ENHANCING THE STABILITY AND FUNCTION OF THE TUMOR SUPPRESSOR PROTEIN P53

By

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Gene therapy is currently used not only to correct the cellular errors associated with tumorigenesis, but to also provide enhanced cellular functions against tumor development. Over half of all known cancers have either a loss of function or inactive tumor suppressor p53. In response to various stress or oncogenic signals, the tumor suppressor protein p53 mediates cellular response via induction or repression of certain genes. Tumor suppressor p53 functions mainly as a transcription factor controlling genes involved in DNA repair, senescence, cell cycle arrest, and most importantly, apoptosis. Accumulation of p53 in the nucleus is necessary for its transcriptional activities; however, inactivation of the transcriptional activity of p53 has been shown to be associated with tumor formation. Correspondingly, loss of structural stability and thermodynamically destabilized p53 mutants are also associated with tumorigenesis. We have analyzed the literature on the stability and function of p53. Several research groups focus on thermodynamically stabilized p53, whereas others primarily targeted the restoration and enhancement of the transcriptional activity of p53. Here, we use a holistic approach by combining both concepts. We have successfully constructed plasmids extracted from literature, including the thermodynamically stabilized p53_N239Y, and the transcriptionally active p53_S46F and p53_S121F. We have also produced novel combinations, p53_S46F_N239Y and p53_S46F_S121F for comparison and transcriptional activity analysis. Furthermore, we have identified two p53 mutants that are thermodynamically stable and with enhanced transcriptional activity compared to wild type p53.
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INTRODUCTION

After sixty years of laboratory research and collaborative biomedical efforts, a clear model for the origin and nature of cancer has been well established. Three models for the origin of cancer have been demonstrated. The first model portrays cancer's origin as a complex process involving changes in cellular behavior—that is, epigenetics. Epigenetics has not only revealed the molecular dynamics and phenotypic characteristics of many cancers, but the changes in cellular behavior and differentiation pathways have also become comprehensible [1]. Virologists, on the other hand, have delineated an alternative model for the origin of cancer.

Distinct cancer-causing viruses with both DNA and RNA have been demonstrated to trigger tumor formation in both humans and different types of animals. Viruses such as the Epstein-Barr Virus, SV40, Human T-Cell Leukemia Virus, Shope Papilloma virus, Kaposi and Rous Sarcoma Virus have been shown to initiate tumor formation [1, 2]. The last and most noteworthy model of cancer's origins was the perspective that cancer is a result of a genetic disorder. This last model linked the relationship between changes in DNA primarily generated by carcinogens and mutagens with an apparent neoplasm [1, 2]. The discovery of these cancer-causing genes, oncogenes, also found inside viruses, united both the concepts of cancer-causing viruses and genotoxically produced oncogenes. Cancer genetics, thus, ubiquitously gained considerable attention and analysis.

The discovery of oncogenes brought about a tremendous shift in cancer research. As the race and search for identifying oncogenes intensified in the early '70's, mutagenic carcinogens and their mechanisms of tumor formation remained unclear. Multistep tumorigenesis, a process by which cells undergo various stages of alteration to become
malignant, was initially proposed by Carl O. Nordling [1]. Multistep tumorigenesis was later verified by several researchers including Peter Armitage and Richard Doll [1, 3-5]. Alfred Knudson furthermore demonstrated that retinoblastoma was caused by at least two independent mutations as cells transformed from a normal cell to a cancerous cell [6, 7]. A single oncogene, unaccompanied with any other genetic mutation, was demonstrated to be insufficient to initiate neoplastic behaviors [1, 3].

Interestingly, similarities were found analyzing the succession of mutations required for the formation of many cancers. As a cell undergoes various stages over time, cancer cells were found to accumulate two significant types of mutations. Subsequent to a primary mutation that produced an oncogene, the second mutations usually affected genes that controlled cellular proliferation. Key mutations were identified in genes that negatively controlled cellular proliferation, named tumor suppressor genes (TSGs). Mutations in TSGs were found to be responsible for the uncontrollable cellular proliferation seen in all cancer cells [1, 3].

A single normal function of TSGs was in some cases sufficient enough to restore some of the normal function of cellular proliferation; however, a loss of function of both normal genes, a recessive trait, severely reduces tumor suppression activity of TSGs [1]. The discovery of TSG’s has been tremendously significant in the progress of cancer therapeutics. Eliminating or suppressing cancers thus requires selective and specific therapeutics that not only target the basis of the tumor formation, but methods that also correct the cellular errors associated with uncontrollable cellular proliferation. Current research in our lab, Dr. Carol Lim’s Lab, is focused on correcting these cellular errors. The Lim Lab has previously shown that in chronic myelogenous leukemia cells (CML),
change in location of the normally oncogenic Bcr-Abl protein from the cytoplasm to nucleus converts Bcr-Abl into an apoptotic factor [8]. Bcr-Abl is itself an oncogene and is a product of the translocation of chromosomes 9 and 22. One goal of our lab is to use a ligand inducing “protein switch” that moves Bcr-Abl from the cytoplasm to nucleus, thus converting the oncogene to an apoptotic factor via cytoplasmic depletion and nuclear trapping of Bcr-Abl proteins [8-10].

Furthermore, and more holistically, we are simultaneously targeting the tumor suppressor protein p53 to the nucleus and the mitochondria for cancer therapy. The human TP53 gene is one of the most frequently altered genes found in all human cancers (Figure 1). Loss of transcriptional activity and structural instability (temperature sensitive mutants) are associated with tumorigenesis. We have analyzed the literature on the stability and function of p53. Several research groups focus on thermodynamically stabilized p53, whereas others primarily targeted the restoration and enhancement of the transcriptional activity of p53. Here, we investigate both the thermodynamically stabilized as well as the transcriptionally active mutant p53 for the capabilities to transcribe the p53-promoter sequence known to correlate with apoptosis. Furthermore, we have generated p53 constructs with both of these combinations. Is the most optimal p53 tumor suppressor protein for future cancer therapeutics a transcriptionally active p53, a thermodynamically stabilized p53, or a combination of both?

The Tumor Suppressor Protein p53

For more than twenty-five years, the tumor suppressor protein p53 has remained the most complex and researched protein in all of cancer therapeutics [11]. The function and integrity of p53 to prevent cancer structure is dependent on the quantity, structure,
and activity of the p53 proteins in the cell (Figure 1). In response to various stress or oncogenic signals, such as DNA damage or hypoxia, the tumor suppressor protein p53 mediates cellular response via induction or repression of certain genes [12]. Tumor suppressor p53 functions as a transcription factor for many genes controlling DNA repair, senescence, cell cycle arrest, metastasis inhibition, and most importantly, apoptosis [12, 13]. Accumulation of p53 in the nucleus is necessary for its transcriptional activities; however, p53 can also induce apoptosis via transcriptionally-independent pathways [12].

![WORLDWIDE DISTRIBUTION OF CANCERS AND p53 MUTATIONS](image)

**Figure 1: Tumor Suppressor p53 Mutations and Cancer.** Greater than fifty-percent of all cancer have a TP53 mutation. TP53 mutations are common in many of the cancers seen both in developing and developed countries. Database maintained at the Institut Curie, Paris France. Reprinted with the permission of Dr. Soussi, website, http://p53.curie.fr, July 2010.

Through the induction of p53, apoptosis can occur via two major pathways, extrinsic and intrinsic. The extrinsic pathway involves three trans-membrane death receptors (Fas, DR5, and PERP) and caspase-8 mediated apoptotic cascade [14]. The
intrinsic pathway, on the other hand, is through the mitochondria and Bcl-2 pro-apoptotic family. Centered on the Bcl-2 family, Bax, Noxa, and PUMA are the pro-apoptotic mitochondrial members [14]. In addition, p53 itself can induce apoptosis by acting directly at the mitochondria [15]. Mitochondrial permeabilization occurs as p53 interacts with Bcl-xL and Bcl-2 to change the mitochondrial membrane potential and release cytochrome c [15].

The TP53 gene is located on human chromosome 17p13 [13, 16, 17]. Tumor suppressor p53 contains 393 amino acid residues, encompassing three distinct regions (Figure 2) [18]. The first region is the N-terminus region with several sub-domains. Of most significant sub-domains in the N-terminus region are the transactivation domain (TAD), and the proline-rich domain (PRD) [12, 13].

**Figure 2: Full Length p53.** The p53 protein holds three distinct regions with several sub-domains in each region. The N-terminus region (residues 1-93), DNA Binding Domain (residues 94-293), and the C-terminus region (residues 294-393). Reprinted with the permission of Mohanad Mossalam, Lim Lab.

The N-terminus region of p53 is approximately 93 amino acid residues in length and the majority of its structure is naturally unfolded [19]. The TAD region (residues 1-
60) is both a negative regulator and activator of transcription activity of p53 via MDM2 and p300/CBP protein interactions, respectively [2, 12]. The N-terminus region that encompasses the TAD section interacts with various proteins including MDM2, a ubiquitin ligase protein that is responsible for tagging p53 for degradation (ubiquitination) [2]. Depending on tissue type, normal half-life of p53 is 6-30 minutes and is auto-regulated by the MDM2 protein [2]. Loss or inhibition of MDM2 through up-regulation mechanisms produced by stress signals significantly extends the half-life of the p53 protein from minutes to hours [2].

Conversely, the proline-rich domain (PRD) (residues 60-93) contributes to the apoptotic function of p53 [12, 13]. This region may contribute to apoptosis in a DNA-damage dependent manner [11-13]. The PRD is a region that has five copies of PXXP (P=proline; X=amino acid residue) and interacts with the co-activator protein p300 [13]. Activation of p53 via the N-terminus region, especially in the PRD region, induces or inhibits the transcription of more than 150 genes [11, 20].

Subsequent to the N-terminus region is the evolutionarily conserved region designated as the DNA binding domain (DBD). The DBD region is the most significant region maintaining p53 structure and function against various different cancers. Although p53 is transcriptionally active as a tetramer, the DBD region consists of the basic region required for DNA binding. The DBD consists of an “immunoglobulin-like β-sandwich” with several loops stabilized by a zinc ion [12]. The folded DBD region is not only responsible for sequence-specific DNA binding, but also protein-protein interactions [11]. Furthermore, the DBD region recognizes and binds to a double-stranded DNA sequence in the nucleus. The DNA sequence of each strand consists of a single 10 base
pair region, producing a palindrome. The palindrome segment is generally a -RRRCWWGYYY- region, where R represents a purine (A, G), Y represents pyrimidines (C, T), and W represents either an A or a T [11-13]. In almost all cancers with a mutated p53, the probability that a missense mutation resides within the DBD of p53 is greater than 80% (Figure 3) [21]. The p53 DBD is quite unstable with a denaturing melting temperature (Tm) of approximately 42°C [18]. A significant number of p53 DBD mutations are responsible for the thermodynamically destabilized protein that unfolds at body temperature [18].

![SOMATIC MUTATIONS - Codon distribution of single base substitutions (n=22356)](C:\\ICAR TP53 Database, R15 release, November 2010)

**Figure 3: Most Common Mutations.** The six mutations above have been designated the ‘hotspot’ mutations. These six mutations (residues 175, 248, 273, 245, 249, and 283) have the highest frequency of occurrence in cancers. Majority of mutations are located in the DBD region. Reprinted with the permission of Dr. Olivier, website, http://www-p53.iarc.fr/, November 2010.

Lastly, the C-terminus region of p53 is primarily responsible for p53 tetramerization [12]. The tumor suppressor protein p53 is only active as a tetramer in the nucleus. The C-terminus is also a regulatory region consisting of three nuclear localization signals (NLS) for the import of p53 into the nucleus [12, 13]. The tetramerization domain itself encompasses a nuclear export signal (NES) that is masked
when p53's homotetramerizes [13]. The natively unfolded region of the C-terminus is also the location where MDM2 is thought to tag lysine residues (ubiquitinates) for proteasomal degradation of p53 [22]. Furthermore, the C-terminus region is considered nonessential for p53 stability but is thought to “fine-tune” p53 transcriptional activity [23].

Understanding the structural and molecular complexity of p53 has provided insight into how we can develop cancer specific therapy. Selective and specific targeting has been described as the ultimate aim of cancer research and therapy [24]. Understanding and targeting the causal events that transform a normal cell into an invasive malignant cell has been shown to be promising, especially with the current computational advancements in research. Chronic myelogenous leukemia (CML) and breast cancer (BC), via targeting Bcr-Abl selective tyrosine kinase inhibitor and HER2 down-regulating targeting drugs, respectively, remain successful examples of selective and specific drug targeting [24]. Furthermore, tumor suppressor p53 has been an effective gene therapy target method against cancers associated with the lungs, head and neck squamous cell carcinoma, and ovaries [25-27].

TP53 and Cancer

According to the International Agency for Research on Cancer (IARC), their TP53 mutation database (www-p53.iarc.fr) encompasses more than 27,000 somatic mutations and 597 germ-line mutations. Of these mutations, over 2000 functional p53 mutant proteins were found. Furthermore, the majority of mutations found not only produced full length p53 proteins but also had various properties and functions. Over 75% of the mutations were reported as amino acid substitutions or missense mutations
Correspondingly, the database at the Institut Curie in Paris, France (http://p53.curie.fr/) has reported over 30,000 TP53 mutations with more than 1500 different types of p53 mutants [28]. Likewise, a majority of the mutations were found to be amino acid substitutions.

Greater than 80% of TP53 mutations reside between residues 100-300 of the DNA binding domain (DBD) of p53 [28, 29]. Breast cancer, the third most common tumor in the world, is the cancer our lab has selected for p53 mutant therapy. Breast cancer (BC) represents the most applicable therapeutic method for all cancers with TP53 mutation because the pattern of mutational events and restoration of p53 parallels the high frequency of missense mutations (Figure 5). Furthermore, mutations in the TP53 gene in BC patients have been shown to have the poorest prognosis in comparison to many other cancer types [21]. Correspondingly p53 mutation in BC is quite complex, especially during metastasis; therefore selective p53-targeted gene therapy is quite promising [30].

Figure 4: Mutation Types. A comparison of the most common somatic mutations seen in human cancers. Base-pair substitutions represent approximately 73% of all mutations in TP53. Reprinted with the permission of Dr. Olivier, website, http://www-p53.iarc.fr/, November 2010.
Breast cancer accounts for 23% of all types of cancers cases globally [31].
According to the American Cancer Society, breast cancer in women living in the United
States accounts for 25% of cancer cases, that is, one in four women with cancer.
Furthermore, in 2010, approximately 208,000 new cases of invasive cancers were
diagnosed and nearly 39,000 women died from breast cancer in the United States.
Although patients with non-invasive cancer have a significantly better treatment and
survival rate in comparison to patients with invasive cancers, the severity of the disease is
dependent on the stage of the cancer. The TNM staging system developed by the
American Joint Committee on Cancer (AJCC) is the most common method currently
used. The first phase of determination is based on letter coding T (tumor size and distance
from organ), N (lymph node connection), and M (metastases). After the initial phase, rise
in stages (0, I, II, III, and IV) correlates with increase severity. The uncontrollable
proliferation and the ability of breast cancer cells to metastasize has been the greatest
challenge to breast-conserving surgery, hormonal therapy, and chemotherapy [32]. The
p53 protein is not only anti-metastasis, but correcting the cellular errors in BC may
perhaps be the most effect method against all stages of breast cancer.

The most common ‘hotspot’ missense mutations of all cancers are located on
residues R175, R248, R273, G245, R249, and R283 (Figure 3) [12, 28]. Of the six most
common TP53 ‘hot spot’ mutations, breast cancer holds three of the six mutations with a
high frequency distinctively at residues 175, 248, and 273 (Figure 5). Each cancer type is
clearly specific and holds a distinctive p53 mutant with corresponding oncogenes;
however p53 mutants have been classified into two different categories. The first class of
mutations are those that affect the transcriptional activity of p53, identified as contact
mutations (CM) [12]. The ‘hot spot’ mutants R273C, R273H, R248W, and R248Q are contact mutations that remove or alter the ability of p53 to interact with essential DNA—that is, these mutants are deficient in their transcriptional abilities in the nucleus of the cell [12]. The second class of mutations as stated earlier are thermodynamically destabilized p53 that carry structural mutations (SM) and affect the overall architecture of p53 [12]. Examples of SMs are R282W, G245S, V143A, and R175H mutations present in various cancers such as lung, breast, colorectal, and prostate and liver cancer [28]. On the basis of these mutational classes and the instability of the p53 protein, restoration and reactivation of p53 require transcriptional reactivation as well as thermodynamic stabilization.

Figure 5: Breast Cancer. Of the six ‘hotspot’ mutations listed earlier for all cancers (residues R175, R248, R273, G245, R249, and R283), three are most common in breast cancer (R175, R248, R273). Reprinted by the permission from Dr. Olivier, website, http://www-p53.iarc.fr/, November 2010.

Restoration: Thermodynamically stabilizing p53
The stability of wild type p53 DBD region is 9.8 kcal/mol approximately at 10°C ($\Delta G_{D,N}^{H_2O}$ free energy of p53 unfolding in the presence of urea) [33, 34]. Several articles have highlighted the irreversible denaturing of p53 DBD region with increased temperature [35]. The DBD region becomes less stable with increased temperature which may lead to loss of function of the protein [35]. The normal human body temperature is 37°C and increases slightly with exercise, thus, the proposal of a ‘superstable’ (thermodynamically stabilized) p53 protein by various researchers. Furthermore, several oncogenic p53 mutants including R175H, C242S and R248Q are destabilized thermodynamically by 3.0, 2.9, and 1.9 kcal/mol, respectively, as compared to wild-type p53 $\Delta G_{D,N}^{H_2O}$ unfolding [35].

Crystal structures of mutant p53 proteins, p53_N239Y and p53_N268D, have been shown to increase the thermodynamic stability of p53 via hydrophobic packing and alternative hydrogen bonding, respectively [33]. Mutant p53_N268D increases the $\Delta G_{D,N}^{H_2O}$ by 1.21 kcal/mol (10.98 kcal/mol at 10°C ) while p53_N239Y increases it by $\Delta G_{D,N}^{H_2O}$ by 1.37 kcal/mol (11.14 kcal/mol at 10°C) [36]. Furthermore, a double mutant construct containing one cancer hot spot mutation G245S and thermodynamically stable p53_N239Y showed a restored activity of the p53 protein—p53_N239Y acts as a second-site suppressors for the cancer hot spot G245S and restores the function of p53 [34].

Additionally, p53 paralogs, p63 and p73, were found to have two mutations that can increase the stability of p53 by 17°C [12]. These two mutations are buried groups located on Y236F and T253I and increase stability of p63 and p73 by 1.6 kcal/mole approximately [12, 37]. Although this double mutation is stated to be additive in its contribution to thermodynamic stability, only the thermodynamic stability of the single
mutation Y236F mutant is known. This mutant, Y236F, holds a thermodynamic stability of 0.27 kcal/mole while the stability of p53_T253I remains unknown or unmeasured for its $\Delta G_{D-N}^{H,0}$ [34, 36]. Furthermore, all thermodynamically stable mutations listed in literature are naturally occurring amino acid substitutions isolated by comparing the stability of various p53s in 23 species [36]. We have therefore engineered the superstable p53_N239Y, p53_Y236F, and p53_T253I mutants to analyze their transcriptional activity which should correlate with p53 apoptosis. These superstable mutants should show enhanced transcriptional activity.

Reactivation: Enhancing Transcriptional Activity

Other super p53 mutants have been identified with enhanced transcriptional activity. Several articles have highlighted the correlation between transcriptional activity and apoptosis [38]. Mutations in the p53 proteins that affect the transcriptional activity usually strengthen, complement, or substitute bonds or different linkages in the p53 protein-DNA contact regions [12]. Key p53 residues (Lys-120, Ser, 241, Arg-248, Arg, 273, Ala-276, Cys-277, and Arg-280) either make direct contact with DNA or through a water mediated DNA contact [12].

Interestingly, a “121F mutant” with decreased MDM2 transcription and enhanced apoptotic ability was one of the first of ‘super’ p53s identified [38]. The 121F mutants demonstrated enhanced apoptotic ability and strong activation for the Rad (a muscle-specific GTPase) and PIR121 (an open reading frame) genes. Although two new genes not previously recognized as p53-targeted genes were identified, selective induction and sequence-specificity of p53 transcription was also observed [38]. Furthermore, 121F mutants were defective on the p2 promoter for the transcription of MDM2 by the auto-
regulatory mechanism of p53 [38]. Extended p53 half-life due to decreased MDM2 was linked to the increased apoptotic function of 121F mutants. Furthermore, 121F mutants are not cell type-specific and can induce apoptosis in cancer cells that are resistant to exogenous introduction of wild-type p53 [38]. Although defective in the p2 promoter for MDM2, the transcriptional activity on a p53 promoter itself remains unknown.

A mutation on the N-terminus region p53_S46F has also been identified as a 'super p53' for a number of p53-targeted genes [39]. p53_S46F was also demonstrated to have enhanced apoptotic ability in various cell lines including H1299, LC176 and A549 (all lung cancer cell lines), HepG2 (hepatoblastoma), and the colon cancer cell lines, LS174T and HCT116 [39]. Furthermore, increased transcriptional activity of proteins involved in the mitochondrial apoptotic pathway including Noxa and two other p53-mediated transcript proteins, p53AIPl, p53RFP were observed [39]. Furthermore, enhanced serine 15 phosphorylation and decrease in p21/WAF1 (regulator of cell-cycle and p53 competitor) were detected, indicating that phosphorylation especially at the serine-15 site increases p53 activity [39]. Increased transcriptional activity of p53 not only correlated with apoptosis, but a wide network of proteins responsible for the regulation of cellular proliferation including cell-cycle arrest are also activated and regulated by p53. Higher p53-targeted transcriptional activity was observed in p53RDL1, p21/WAF1, p53RFP, p53AIPl, Bax, Puma, Noxa, p53DINPl, and p53R2 [39].

We have generated both the p53_S12F and p53_S46F mutant constructs. Furthermore, we have also produced a novel combination of two mutants: p53_S46F_S121F and p53_S46F_N239Y. In theory, phosphorylation of serine 15 as well as the DNA affinity of 121F in our p53_S121F_S46F should create a highly optimal p53
that can not only induce apoptosis in various cell lines, but also hold higher transcriptional activity across a broader spectrum of genes that regulate the cell-cycle. Clearly the most favorable and perhaps the best p53 for gene therapy is the introduction of a thermodynamically stable as well as transcriptionally active protein: p53_S46F_N239Y. Table 1 below summarizes a list of our mutations and constructs.

Table 1.

*Restoration and Reactivation: p53 constructs*

<table>
<thead>
<tr>
<th>Type</th>
<th>Construct</th>
<th>Mutation*</th>
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<tbody>
<tr>
<td>Thermodynamically Stabilized</td>
<td>p53_N239Y</td>
<td>N→Y</td>
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<tr>
<td></td>
<td>p53_Y236F</td>
<td>Y→F</td>
</tr>
<tr>
<td></td>
<td>p53_T253I</td>
<td>T→I</td>
</tr>
<tr>
<td>Transcriptionally Active</td>
<td>p53_S12F</td>
<td>S→F</td>
</tr>
<tr>
<td></td>
<td>p53_S46F</td>
<td>S→F</td>
</tr>
<tr>
<td>Combinations</td>
<td>p53_S46F_N239Y</td>
<td>S→F and N→Y</td>
</tr>
<tr>
<td></td>
<td>p53_S46F_S121F</td>
<td>S→F and S→F</td>
</tr>
</tbody>
</table>

*Amino acid substitution of corresponding numbers in respective order.
MATERIALS AND METHODS

LB Broth and Agar Plates

Bacterial medium was prepared by using 12g of LB Broth (SIGMA Life Sciences) in 600 ml of double-distilled water (ddH₂O) followed by autoclaving. After cooling, 3 ml of kanamycin was added to complete the broth. Correspondingly, LB agar plates (batches of 20 plates) contained 24 g of LB agar in 600 ml of ddH₂O and autoclaved for 20 min at 121°C. 3ml of kanamycin was added.

Plasmid Constructs

Specific mutations in full length p53 plasmid (a gift from Dr. S. J. Baker, Addgene) containing ColE1 origin, MCS, F1 ori, SV40 early promoter, and KanR) were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Agilent Tech.). Mutagenic primers were designed on ApE Plasmid editor for length, Tm, and %GC (see primer list below). PCR matching (PTC-200 Peltier Thermal) and BIO-RAD (C1000) thermal cycler instruments were used for DNA amplification. PCR reaction conditions are listed below in Table 2. After temperature cycling, the reaction was placed on ice for 2 minutes to cool the reaction to an approximate temperature of 37°C.

Table 2.
**PCR Reaction Conditions: QuikChange Site-Directed Mutagenesis**

<table>
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<td>30 sec</td>
</tr>
<tr>
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<td>12</td>
<td>95°C</td>
<td>30 sec</td>
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<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

*Minutes/kb of plasmid length, p53 plasmid was used for all missense mutation. The length of p53 plasmid is 5151 bp in length.*
Oligonucleotide primers, complementary to strands of the vector, were extended via *PfuTurbo* DNA polymerase. Mutated plasmids are thus obtained by incorporating the oligonucleotide primers in the extended DNA. Parental DNA is digested by the *Dpn I* endonuclease, 5'-Gm6ATC-3', specific for methylated and hemimethylated DNA. The circular, nicked double-stranded DNA was then transformed into XL1-Blue supercompetent cells, and subsequently plated. Prepared controlled reaction mix can be found in Table 3 below.

**Table 3.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer</th>
<th>Backward Primer</th>
<th>PfuTurbo DNA polymerase</th>
<th>dNTP</th>
<th>Dpn I</th>
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<tbody>
<tr>
<td>p53_N239Y</td>
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<td>125 ng</td>
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</tr>
<tr>
<td>p53_Y236F</td>
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<td>125 ng</td>
<td>2.5 U/µl</td>
<td>1 µl</td>
<td>10 U/µl</td>
</tr>
<tr>
<td>p53_T253I</td>
<td>125 ng</td>
<td>125 ng</td>
<td>2.5 U/µl</td>
<td>1 µl</td>
<td>10 U/µl</td>
</tr>
<tr>
<td>p53_S12F</td>
<td>125 ng</td>
<td>125 ng</td>
<td>2.5 U/µl</td>
<td>1 µl</td>
<td>10 U/µl</td>
</tr>
<tr>
<td>p53_S46F</td>
<td>125 ng</td>
<td>125 ng</td>
<td>2.5 U/µl</td>
<td>1 µl</td>
<td>10 U/µl</td>
</tr>
</tbody>
</table>

**DNA Purification**

The next day, single colonies in 5 ml of LB-kanamycin broth were incubated at 37°C for 24 hours, spun for 15 min at 35000 rpm at 4°C. Zippy Plasmid miniprep kit was used to
isolate plasmid as follows. After centrifugation of the 5ml bacterial culture grown in LB-kanamycin broth, 600 μl of ddH₂O was resuspended in the bacterial pellet. 100 μl of 7X lysis buffer was added after transfer in a 1.5 ml microcentrifuge tube. 350 μl of cold neutralization buffer followed by 16,000 g for 4 minutes pelleted the debris. 200 μl of Endo-Wash Buffer, 400 μl of Zyppy™ wash buffer were used further to wash and collect the purified DNA. 30 μl of ddH₂O and 30 seconds centrifuge eluted the plasmid DNA.

Plasmid concentrations were measured using the Thermo-Scientific NanoDrop 2000 spectrophotometer. Using 1.0μg of plasmid and 10 pmol of primer; mutations in the plasmids were verified by sequencing from The University of Utah HSC Core Research Facilities. Subsequently, isolation of DNA in greater amounts was carried out using HiPure MaxiPrep Kit (Invitrogen). Colonies obtained from master plates were grown in 150 ml of LB-kanamycin broth for 24hr at 37°C and centrifuged, the bacterial lysate were cleared via the binding the DNA directly to the chromatographic anionic resin. Following centrifugation at 4,000 × g for 10 minutes purification preceded similar to the miniprep protocol and using the Precipitating DNA Using Precipitator Module, and with 500μl of ddH₂O as the final step.

Cell Culture

Invasive breast ductal carcinoma, MCF-7 cells (http://www.atcc.org/), were prepared and cultured in RPMI 1640 for transcriptional analysis. Adherent MCF-7 with mislocalized p53 was maintained at 37°C in air, 95% and carbon dioxide, 5% (http://www.atcc.org/). Doubling time of MCF-7 is approximately 29 hours. RPMI 1640 containing 10% FBS, 1% L-glutamine, 1% penicillin, 0.1% streptomycin, and 4mg/L of insulin was used to grow MCF-7 cells. MCF-7 cells were split 1:3 every 48 hours. Cells were first washed
with 10 ml PBS and treated with 3 ml of trypsin for a 5 minutes (to detach cells), followed by the addition of 7 ml RPMI medium and subsequent splitting.

**Plating**

After trypsinization, MCF-7 cells were placed in 50ml tubes then centrifuged for 7 min at 3000g at 4 °C. Cells were counted using a hemacytometer. Approximately 1x10^5 cells were plated into three wells for each construct in a 96 well-plate (white) for 24 hour before lipofectamine transfection.

**Lipofectamine and Luciferase activities**

24 hours after plating, media was removed, and 80ul of RPMI with FBS only was added to each well. Optimem (80 μl), DNA plasmid (175ng), reporter master mix (8 μl), Plus reagent (1 μl), and LTX (2.5 μl) were all mixed in a microfuge tube multiplied by four (three wells were used, however, all solutions were divided by four to prevent shortage) and placed in each of the wells. Reporter mix was prepared by adding 10 μl of p53-reporter plasmid in 90 μl of ddH2O with 1 μl of renilla in 99 μl of ddH2O. To examine the transcriptional activity of p53, PathDetect® p53 Cis-Reporter plasmid (Stratagene) was co-transfected with plasmid with respective mutations. The PathDetect® reporter contains a synthetic promoter with repeats of the transcription recognition sequences for p53 fused to the luciferase gene. Firefly luciferase was normalized to renilla luciferase. Dual-Glo Luciferase Assay System® (Promega) was used for cell lysates and quantitative analysis used Lumino-program system. Luciferase assays was performed 2 times in triplicate for each plasmid transfected per cell line.
RESULTS

I have successfully constructed all seven plasmids extracted from literature and novel constructs listed above containing their following respective mutations (Figure 6). We expected our novel p53_S46F_S121F to have the highest transcriptional activity. The double mutant, p53_S46F_S121F, should not only exhibit elevated transcriptional activity due to the enhanced serine 15 phosphorylation by the addition of the S46F mutation but the S121F mutant should also contribute to transcriptional activity via its DNA affinity by for p53 binding sites [38, 39]. An increased level in serine 15 phosphorylation has been suggested to stabilize and enhance the transactivation of the p53 protein [39]. Conversely, we expected the thermodynamically stabilized p53_N239Y constructs to have a similar or slightly greater transcriptional activity than the wild-type perhaps due to additional “hydrophobic packing interactions at the DNA-binding surfaces” [12, 35, 36]. However, we expected our double mutant constructs p53_N239Y_S46F to demonstrate enhanced transcriptional activity because of the reactivation abilities of S46F mutants discussed previously and the stabilized DBD core domain contributed by N239Y.

Fire-fly reporter vector containing the p53 promoter-gene was used for transcriptional analysis. PathDetect\textsuperscript{®} reporter containing a synthetic sequence of the p53 promoter was fused to the luciferase gene. PathDetect\textsuperscript{®} p53 Cis-Reporter plasmid (Stratagene) was co-transfected with plasmid constructs. The p53-reporter gene is mainly used to determine a constructs interaction and regulatory potential of the DNA-sequence. This reporter gene also identifies constructs that are sequence specific for the p53 promoter.
Figure 6: Plasmid Constructs. Full length p53 plasmid containing ColE1 origin, MCS, F1 ori, SV40 early promoter, and KanR was used to introduce the mutations. ApE Plasmid Editor generated images, http://biologylabs.utah.edu/jorgensen/wayned/ape/.
Thermodynamically stabilized p53_T253I has shown the highest transcriptional activity (Figure 7). The relative transcriptional activity of p53_T253I is higher than the wild-type p53. p53_T253I is a thermodynamically stabilized construct with unknown free energy change, $\Delta G^\bullet_{-NH\text{\textsubscript{2}}}$ [35]. Furthermore, the second p53 paralog, p53_Y236F has also shown a higher transcriptional activity than wild type p53, suggesting that perhaps p53 paralogs are not only thermodynamically stable but may also contribute superior interactions with the p53 promoter than the wild-type.

**Luciferase Assay**

![Figure 7: Transcriptional Activity of the p53-reporter Gene.](image)

Conversely, the five other p53 constructs yielded surprising results. p53_S46F_S121F, the construct with two known mutants that enhanced transcriptional activity showed almost a 3-fold lower relative activity in comparison to the wild-type.
p53_S121F showed equal activity as the wild-type p53, however, the introduction of the second mutations, p53_S46F, lowered the transcriptional activity. Although the DBD region is where DNA is attached, lowered transcriptional activity in this double mutant is mostly attributed to p53_S46F. Suggestions have been made by other investigators that perhaps the substitution of S→F in p53_S46F causes favorable structural changes in the p53 protein [39]. However, perhaps the replacement of the two polar proteins in p53_S46F_S121F to bulky aromatic ones (S→F) induce conformational changes to a greater degree and in an unfavorable manner [39].

Poorer is yet the p53_N239Y_S46F, which showed no activity. Although there is some activity in p53_S46F, it appears as if p53_N239Y inhibits the transcriptional activity of p53_S46F. p53_N239Y acts as a second-site suppressors for the cancer hot spot G245S and is transcriptionally active for the gadd45 DNA region [12, 33]. However, our data shows that this mutant is transcriptionally deficient for the p53 promoter sequence. Further studies verifying transcriptional studies are required noting our preliminary studies, particularly since the experiments were only performed twice (albeit in triplicate).
DISCUSSION

Identifying Super p53s

Initial studies of our p53 mutants have identified two possible p53 mutants with enhanced activity. These two mutants (Y236F and T253I) have been shown by others to be thermodynamically stable, and show improved reporter gene activity in these preliminary studies. Our ultimate goal is to enhance apoptosis. We are currently in the process of analyzing these constructs for apoptotic abilities in caspase 3 and 9 assays in T47D and MCF-7 cell lines, respectively.

Apoptosis

Although determination of the most optimal p53 tumor suppressor protein in regards to the transcriptional activity and thermodynamic stability require further research, Y236F and T253I apoptotic analysis may reveal the potency of our combination of mutations against cancer. Several investigators have argued that there are no significant correlations between enhanced transcriptional activity and apoptosis, and that transactivation independent mechanism may be equally important [40]. Therefore, to determine apoptotic activity, we will consider caspase assays, DNA segmentation cell analysis, morphology, and cytochrome c release in breast cancer cells. Furthermore, we aim to validate the combination effect in tumors via adenovirus vector delivery in a human xenograft solid tumor murine model in vivo. As our current aim, the therapeutic effect of super p53 delivery will be tested in athymic nude mice subjected to right dorsa subcutaneous injection of either T47D or MCF-7 cells.

Finally, utilizing the restoration and reactivation approaches of cellular mechanisms described here is more advantageous than strategies involving
chemotherapy, small molecule inhibitors, surgery, and hormonal therapy because correcting p53 associated tumorigenesis will allow the restoration and reactivation of the tumor suppressor activity of p53 in the cells and will also trigger apoptosis. Stabilizing and reactivating the tumor suppressor protein p53 provides a more effective strategy against aggressive cancers that presently have no effective therapies.
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