, or complementary oligonucleotide for one week. After one week, medium was removed and cells

were washed two times with PBS. Cells were then fixed at room temperature using 3% formaldehyde for 5 minutes, after which 3% formaldehyde was removed and cells were washed two times with PBS. The β-galactosidase solution prepared as previously shown was added to each well and kept at 37°C for 24 hours. Cells were then counted in 15 different microscopic fields using 40X magnification on a Nikon Diaphot (Nikon, Mellville, NY) and the mean number calculated for each sample.

3.4 ***in vivo* Experiment**

3.4.1 **Tumorigenicity Experiment in a Mouse Model**

SW1573 and H358 cells were cultured following standard protocol, harvested with indicated concentrations of trypsin/EDTA for each cell line, and viability was determined by trypan blue exclusion. Cell populations with 90% or greater viability were used for this procedure. Cells were suspended in 1X HBSS (Hank's Balanced Salt Solution) (Invitrogen, Carlsbad, CA) and $5x10^6$ viable cells were injected subcutaneously into the flank or leg region of five-week old Nu-/Nu- nude mice (Taconic, Hudson, NY) to produce tumors. After 72 hours, mice were divided into 2 subgroups of 10. T-oligo treatment group received daily intratumoral injections of 40 μM (210μg in 150μl of PBS) of T-oligo, and the control group received daily intratumoral injections of 40 μM (210μg in 150μl of PBS) complementary oligonucleotide. Mice were weighed weekly for signs of excessive weight loss, and weekly tumor measurements were performed with digital calipers (Fisher Scientific, Hampton, NH). Tumor volume was calculated according to the formula volume (mm^3)=(length x $width^2$)/2. After seven weeks, mice were euthanized and tumors were excised and preserved in either 10% formalin for subsequent

paraffin embedding and immunohistochemical analysis or placed in OCT solution and flash frozen in an ethanol/dry ice solution and stored at -70°C.

3.4.2 **Immunohisto Analysis of Senescence Associated Proteins of Nude Mice Tumor Sections**

SW1573 paraffin sections obtained from the intratumoral treatment experiment were rehydrated by 15 dips in xylene, 100%, 95% and 70% ethanol. Antigen retrieval was done by incubating sections in 0.05% trypsin solution for 10 minutes after which sections were washed in running tap water. Sections were then blocked using 0.3% H_2O_2 in 100% methanol for thirty minutes. Following blocking, sections were washed two times for two minutes in PBS. Sections were then blocked using a M.O.M. mouse IgG blocking reagent prepared following manufacture protocol by adding two drops of IgG stock solution to 2.5 ml PBS (Vector Labs, Burlingame, CA) overnight in the cold room. Sections were then removed from the cold room and were washed two times for two minutes with PBS, and then incubated at room temperature with M.O.M diluent solution prepared by adding 600 μL protein concentrate stock solution to 7.5 mL PBS as found in the manufacture's protocol (Vector Labs, Burlingame, CA). Excess diluent solution was drained off and the primary antibody solution was prepared by adding the appropriate dilution of primary antibody to M.O.M. diluent solution, after which it was added to sections and incubated at room temperature for 30 minutes. Primary antibody was subsequently removed and sections were washed two times for two minutes in PBS. M.O.M. biotinylated anti-mouse IgG reagent was added to sections for 10 minutes and was prepared by adding 10 μL of stock solution to 2.5 mL of M.O.M. diluent following manufacture protocol. Sections were then washed two times for two minutes in PBS. Following these washings, Vectastain ABC

reagent, prepared by adding two drops of reagent A to two drops of reagent B in 2.5 mL of PBS (Vector Labs, Burlingame, CA), and added to sections for ten minutes. The sections were then washed two times for five minutes in PBS. A peroxidase substrate solution prepared by adding one drop of chromagen to 1 mL of substrate solution (Vector Labs, Burlingame, CA) was then added to the sections for about seven minutes while monitoring the intensity of the staining. Once desired staining intensity was obtained, slides were washed under running tap water and counterstained using hemotoxylin (Sigma, St. Louis, MO) for 15 seconds. Hemotoxylin was then washed off under running tap water. Samples were dehydrated following a procedure of 15 quick dips in 70% ethanol, followed by 95% ethanol repeated twice, and 100% repeated twice, and were then kept in xylene (Sigma, St. Louis, MO) for five minutes. Slides were mounted with a cover slip using Permount (Fisher, Rockford, IL). Slides were viewed under an Olympus BH-2 microscope (Olympus, Center Valley, PA) at 10x magnification and photographs were taken of the sections.

4. RESULTS

4.1 Experimental Studies

4.1.1 Effect of T-oligo on Proliferation of Normal Bronchial Epithelial Cells

To see if T-oligo's DNA damage responses were specific to cancer cells, we examined the effect of T-oligo on proliferation of NBEC, which were treated with diluent, complementary oligonucleotide, or T-oligo for 72 and 96 hours. Cells were then trypsinized and number of cells evaluated after staining with trypan blue. We found that there was no statistically significant difference ($p>0.05$) in cell viability between diluent, T-oligo, and complementary oligonucleotide treatment groups, implying that T-oligo as shown in figure 1, did not inhibit proliferation of NBEC.

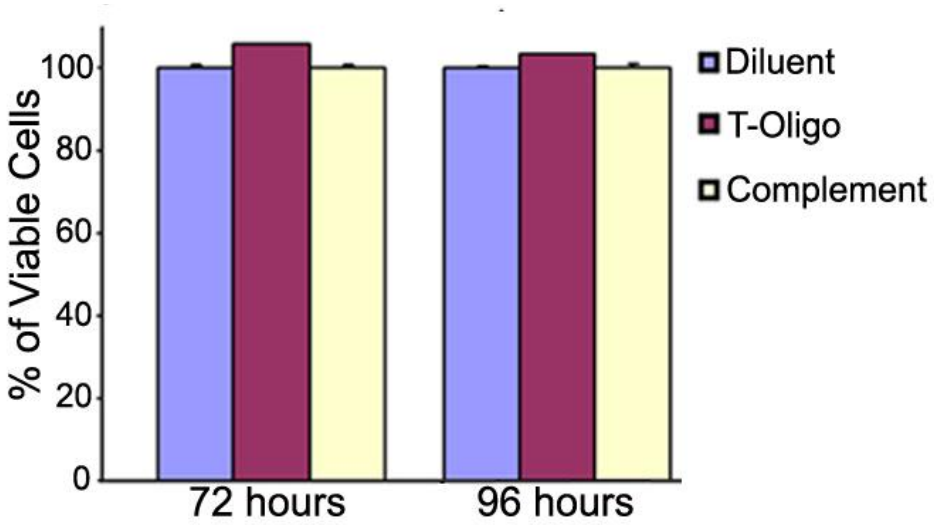

Figure 1. T-oligo Does Not Inhibit Proliferation of NBEC. NBEC were plated in triplicate in 6 well plates and treated with diluent, 40µM T-oligo or complementary oligonucleotide for 72 and 96 hours for proliferation studies. Cells were harvested with trypsin and counted using a hemocytometer. It was found that T-oligo did not inhibit proliferation in NBEC at 72 and 96 hours. $p>0.05$

4.1.2 **Effect of T-oligo on Apoptosis in Normal Bronchial Epithelial Cells**

We also examined the effect of T-oligo on apoptosis in NBEC. NBEC were plated and treated with diluent, complementary oligonucleotide, or T-oligo for 48, 72, and 96 hours. The number of apoptotic cells was determined by fluorescence-activated cell sorting (FACS) analysis by propidium iodide staining. It was determined that T-oligo did not induce significant apoptosis in NBEC as seen in figure 2, and there was no statistically significant difference between diluent, T-oligo, and complimentary oligonucleotide groups ($p>0.05$).

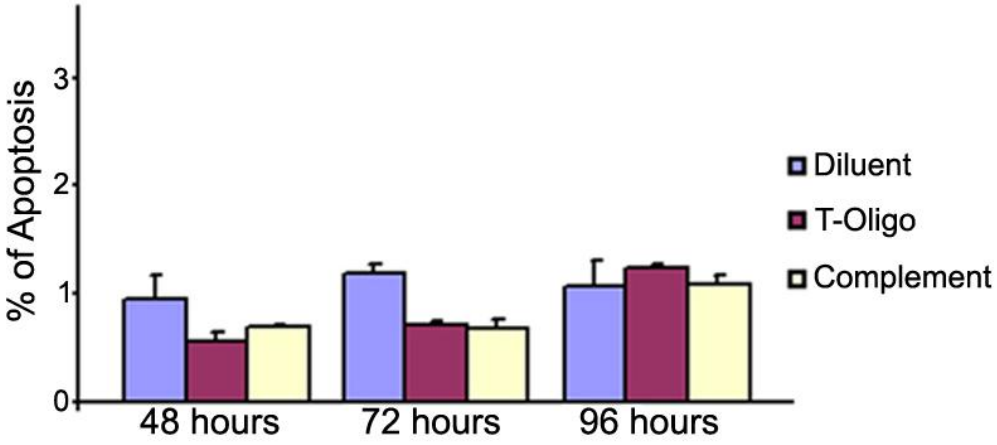

Figure 2. T-oligo Does Not Induce Apoptosis in NBEC. NBEC were plated in triplicate in 6 well plates and treated with diluent, 40µM T-oligo or complementary oligonucleotide for 48, 72, and 96 hours for determining apoptosis. Cells were harvested, fixed in 70% ethanol and then stained with propidium iodide and analyzed with FACS scan. It was found that T-oligo did not induce apoptosis in NBEC at 48, 72, and 96 hours. $p>0.05$

4.1.3 **Effect of T-oligo on Senescence in Normal Bronchial Epithelial Cells**

To see if T-oligo's DNA damage responses were specific to cancer cells, we examined the effect of T-oligo in inducing senescence in NBEC. Cells were treated for one week with diluent, complementary oligonucleotide, or T-oligo. After treatment for one week cells were stained with β-galactosidase, a known marker of senescence that stains senescent cells blue. It was found that T-oligo did not induce senescence in NBEC. We found that there was no statistically significant differences between the three groups described above ($p>0.05$).

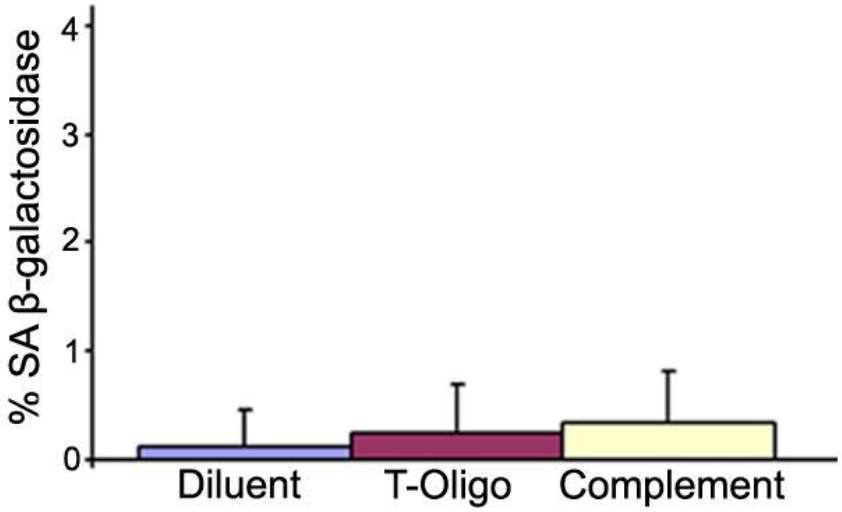

Figure 3. T-oligo Does Not Induce Senescence in NBEC. NBEC were plated in triplicate in 6 well plates and treated with diluent, 40µM T-oligo, or 40µM complementary oligonucleotide for one week. Cells were stained with β-galactosidase, a known marker of senescence. Cells that stained blue indicated senescence. It was found that T-oligo does not induce significant senescence in NBEC. $p>0.05$

4.1.4 **Studying the Effect of T-oligo on p53 and p21 Levels in Normal Bronchial Epithelial Cells**

To understand why T-oligo did not significantly induce apoptosis and senescence in NBEC, the proteins p53 and p21were analyzed. NBEC were treated with diluent, T-oligo, or complementary oligonucleotide, for 12 and 24 hours. Cells were then collected and analyzed using immunoblotting to identify the effect of T-oligo on p53 and p21 levels in NBEC. It was observed that T-oligo did not modulate p53 or p21 in NBEC at 12 and 24 hours as seen in figure 4. These results indicate that T-oligo did not induce DNA damage responses or senescence associated pathways in NBEC. Densitometric analysis was performed using NIH ImageJ analysis and confirmed observations seen in figure 4.

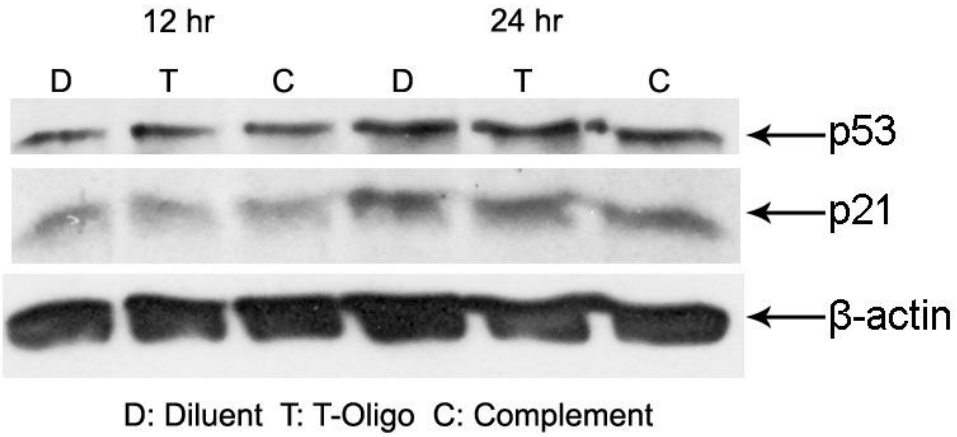

Figure 4. T-oligo Does Not Affect p53 and p21 in NBEC. NBEC were plated in 6 well plates and treated with diluent, T-oligo, or a complementary oligonucleotide for 12 and 24 hours. Cells were harvested and analyzed using immunoblotting techniques. In NBEC it was found that T-oligo did not modulate p53 or p21 at 12 and 24 hours respectively.

4.1.5 **Effect of T-oligo on Clonogenicity in Melanoma and Lung Cancer Cells**

Clonogenecity is an indicator of senescence (146). To determine the effect T-oligo has on clonogenic capacity, MU and MM-AN melanoma cells were plated at 25,000 cells in 60 mm^2 dishes and were treated with a complementary oligonucleotide or T-oligo for one week. Cells were harvested with trypsin/EDTA and plated at 500 and 1,000 cells respectively in 60 mm^2 dishes in growth medium for 10 days. H358 and SW1573 cells were plated at 25,000 cells in 60 mm^2 dishes and were treated with a complementary oligonucleotide or T-oligo for one week. Cells were then harvested and treated using the same method as above. As shown in figure 5, MU cells treated with T-oligo exhibited a 3.7-fold decrease in clonogenic capacity, whereas MM-AN cells treated with T-oligo showed a 4.4-fold decrease in clonogenic capacity respectively. As shown in figure 6, H358 cells treated with T-oligo exhibited a 12-fold decrease in clonogenic capacity, whereas SW1573 cells treated with T-oligo showed a 4-fold decrease in clonogenic capacity.

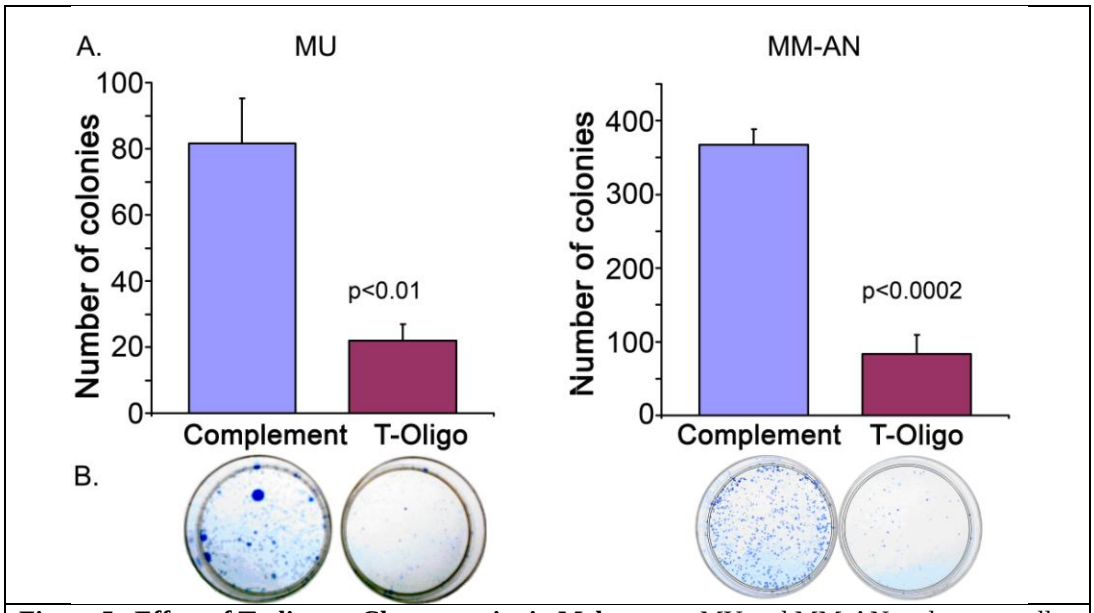

Figure 5. Effect of T-oligo on Clonogenecity in Melanoma. MU and MM-AN melanoma cells were plated at 25,000 cells/60 mm^2 dishes and treated with 40µM T-oligo or 40µM complementary oligonucleotide. After one week, cells were harvested with trypsin/EDTA and plated at 500 cells for MU and 1,000 for MM-AN in 60 mm^2 plates and incubated for 10 days in growth medium. Cells were fixed in 100% ethanol and stained with 1% methylene blue in PBS for 10 minutes. Cell colonies were counted using Kodak ROI analysis. **A.** Statistical analysis shows that T-oligo reduced clonogenic capacity by 3.7-fold in MU and 4.4-fold in MM-AN respectively. **B.** Appearance of stained dishes of MU and MM-AN after staining of colonies as described above. Plates were counted using Kodak ROI analysis software

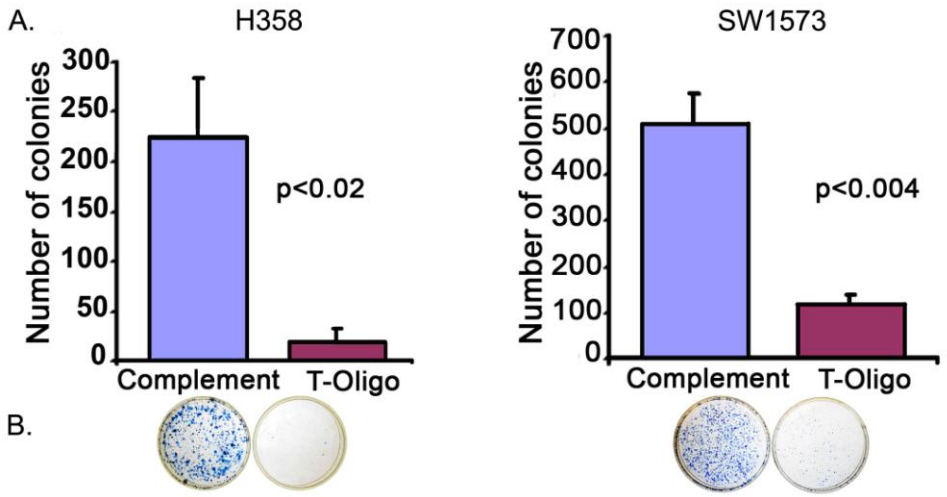

Figure 6. Effect of T-oligo on Clonogenecity in Lung Cancer. H358 and SW1573 cells were plated at 25,000 cells/60 mm^2 dishes and treated with 40µM T-oligo or 40µM complementary oligonucleotide. After one week, cells were harvested with trypsin/EDTA and plated at 2,000 cells/60 mm^2 dishes and incubated for 8 days in growth medium. Cells were fixed in 100% ethanol and stained with 1% methylene blue in PBS for 10 minutes. Cell colonies were counted using Kodak ROI analysis. **A.** Statistical analysis shows that T-oligo reduced clonogenic capacity by 12-fold and 3-fold in H358 and SW1573 respectively. **B.** Cell colonies in Petri dishes stained with methylene blue.

Clonogenic capacity was first studied in melanoma cell lines as it is a well established T-oligo model. It is also the first time clonogenicity has been studied in melanoma. In summary we found that MU cells treated with T-oligo exhibited a 3.7-fold decrease in clonogenic capacity, whereas, MM-AN cells treated with T-oligo showed a 4.4-fold decrease in clonogenic capacity. We confirmed these results by conducting the same experiment in H358 and SW1573 lung cancer cells. We found that H358 cells treated with T-oligo exhibited a 12-fold decrease in clonogenic capacity, and a 4-fold decrease in SW1573 cells. These results solidify our findings in lung cancer.

4.1.6 **Effect of T-oligo in Inducing Senescence in Melanoma and Lung Cancer Cells**

To determine the effect of T-oligo on senescence in melanoma and lung cancer cells, MU melanoma cells and H358 and SW1573 were plated at 25,000 cells/60 mm^2 dish and treated with T-oligo or complementary oligonucleotide for one week. Cells were then analyzed under a microscope after staining with senescence-associated β-galactosidase to determine if T-oligo did induce senescence. As seen in figure 7, T-oligo treated cells exhibited a senescent phenotype and there was a 10-fold increase in senescent cells in cells treated with T-oligo compared to diluent. As seen in figure 8, T-oligo induced a 10-fold and 4-fold increase in senescence associated β-galactosidase positive cells in H358 and SW1573, respectively.

In summary, these results demonstrate that T-oligo induces senescence in lung cancer and melanoma. These results contrast with the observations in NBEC, where senescence was not observed. These findings indicate that T-oligo mediates senescence specifically in cancer cells.

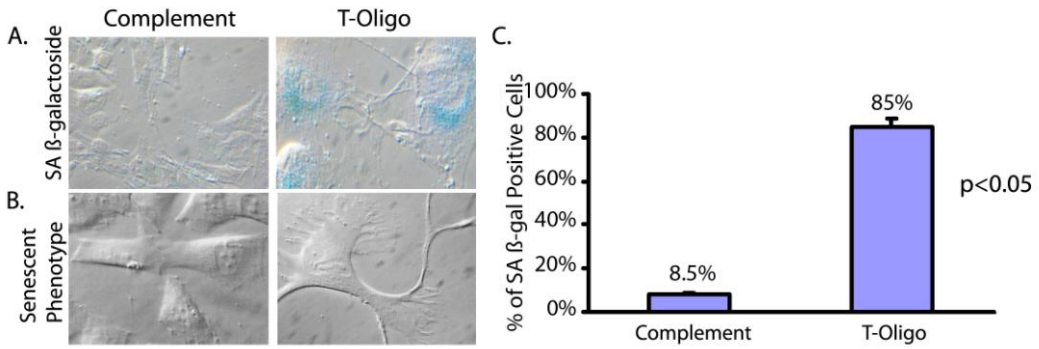

Figure 7. T-oligo Induced Senescence in MU Cells. MU melanoma cells were treated for 1 week with T-oligo, complementary oligonucleotide, or diluent, after which the cells were stained for senescence associated β-galactosidase which appears in **B** as blue color. **A.** MU melanoma cells were stained with β-galactosidase. T-oligo treated cells have a blue color indicating that these cells are positive for senescence. **B.** MU melanoma cells after treatment with T-oligo show a senescent phenotype and cells became flatter and had a dendritic appearance. **C.** MU melanoma cells were counted under 40x magnification in 15 microscopic fields and it was found that there was a 10-fold increase in β-galactosidase positive cells when treated with T-oligo. These results indicate that T-oligo induces senescence in melanoma.

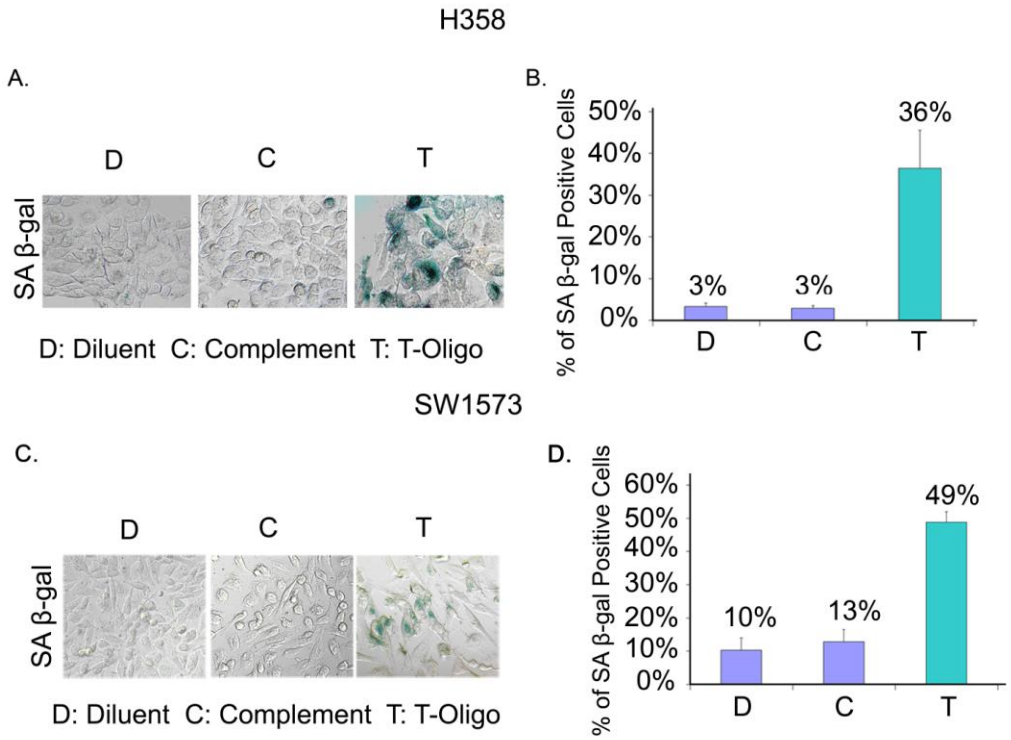

Figure 8. T-oligo Induces Senescence in H358 and SW1573 Cells. H358 and SW1573 cells were treated for 1 week with T-oligo, complementary oligonucleotide, or diluent, after which cells were stained for senescence-associated β-galactosidase which appears blue. **A.** Shows altered cell morphology in T-oligo treated H358 cells similar to senescent fibroblasts, and positive for β-galactosidase. **B.** H358 shows a 10-fold increase in β-galactosidase positive cells. **C.** Shows altered cell morphology in T-oligo treated SW1573 cells similar to senescent fibroblasts, and are positive for β-galactosidase. **D.** SW1573 shows a 4-fold increase in β-galactosidase positive cells. These results indicate T-oligo induces senescence in lung cancer.

4.1.7 <u>**T-oligo Induces Apoptosis in H358 Cells**</u>

Apoptosis has been observed in melanoma; therefore, we investigated if T-oligo induced apoptosis in H358 cells. H358 cells were treated with T-oligo or complementary oligonucleotide for 96 hours. Cells were harvested and analyzed with FACS scan. As seen in figure 9, T-oligo caused a 4-fold increase of apoptosis in H358 cells.

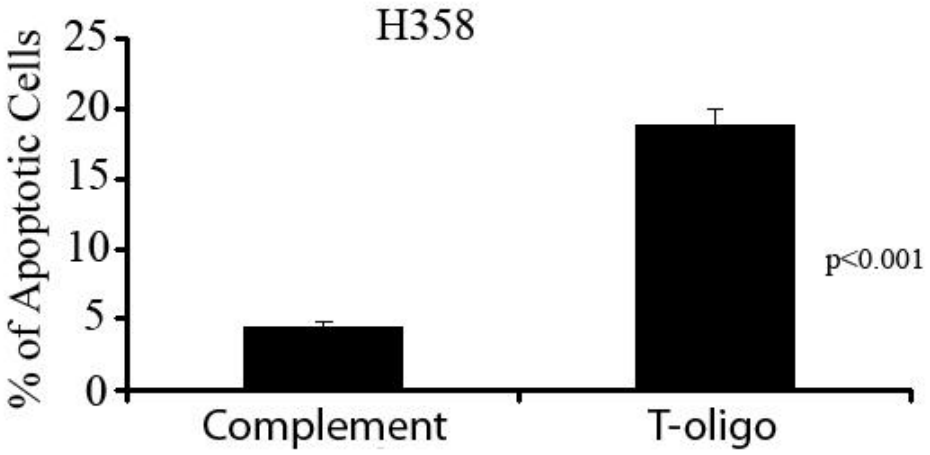

Figure 9. T-oligo Induces Apoptosis in H358 cells. H358 cells were treated with T-oligo or complementary oligonucleotide for 96 hours. Cells were harvested and fixed in 70% ethanol, and then stained with propidium iodide and analyzed with FACS scan and CellQuest software. Figure 9 shows a 4-fold increase of apoptosis in H358 cells when treated with T-oligo. $p < 0.001$.

4.1.8 **T-oligo Upregulates Proapoptotic Proteins p53, phospho (ser-15) p53, p21, and p73**

Seeing that T-oligo induced apoptosis in H358 cells, we wanted to determine what apoptotic proteins were modulated by T-oligo. H358 and SW1573 cells were treated with diluent, T-oligo, or complimentary oligonucleotide for 12-24 hours. Cells were harvested and western blotting was performed. As seen in figure 10, it was also observed in SW1573 cells that T-oligo upregulated p53 and phosphorylation of p53 at ser 15 at 12 hours. It was further found that T-oligo upregulated p21 and E2F1 at 24 hours. E2F1 transcirption factor is known to cooperate with p53 or p73 in induction of apoptosis and to induce a senescent phenotype in human fibroblasts in a p53-dependent manner (19). It was also determined that in H358 cells, which have a mutated p53 and non-functional p53 (147), T-oligo upregulated p73 at 12 hours. Densitometric analysis was performed using NIH ImageJ analysis and confirmed observations seen in figure 10.

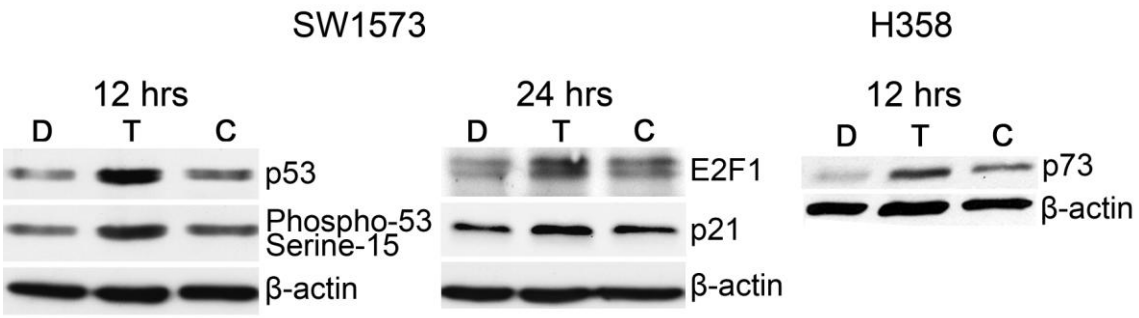

Figure 10. T-oligo Upregulates Proapoptotic Proteins in SW1573 and H358. SW1573 and H358 cell lysates were prepared after 12 and 24 hours of exposure to diluent, T-oligo, or complementary oligonucleotide, and subsequently immunoblotted. SW1573 cells treated with T-oligo exhibited upregulation of p53 and activated phospho-p53 (ser-15) at 12 hours. Upregulation of p21 was seen at 24 hours in addition to an upregulation of E2F1. H358 cells have a mutated p53, but exhibit upregulation of p73, a p53 homologue upon treatment with T-oligo for 12 hours. D:Diluent T:T-oligo C:Complement

4.1.9 **Proposed Pathway of T-oligo Induced Responses**

To elucidate how T-oligo induces senescence and apoptosis, our laboratory identified proteins that bind to T-oligo. Cancer cells were treated with biotinylated T-oligo or complementary oligonucleotide. Subsequently, T-oligo associated proteins were immunoprecipitated from cell lysates with streptavidin beads and further analysis showed three unique groups of proteins. Mass spectrometry was used to identify these proteins which were determined to be Pur-β, hnRNP C1/C2, and Msi-1. These are potential target proteins which increase senescence and apoptosis. Following identification of proteins that bind to T-oligo using literature, Ingenuity Pathways Analysis and other pathways software were used to identify how T-oligo could mediate modulation of its associated proteins. Figure 11 is a proposed pathway developed in our laboratory in which T-oligo may induce its anti-cancer effects through T-oligo associated proteins. The following experiments in 4.1.10 seek to confirm the proposed pathways.

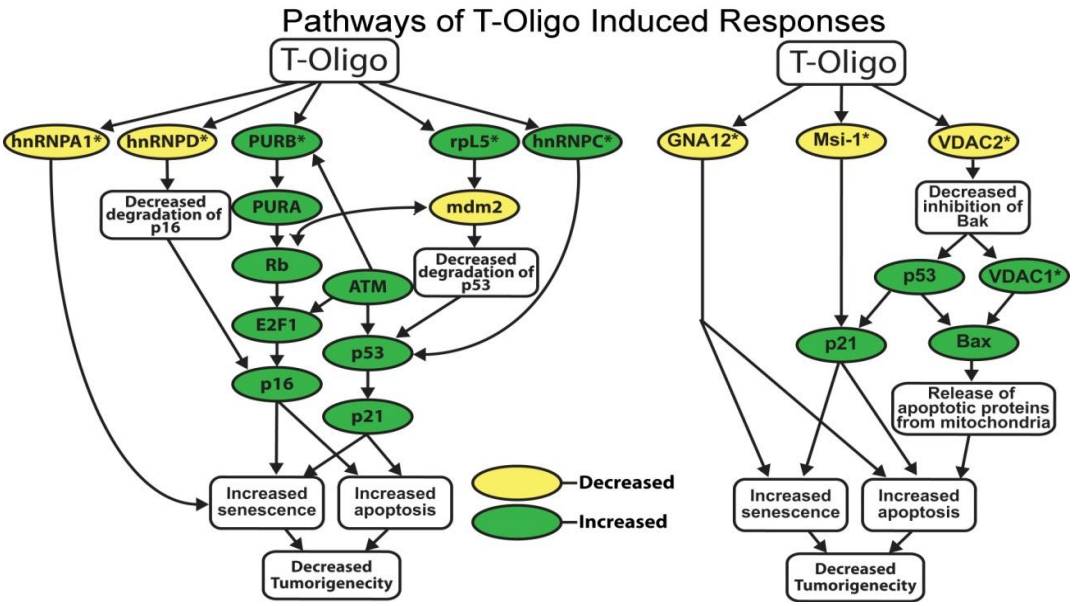

Figure 11. Pathways of T-oligo Induced Responses. A literature search was performed to identify how T-oligo might modulate these T-oligo associated proteins.

4.1.10 <u>**Effect of T-oligo on T-oligo Associated Proteins in Melanoma and Lung Cancer**</u>

hnRNP C and Purβ, which are involved in anti-cancer responses, bind to T-oligo. We wanted to investigate whether T-oligo could modulate hnRNP C and Pur-β in melanoma and lung cancer. Another protein found to bind to T-oligo, Msi-1, was also examined. Msi-1 is known to play a role in anti-cancer responses (148). MU and MM-AN melanoma cell lines were treated with diluent, complementary oligonucleotide, or T-oligo for 6, 18, 24, and 48 hours respectively. Cells were harvested and analyzed using immunoblot procedures. As shown in figure 12, it was observed that T-oligo upregulated Pur-β and hnRNP C at 18, 24, and 48 hours respectively. In MU melanoma cells, it was found that T-oligo downregulates Msi-1 at 6 hours and upregulates hnRNP C at 6 hours. Densitometric analysis was performed using NIH ImageJ analysis and confirmed observations seen in figure 12. H358 and SW1573 cells were treated for 18 and 24 hours with diluent, T-oligo, or complementary oligonucleotide. Cells were harvested

using immunoblot procedure. It was found that T-oligo upregulates hnRNP C and Purβ in H358 at 18 hours, and upregulates hnRNP C in SW1573 at 18 and 24 hours as seen in figure 13. Densitometric analysis was performed using NIH ImageJ analysis and confirmed observations seen in figure 13.

To summarize T-oligo upregulated several T-oligo-associated proteins which have a role in senescence, growth, and telomere biology. These studies confirm their potential involvement in T-oligo mediated responses.

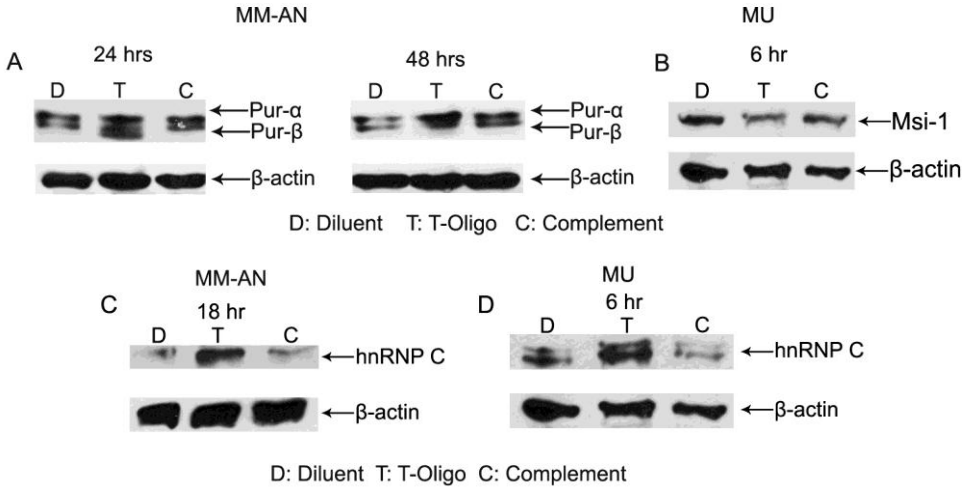

Figure 12. T-oligo Modulates hnRNP C, Purβ, and Msi-1 in Melanoma. Cells were treated with diluent, T-oligo, or complementary oligonucleotide, and collected at the indicated times. Cells were then analyzed using immunoblot techniques. **A.** T-oligo upregulated Pur-β in MM-AN melanoma at 24 and 48 hours. **B.** In MU melanoma Msi-1 was downregulated at 6 hours. **C.** In MM-AN melanoma hnRNP C was upregulated at18 hours. **D.** T-oligo upregulated hnRNP C at 6 hours in MU melanoma cells.

Figure 13. T-oligo Upregulates hnRNP C and Purβ in Lung Cancer. H358 and SW1573 lung cancer cell lines were treated with diluent, T-oligo, or complementary oligonucleotide for 18 and 24 hours. Cells were harvested and analyzed using immunoblotting techniques. **A.** In H358 cells, hnRNP C was upregulated after 18 hours of treatment with T-oligo. **B.** In H358, it was found that treatment with T-oligo also upregulated Pur-β at 18 hours. **C.** In SW1573 cells, T-oligo upregulated hnRNP C at 18 and 24 hours.

4.1.11 **Effect of T-oligo in Reducing Tumorigenicity *in vivo***

To determine if T-oligo can be used as a cancer therapeutic, we examined the effects T-oligo had in reducing tumorigenicity in a mouse model. 5 million H358 and SW1573 lung cancer cells were injected subcutaneously into the flanks of nude mice. Tumors grew for one week and then were treated daily with T-oligo or complementary oligonucleotide for seven weeks. As shown in figure 14, T-oligo treated tumors were reduced significantly in size. Statistical analysis was performed indicating that T-oligo reduced tumors by 4.3-fold in H358 ($p<0.0001$) and 5.6-fold in SW1573 cells ($p<0.001$).

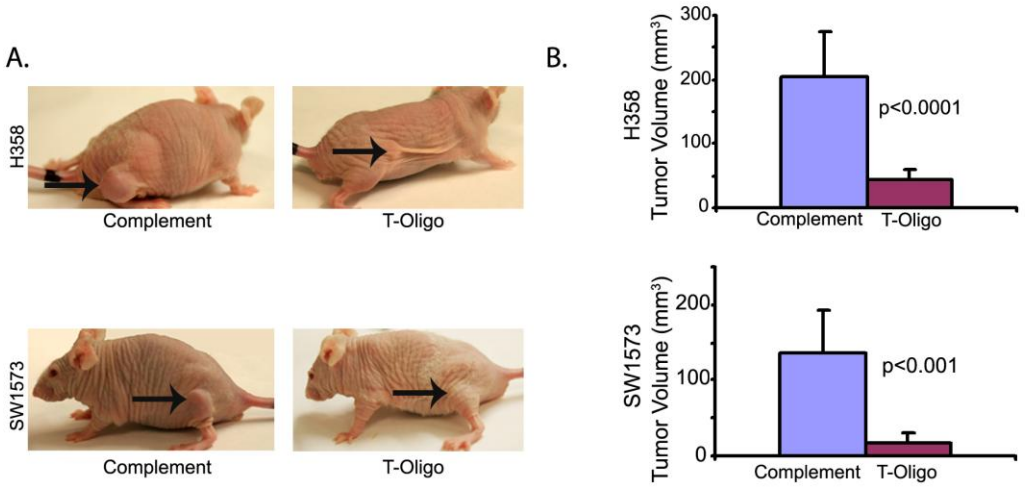

Figure 14. T-oligo Reduces Tumorigenicity in Mice. H358 and SW1573 lung cancer cells ($5x10^6$) were injected subcutaneously into the flank of nude mice. Tumors were allowed to grow for one week and then treated daily with T-oligo or complementary oligonucleotide in PBS for seven weeks. **A**. Mice treated with T-oligo had either no tumors or very small tumors. **B**. T-oligo reduced tumor volume by 4.3 and 5.6-fold in H358 and SW1573, respectively.

4.1.12 **Effect of T-oligo on Senescence-Associated Proteins *in vivo* and *in vitro***

To determine if T-oligo modulates senescence-associated proteins *in vivo* and *in vitro*, known senescence proteins p21, p27, and p33 which induces senescence were examined. Tumors grown in nude mice were excised and preserved for analysis of senescence-associated proteins. Paraffin sections of SW1573 tumors were prepared following the protocol described in section 3.4.2. Sections were stained using a M.O.M. kit (Vector) and stained with p21, p27, and p33 antibodies. As shown in figure 15, tumor sections treated with T-oligo show upregulation of p21, p27, and p33, demonstrating that T-oligo induces senescence *in vivo*.

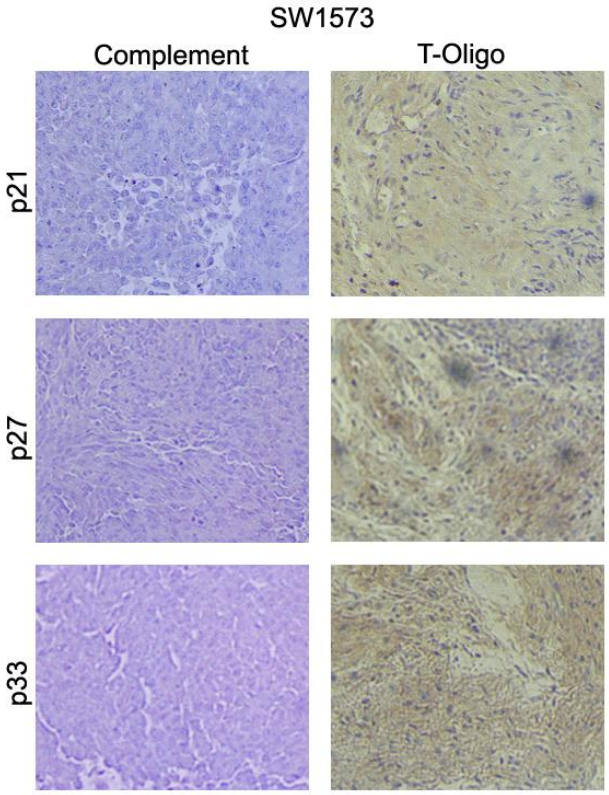

Figure 15. T-oligo Induces Senescence-Associated Proteins *in vivo*. SW1573 lung cancer cells ($5x10^6$) were injected into the flank of nude mice. Tumors were allowed to grow for one week and then treated daily with T-oligo or complementary oligonucleotide in PBS for seven weeks. Paraffin sections of SW1573 were used and prepared following standard immunohistochemical procedures. Tumor sections were stained using M.O.M. kit (Vector) with p21, p27, and p33 antibodies. As seen, tumor sections treated with complementary oligonucleotide showed no induction of senescence associated proteins. However, tumors treated with T-oligo show induction of p21, p27, and p33 as indicated by the light brown staining. This demonstrates that T-oligo induces senescence-associated proteins *in vivo*.

As previously seen in figure 8, T-oligo induces senescence in H358 and SW1573 cells, we wanted to determine which senescence associated proteins T-oligo was modulating. H358 and SW1573 cells were treated with diluent, T-oligo, or complementary oligonucleotide for 48-96 hours. As seen in figure 16, T-oligo induced upregulation of p21 and p27 at 72 hours, and p33 at 96 hours in SW1573 cells. In H358 cells, T-oligo induced upregulation of p33 at 48 and

96 hours, and p27 at 96 hours. Densitometric analysis was performed using NIH ImageJ analysis and confirmed observations seen in figure 16.

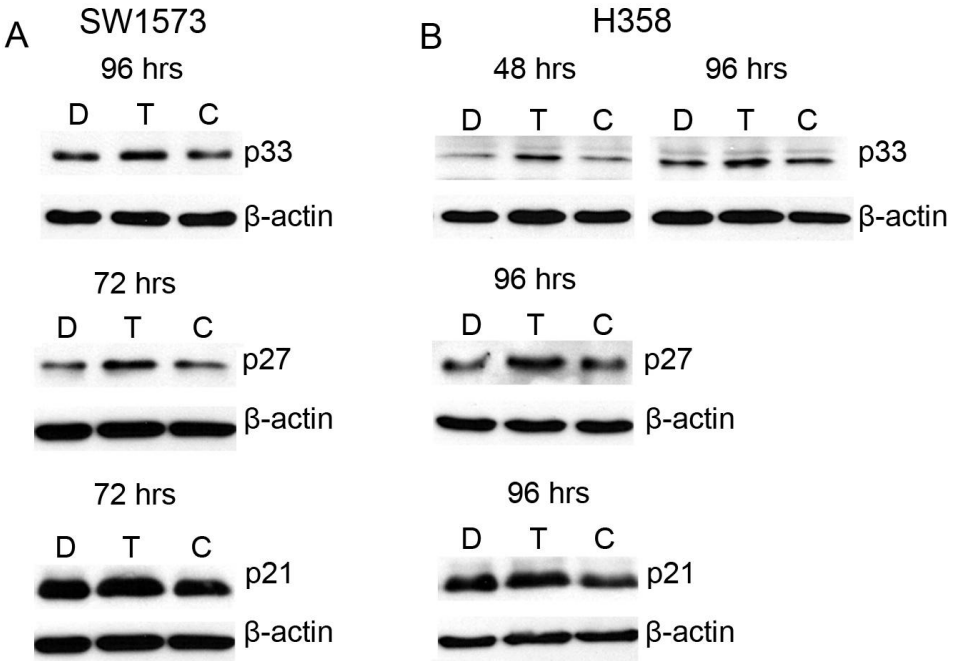

Figure 16. T-oligo Upregulates Senescence-Associated Proteins in SW1573 and H358. H358 and SW1573 NSCLC cell lysates were evaluated for a change in expression of senescence inducing proteins p33, p27, and p21. **A.** SW1573 cells showed a significant increase in expression of p33 at 96 hours only, and increase in p27, and p21 at 96 hours. **B.** Treatment with T-oligo increased the expression of p33 at 48 and 96 hours, and increased the expression of both p27, and p21 at 96 hours in H358 cells**.**

5. DISCUSSION

5.1 Overall Rationale

T-oligo has been shown to induce anti-cancer responses such as apoptosis and senescence in various types of cancer cell lines (9, 16, 17, 21, 22, 144, 145). As lung cancer and melanoma are two of the deadliest cancers in the U.S. (1), we wanted to investigate the effect T-oligo had on these two cancers, particularly in inducing senescence, apoptosis, and inducing their respective proteins to gain a better understanding of the mechanism of T-oligo. We were also interested in examining if T-oligo treatment modulates proteins associated with it. We further wanted to examine the potential of T-oligo as a cancer therapeutic by testing T-oligo's effects *in vivo*.

5.2 Effect of T-oligo on NBEC

Before testing to study whether T-oligo induces senescence and apoptosis in lung cancer, we first tested the effect T-oligo has on NBEC to determine if T-oligo would induce anti-cancer responses in non-cancerous normal bronchial epithelial cells. Figure 1 demonstrates that when NBEC were treated with T-oligo, complementary oligonucleotide, or diluent, T-oligo did not inhibit the proliferation of the NBEC at 72 and 96 hours and its effects were comparable to those observed in complementary oligonucleotide or diluent treated cells. It was determined that there was no statistically significant difference between the three treatment groups ($p>0.05$).

After analyzing the effect of T-oligo on the growth of NBEC, the effect of T-oligo in inducing apoptosis was examined. Figure 2 demonstrates that treating NBEC with T-oligo did not inhibit apoptosis at 48, 72, and 96 hours. No significant differences ($p>0.05$) in induction of

apoptosis were found when comparing effects of diluents, T-oligo, and complementary oligonucleotide treatments.

Figure 3 shows the effect of T-oligo in inducing senescence in NBEC. It was found that treating cells for seven days with T-oligo did not induce a statistically significant increase in senescence in NBEC when compared to the diluent and complement treated cells ($p>0.05$).

Having determined that T-oligo did not inhibit proliferation and induce apoptosis and senescence in NBEC, we examined whether T-oligo had any effect on p53 and p21. p53 targets many genes and has a key role as a tumor suppressor to block cell cycle progression and/or to induce apoptosis in response to cellular stresses such as DNA damage (149). p53 also induces G_1 arrest through activation of transcription of the cyclin-dependent kinase inhibitor p21, which also mediates cellular senescence (149). Thus, activation of p53 and p21 are excellent markers for DNA damage responses and induction of senescence. Figure 4 shows that at 12 and 24 hours T-oligo did not modulate p53 or p21. These results confirm our previous results where we show that T-oligo did not inhibit proliferation and did not induce DNA damage responses such as apoptosis and senescence in NBEC.

These findings demonstrate that T-oligo has no negative effects on NBEC, and are encouraging as T-oligo could prove to be a useful therapy in the fight against cancer. These results validate other studies by Longe et al. who showed that T-oligo did not induce apoptosis in normal lymphoid cells (22, 144), and Puri et al. who demonstrated that T-oligo did not induce apoptosis in normal human melanocytes, while inducing apoptosis in 65% of melanoma cells (9).

To our knowledge, this is the first study which demonstrates that T-oligo has no effect in NBEC. Since T-oligo has the ability to spare normal cells from cytotoxic effects, while inducing senescence and apoptosis in cancer cells, this suggests T-oligo could be a potential cancer therapeutic with very few side effects.

5.3 **Effect of T-oligo on Clonogenecity in Lung Cancer and Melanoma Cell Lines**

Clonogenic capacity is considered a good marker for predicting cancer treatment responses (17, 150)(146), and Roninson et al. showed that loss of clonogenic capacity is an indicator of senescence (146). Prior studies involving T-oligo treated fibrosarcoma cells demonstrated a pronounced reduction in clonogenic capacity (17). Thus, we wanted to verify these findings in our NSCLC and melanoma models. In figure 5, we show that T-oligo treated H358 cells, which have a homozygous deletion of p53 (151), exhibited a 12-fold reduction of clonogenic capacity, suggesting that T-oligo may reduce clonogenic capacity through other pathways such as the pRb pathway; while T-oligo treated SW1573cells, having wild-type p53, exhibited a 3-fold reduction of clonogenic capacity. Melanoma cell lines, MU, which has wild-type p53, and MM-AN, which has undetectable levels of p53, exhibited a 3.7-fold decrease and a 4.4-fold decrease of clonogenic capacity, respectively. Since clonogenic capacity is considered a reliable marker for predicting cancer treatment response, these results indicate that T-oligo could be effective against lung cancer and melanoma by mediating senescence in the presence/absence of mutant/wild type p53.

5.4 **Effect of T-oligo Inducing Senescence in Melanoma and Lung Cancer Cells**

Senescence is an important DNA damage response pathway and a vital modality for prevention and treatment of cancer (146). Previous evidence has shown that senescent cells are known to exhibit p53 activation and induction, upregulation of the cyclin-dependent kinase inhibitors p21 and p16, and subsequent reduction in pRb phosphorylation (which is the active tumor suppressing form of pRb) (16).

To see if exposure to T-oligo could induce senescence in the melanoma cell line MU, and lung cancer cell lines H358 and SW1573, cells were treated with T-oligo or a complementary oligonucleotide. It was observed that cells exposed to T-oligo exhibited an altered morphology and increased in size (figure 7) similar to that of senescent fibroblasts (16). Following this observation, cells were stained for the presence of senescence associated β-galactosidase, a senescence specific marker (152). MU melanoma cells treated with T-oligo showed a 10-fold increase in cells positive for senescence specific β-galactosidase. Similar results were observed in lung cancer cells, where H358 and SW1573 cells treated with T-oligo showed a 12-fold increase and 4-fold increases in cells positive for β-galactosidase respectively (figure 8). Interestingly, β-galactosidase staining intensity was greater in H358, which is deficient in p53 (9) compared to SW1573 and MU cells, suggesting a strong link between the p53-independent mechanism of DNA damage responses and senescence. Studies within p53-deficient fibroblasts and melanoma cells indicate that these mechanisms could be through p73 or pRb (17, 19).

Furthermore, as seen in figure 16, immunoblotting of H358 and SW1573 cell lysates confirmed the roles of p21, p33, and p27 which activate specific DNA damage responses in T-

oligo mediated senescence in NSCLC in culture. p27 functions by causing cell cycle arrest in the G_1phase (153), by inhibiting the catalytic capacity of CDK4, by preventing CDK4 from phosphorylating pRb, thus causing cell cycle arrest in the G_1 phase (153). p33 is downstream of the p53 tumor-suppressor and causes cell cycle arrest and senescence upon upregulation of p53 (16). p21 is also upregulated by p53 signaling and functions in a similar manner as p27, causing cell cycle arrest in G_1 (154). Previous studies have shown that T-oligo induced p27, p21, and p33 in human fibroblasts (16) which we have confirmed in our studies on lung cancer.

Interestingly, β-galactosidase staining intensity was greater in H358 cells, which are deficient in p53 (9) compared to wild-type p53 SW1573 and MU cells. This suggests a strong link between p53-independent mechanisms of DNA damage responses and senescence. Studies within p53-deficient fibroblast and melanoma cells indicate that these mechanisms could be through p73 or pRb (17, 19). This coincides with our study where we show upregulation of p53 and induction of senescence in SW1573 (figure 10) with wild-type p53, and upregulation of p73 (figure 10) and induction of senescence through p73 in p53 deficient H358 cells. Our findings further validate these results that T-oligo may mediate senescence through p53 or through some other pathway (possibly the Rb or p73 pathway). However, future studies that knock down p53, p73 and Rb would be needed to confirm that T-oligo works through these pathways.

5.5 **Effect of T-oligo in Inducing Apoptosis and Induction of DNA Damage Response Proteins in NSCLC**

As seen in figure 9, T-oligo induced a 4-fold increase in apoptosis in H358 cells. Apoptosis is proposed to mediate the malignancy-specific effects of T-oligo through activation of multiple independent DNA damage pathways including ATM, E2F1, p73, and p53 (9). Our results (figure 10) show that administration of T-oligo induces DNA damage responses in SW1573 (WT p53) by upregulation of DNA damage responses proteins p53, p21, and E2F1, and in H358 (mutated p53) by upregulation of p73. These results are consistent with previous findings (16, 17) that suggest an important role for a p53-dependent DNA damage responses. Eller et al. have found that T-oligo could also mediate apoptosis through the p53 homologue, p73, in MM-AN melanoma cells (19). They also found that in MM-AN cells dominant negative p73 decreased T-oligo induced apoptosis by 50% (9). Our results substantiate these findings of T-oligo mediating p73/p53 dependent mechanism of induction of apoptosis and senescence in NSCLC.

5.6 **Effect of T-oligo on T-oligo Associated Proteins in Melanoma and Lung Cancer**

As shown in figures 12 and 13, it can be observed that T-oligo upregulates Pur-β and hnRNP C at several different time points in lung cancer and melanoma cells, suggesting that telomere-associated proteins may play an important role in mediating T-oligo induced responses. Of particular interest are hnRNP proteins, and Pur-α/β. Pur-β may increase senescence through the formation of a heterodimer with Pur-α, which then binds Rb, leading to increased senescence and apoptosis (155). Proteins from the hnRNP family are RNA binding proteins that complex with heterogenous nuclear RNA (hnRNA). Members of the hnRNP family have been implicated

in many aspects of mRNA maturation/turnover and in telomere and telomerase regulation (156). DNA damage, which T-oligo is mimicking, increases hnRNP C activity, which upregulates p53 activity and causes apoptosis in tumor cells (157). Future studies could determine the role of these proteins in mediating T-oligo induced DNA damage responses.

5.7. **T-oligo Reduces SW1573 and H358 Tumors *in vivo***

In figure 14, we show that lung cancer cells H358 and SW1573 when treated with T-oligo reduced tumor size in nude mice by 4.3-fold and 5.6-fold respectively when compared to cells treated with the complementary oligonucleotide. Further tumor analysis demonstrated that daily injections of T-oligo, but not the complementary oligonucleotide, can reduce NSCLC tumor volume by strong induction of senescence as evidenced by β-galactosidase staining and expression of p33, p27, and p21. These *in vivo* results are the first to demonstrate T-oligo induced senescence *in vivo* and are in concordance with our *in vitro* results suggesting that the induction of senescence may be an important route by which T-oligo prevents tumor growth or metastatic potential (9).

The following studies confirm the effects of T-oligo in other tumor xenograft models. In a study by Yaar et al. on MCF-7 metastatic breast cancer tumors treated with intravenous T-oligo, 75% of T-oligo treated mice lived through 30 weeks without developing weight loss or pathological symptoms (8, 22) in comparison to diluent treated mice which survived for only 6 weeks. In studies on melanoma by Puri et al., it was found that pre-treatment of melanoma cells with T-oligo displayed an 85% reduction in metastatic tumor volume (9). They also found that intratumoral T-oligo treatment reduced tumor growth in pre-existing tumors by 84% compared

with diluent or control oligonucleotide (9). In another study by Coleman et al., using a melanoma xenograft model, intravenous T-oligo decreased tumor volume by 60%, and also reduced total tumor microvascular density and functional vessels density by 80% (20). In a study of a glioma mouse xenograft model treatment with T-oligo reduced size of glial cell tumors and it was found that four of six mice treated with T-oligo survived to day 72, whereas mice treated with diluent or complementary oligonucleotide cells died by day 42 (8, 158). Interestingly, mammary tumors that were treated with T-oligo combined with 3 Gy irradiation were almost completely eradicated (159). Our *in vivo* studies and published investigations indicate that future work on T-oligo should be focused on increasing the clinical relevance of telomere oligonucleotides as novel cancer therapeutic.

5.8 **Conclusion**

This study sought to investigate T-oligo's ability to induce DNA damage responses in lung cancer and melanoma. It also investigated the ability of T-oligo to induce DNA damage response proteins in cancer cells, and the effects of T-oligo in a xenograft mouse model. We found that T-oligo did not reduce proliferation, induce apoptosis or senescence in NBEC, nor did it upregulate key DNA damage response proteins such as p53 and p21 in NBEC. It was found that T-oligo was able to reduce clonogenic capacity in lung cancer and melanoma cells lines. Reduction of clonogenic capacity is an indicator of senescence (146). It was further found that after treatment of MU melanoma cells and lung cancer cell lines with T-oligo, they exhibited a senescent morphology and were positive for senescence-associated β-galactosidase, a common marker of senescence. It was also demonstrated that T-oligo associated proteins such as hnRNP

C and Pur-β were upregulated by T-oligo. The discovery that T-oligo modulates hnRNP C and Pur-β is interesting and requires future investigation of their roles in anti-cancer responses.

This study also demonstrates the feasibility of using T-oligo as a cancer therapeutic. It was found that T-oligo is able to reduce tumorigenicity in a lung cancer xenograft, and further established that important senescent associated proteins p21, p27, and p33 were upregulated *in vivo*. As this study has highlighted the ability of T-oligo to induce senescence only in malignant cells, and since T-oligo has no cytotoxic effects on normal cells, these results suggest that T-oligo could be a novel molecularly targeted cancer therapeutic. Further research must be conducted to see how T-oligo could be stabilized and delivered to ensure its full potential as a standard treatment for cancer.

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