Mutational Analysis of *TEP1*, a Budding Yeast Homologue of the Human Prostate Cancer Factor *PTEN*

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INTRODUCTION

Prostate cancer, a particularly large part of men's health, either affects or kills upward of 30,000 men in the United States alone. The goal of this research project was to study the growth of the *PTEN*(*MMAC1*/*TEP1*) gene under a variety of different controlled environmental factors. Using previous research, these environmental factors were chosen on their noted presence or absence in the growth and development of prostate cancer. An additional aspect of this research was to create a bacterial plasmid of the gene so that duplicates can be made for further study.

Since the development of prostate specific antigen (PSA) tests in the 1960s, prostate cancer has had a significant increase in positive diagnoses. It wasn't until around 1980 that PSA tests were given more liberally, however the United States Preventative Services Task Force contends that PSA testing is not optimal because of its potential to lead to over-diagnosis and over-treatment thus furthering their notion that "the potential benefits of testing do not outweigh the expected harms" (USPSTF). Prostate cancer is the second most common type of cancer and is the fifth leading in cancer-related deaths among men. For the year 2015 alone, it is estimated that there will be 220,800 new cases and 27,540 deaths from prostate cancer (ACS). With this being said, there is relatively little information known about the pathways and overall nature of the cancer. Once a patient has received a diagnosis, treatment options remain limited. An area of interest regarding the development of prostate cancer is the *PTEN* (MMAC1/*TEP1*) gene. *PTEN* is a tumor suppressor gene and has been supported by the evidence of their association with autosomal dominant hamartomatous/cancer-predisposing syndromes, the gene is homozygously inactivated in numerous human forms of cancer, and the gene is a highly conserved protein (Ali). Studies thus far have shown that implementing the *PTEN*/MMAC1 gene into cancer cell lines will lead to suppression, and the heterozygous or homozygous inactivation of the gene in mice will lead to increased tumor susceptibility (Ali). The *PTEN* gene is located in the region, 10q23.3—a region that is typically deleted in individuals with prostate cancer (Suzuki). The *PTEN* gene's role in current understanding is that it functions as an acting component in a cells pathway when the process of apoptosis is occurring. It has been noted that the *PTEN* gene is usually silenced or mutated when prostate cancer is identified in the living system. Furthermore, research has shown that *PTEN* does contribute to the metastasizing of the cancer. When tumor-suppressing genes, such as *PTEN*, are missing/silenced/deleted, the tumor(s) has a greater chance of growing/developing and resulting potentially into malignancy.

We proposed a research project that looks to further investigate the role that *PTEN* plays in formation and development of cancerous cells and the cancer's metastasis throughout the body. During this project, we isolated functional regions in the gene using multiple site directed alanine mutations. These mutations assisted in studying the downstream molecular pathways, biomarker expression, and tumorigenesis. Alanine mutations tend to be an effective way to silence targeted regions of the *PTEN* gene. As surrounding regions were silenced, distinguished individual regions were amplified and were studied and compared. This research examined the *PTEN* homologue in saccharomyces (yeast), which is known as *TEP1*. OneShot TOP10 competent cells were used in order to quantify the growth rate of cells. These cells are chemically pre-treated in order to allow them to accept DNA more readily. The characterizations of these cells were done using methods learned in previous biology lab courses—these techniques include PCR and gel electrophoresis, among others. In addition, QuikChange protocol will be used in order to establish single and multiple site-specific mutations. Environmental stress testing was executed in order to gain further insight into how the cells would react under in vivo conditions. The *PTEN* mechanism of suppressing tumors acts by limiting the rate of cell growth and cell division—the primary mechanism in mutation, which can subsequently lead to cancerous cell formation. The ultimate purpose of this research was to characterize the *TEP1* homolog of the human cancer factor *PTEN* in order to gain a better understanding of the role it plays in tumor suppression. Taking a further look at these site-specific mutations can help further expand the knowledge regarding the role that the specific regions have in the formation and development of tumor cells, production of cancer specific antigens, and changes in/of the biological pathways.

Principal Approaches

Growth Experiments: Growth experiments were conducted on a variety of differently composed media. All liquid broth was autoclaved for 12 minutes before allowing cooling prior to plating.

Creation of Parent Plates: Parent plates for these experiments were created using pre-grown samples of FY86 wild type yeast, TEPΔ1, and TEPΔ6 mutant type strains. After having prepared test tubes filled with 5 milliliters of YPD broth, sterile toothpicks were used to scrape a small sample of the yeast off of the pre-existing plates. The toothpicks were placed in individual test tubes, which were then placed in a spin column in a 32℃ incubator. The samples were allowed to incubate for 24 hours before being transferred to an YPD plate. Samples were allowed to grow in a 32℃ incubator for two days, upon which they were transferred to a refrigerator to stop growth. It is from these plates that all other experiments received the starting yeast.

PH Growth Experiments: After researching environmental factors in the human body that could lead to the presence of prostate cancer, it was determined that slight variations of pH could play a role in the formation and spread of cancerous cells. Thusly, a pH range of 6 to 7.5 was chosen for study. The pH plates were created by first mixing the necessary ingredients to create an YPD broth in a 500mL beaker. In order to make plates that were more acidic (plates with a pH of less than 7), hydrochloric acid was added drop-wise using a pipette. The pH of the broth was confirmed using a standard pH laboratory reading kit. In order to make broths that were more basic, sodium hydroxide was added drop-wise using a pipette. The pH of the broth was confirmed using a standard pH laboratory reading kit. Once the broths were at the desired pH they were placed into an autoclave for twelve minutes. After autoclaving, they were allowed to cool before being transferred to plastic molds. These molds were placed in a refrigerator and allowed to harden. Using the pre-created parent plates, a sterile toothpick was used to transfer a small sample to the plates. This sample was then streaked along the edges of the plates. The plates were then transferred to a 32℃ incubator and allowed to grow for two days. Dilution plates were also created for this experiment. First, a toothpick of each type of yeast was placed in a test tube of 2mL of YPD broth. This sample was then diluted ten-fold five times into five separate test tubes using a ratio of ten units of sample to ninety units of water, ultimately creating a liquid sample that was 1/10,000 of the original liquid sample. Starting with the full liquid sample, the plates were dotted with 2μL of each dilution of sample in a straight row. All three types of yeast were grown on the same plate. The plates were allowed to grow for two days in a 32℃ incubator before being transferred to a refrigerator to stop growth.

Bacterial Growth Experiments: Using existing cultures of E. coli and *Staphylococcus A.*, liquid cultures were made by placing a sterile toothpick with sample on it into a test tube that had 5mL of LB broth. The test tubes were placed in a 37℃ shaker and allowed to grow overnight. In order to create parent plates of the bacteria, a sterile toothpick was used to spread 5μL of the liquid sample across a pre-made LB plate. These plates were then placed in a 37℃ incubator and allowed to grow for one day before being moved to a refrigerator to stop growth. It is from these parent plates that following bacterial growth experiments were conducted.

The first bacterial growth experiment involved growing both the bacteria and each type of yeast on the same plate. First liquid cultures were prepared from the parent plates. For the yeast, a sterile toothpick was used to transfer a small sample of each type of yeast, FY86, *TEP1*, and TEP6, to three separate test tubes that had 5mL of YPD broth. For the bacteria, a sterile toothpick was used to transfer a similar sized sample to a test tube that held 5mL of LB broth. Each of the test tubes were allowed to grow overnight in their respective incubator, 32℃ for the yeast and 37℃ for the bacteria. During this period of growth, YPD plates were made and labeled for the plating process. It was decided that each type of yeast would be paired with each bacteria. Thus, plates were labeled: FY86-E.coli, FY86-*Staph A.*, *TEP1*-E.coli, *TEP1*-*Staph A.*, TEP6-E.coli, and TEP6-*Staph A.*. Two microliters of each corresponding sample was transferred to the correct plate and a sterile toothpick was used to spread the samples around the corners of the plate. The plates were

transferred to a 32℃ incubator and allowed to grow for two days. The plates were examined after the end of each day.

The second bacterial growth experiment involved growing the bacteria and yeast in the same test tube prior to plating. In this experiment, liquid samples were prepared by placing sterile toothpicks of each matching of bacteria and yeast in a test tube with 5mL of YPD broth. The test tubes were then placed in a 32℃ shaker and allowed to grow overnight. Afterwards, 5μL of the liquid samples were streaked across YPD plates. These plates were allowed to grow for two days in a 32℃ incubator before being placed in a refrigerator. The plates were studied at the end of each day.

The third growth experiment involved growing the mixed bacteria and yeast liquid solutions on media of different mixed composition. For this experiment, it was decided that three additional types of media would be used in addition to LB and YPD. A mixed ratio of 75 LB/ 25 YPD, 50 LB/ 50 YPD, and 25 LB/ 75 YPD were produced using the chart listed above. The broths were all autoclaved for 12 minutes. They were allowed to cool before being poured into separately labeled molds. Plates were allowed to cool and harden overnight. Liquid cultures of mixed yeast and bacteria were created using the previous experiment as a guide. Small samples of each type of bacteria were paired with each type of yeast and were transferred to test tubes that held 5mL YPD using a sterile toothpick. Enough samples were made so that half could be placed in a 32℃ incubator and the other half could be placed in a 37℃ incubator. Each set of samples was allowed to grow over night before being plated on each style of created plate. The plates were allowed to grow for two days, being studied at the end of each day.

Bacterial Plasmid Experiments: Bacterial strands YIplac33, YIplac195, and YIplac211 were used in the bacterial plasmid experiments. These strands were chosen because they contain the Leucine2 and ampicillin gene for selective plate selection.

Yeastar Genomic DNA Kit: Using a standard laboratory vortex, approximately 1.5mL of FY86 was spun briefly. The excess supernatant was removed and discarded. To the cells, 120μL of YD digestion buffer and 5μL of R-Zymolyase was added. The pellets were suspended by vortexing and were incubated for one hour at 37℃. 120μL of YD Lysis Buffer was added to the sample. The sample was vortexed for five seconds immediately after addition of the YD Lysis Buffer. It was then centrifuged at 13,000 RPM for two minutes. From this, the supernatant was transferred to a Zymo-spin III column and centrifuged at 13,000 RPM for one minute. To this, 300μL of DNA Wash Buffer was added and centrifuged for one minute at 13,000 RPM. After, an additional 300μL of DNA Wash Buffer was added and centrifuged for one additional minute at 13,000 RPM. The Zymo-spin III column was transferred to a fresh and clean 1.5mL centrifuge column. 60μL of Elucidation Buffer was added to the column. The column sat for one minute then was centrifuged for ten seconds to elucidate DNA.

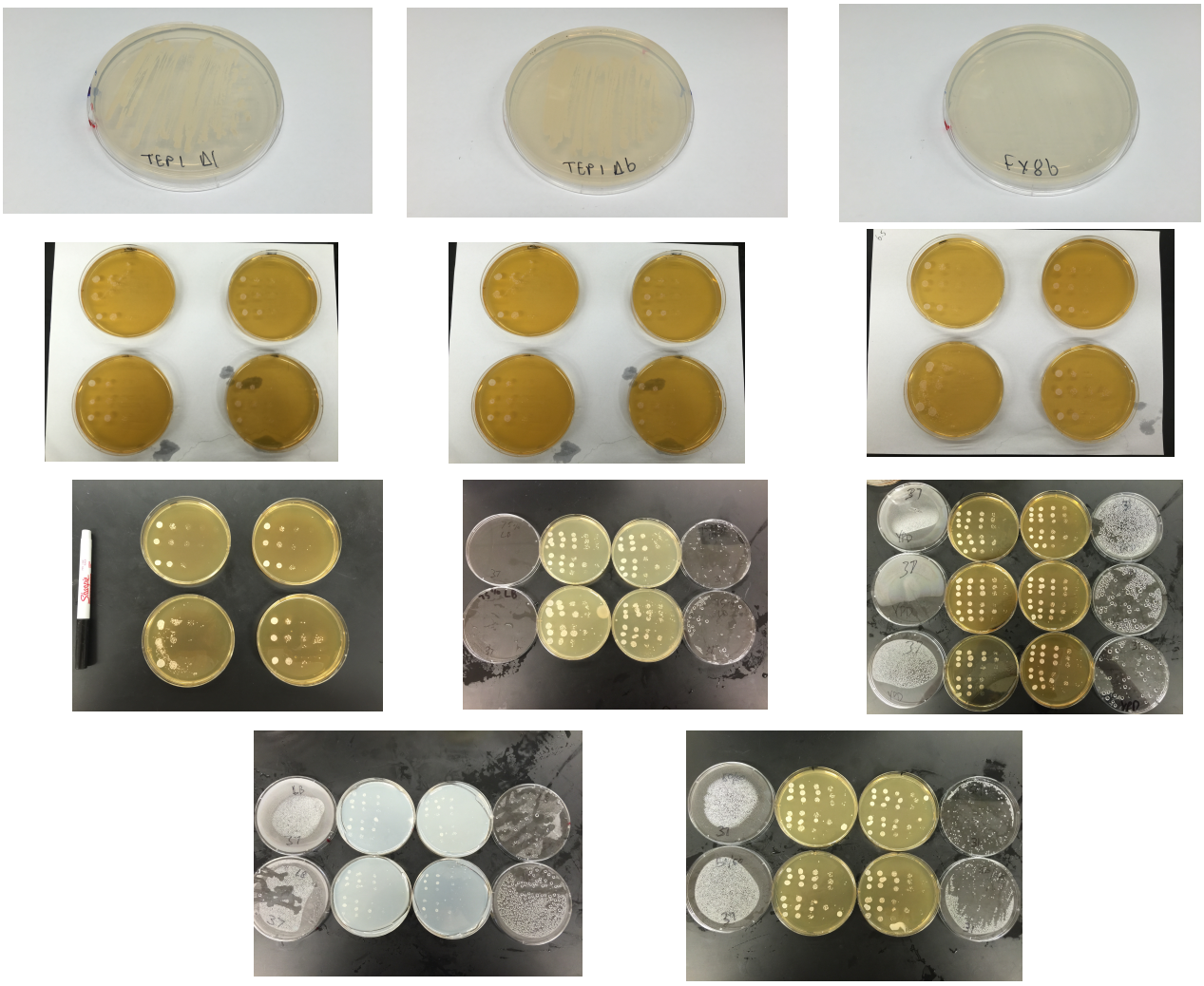
QIAprep Spin Miniprep Kit: Five milliliters of bacteria were centrifuged at 10,000 RPM for three minutes at room temperature. They were stored overnight. The pelleted bacterial cells were re-suspended in 250μL P1 Buffer and transferred to a microcentrifuge tube. 250μL of P2 Buffer were added. The solution was inverted six times so that it became clear (light vortexing assisted in the process). 350μL of N3 Buffer was mixed immediately and the tube was inverted six times. The tube was then centrifuged for ten minutes at 13,000 RPM. The supernatant was transferred spin column and centrifuged for one minute. The flow-through was discarded. The column was washed by adding 750μL PE Buffer. The column was centrifuged for one minute and flow-through discarded. The column was centrifuged for one additional minute to remove any residual wash buffer. The column was then transferred to a clean 1.5mL microcentrifuge tube. 50μL EB Buffer was added to the center of the column. It was allowed to stand for one minute, and then centrifuged for one minute.

QIAquick Gel Extraction Kit: Using a sharp, clean scalpel, the DNA fragments were removed from the agarose gel. The gel slices were weighed and placed into a microcentrifuge tube. To each tube, three volumes of QG Buffer were added per one volume of gel. These were then incubated for ten minutes at 50℃. After the gel had completely dissolved, one volume of isopropyl alcohol was added to each tube and the tubes were vortexed. The spin column was placed in 2mL collection tube. The sample was then centrifuged for one minute. Flow-through was discarded. The spin column was placed back into the same collection tube. In order to wash the sample,

750µL of PE Buffer was added to the column. The column was centrifuged for one minute. Flow-through was discarded and the spin column was placed in the same collection tube. The column was centrifuged for one additional minute in order to remove any residual wash buffer. The spin column was transferred to a clean and new collection tube. To elucidate the DNA, 50µL of EB Buffer was added to the center of the column. The column was allowed to sit for one minute, and was then the column was centrifuged for one additional minute.

QIAquick PCR Purification Kit: For every one volume of the PCR reaction, five volumes of PB Buffer was added and mixed. A spin column was placed in a 2mL collection tube. Sample was placed in the spin column and centrifuged for one minute at 13,000 RPM. Flow-through was discarded and the spin column was placed in the same microcentrifuge tube. To wash the sample, 750µL of PE Buffer was added. The column was then centrifuged for one minute, and flow-through was discarded. In order to remove excess wash buffer, the column was spun for an additional minute. The column was transferred to a clean and fresh collection tube. 50µL of EB Buffer was added to the center of the column to elucidate DNA. The column was allowed to stand for one minute then centrifuged for one additional minute.

Present Knowledge



Pictures from left to right:

Row 1: Picture 1 – TEP1 Colony; Picture 2 – TEP6 Colony; Picture 3- FY86 Colony

Row 2: Pictures 1 and 2 – Controlled Growth Dilutions (FY86, TEP1, TEP6); Picture 3 – pH Test (6.5)

Row 3: Picture 1 – pH Test (7.5); Picture 2 – Bacteria Duel Growth Test (37°C left) and (31°C right), 75% LB and 25%YPD; Picture 3 – Bacteria Duel Growth Test (37°C Left) and (31°C Right), 100% YPD

Row 4: Picture 1 – Bacteria Duel Growth Test (37°C Left) and (31°C Right), 100% LB; Picture 2 – Bacteria Duel Growth Test (37°C Left) and (31°C Right), 50% YPD and 50% LB

The initial efforts of the research were to successfully breed colonies of *Saccharomyces cerevisae* FY86, *TEP1*, and TEP6. The colonies were continually used throughout the summer for testing and regrowth purposes. Next we chose to run controlled, diluted growth trials to examine how well the cells grow naturally under optimal conditions. The dilutions (1:1, 1:10, 1:100, 1:1000, 1:10,000) are ordered from top (black stripe) to bottom in the order: FY86, *TEP1*, TEP6. The stress tests were the primary concern of the various colonies. The first stress test observed the potential for pH to affect the growth rate at which the yeast would grow. For in vivo purposes, the pH levels selected were chosen in order to closely reflect the slight acid/base changes occurring in the body. Picture 3 in row 2 reflects a pH of 6.5 and picture 1 in row 3 reflects a pH of 7.5. Following the pH stress test, the next stress test completed was the bacterial dual growth stress test. For this stress test, the bacteria strains *Staphylococcus aureus* and *Escherichia coli* were chosen because of their gram-positive and gram-negative characteristic respectively. From top (black stripe) to bottom, all plates follow the order: FY86/E. Coli, FY86/Staph A., TEP6/E. Coli, TEP6/Staph A., *TEP1*/E. Coli, *TEP1*/Staph A. To limit growth advantages, trials were conducted on various medias (100% LB, 100% YPD, 75/25% LB/YPD, 75/25% YPD/LB, 50/50% LB/YPD.

DISCUSSION

From analyzing the results from the dual growth part of the experiment, it was inconclusive as to whether the presence of the bacteria, either the *E. coli* or the *Staph. A* had any effect on the growth of the *TEP1* and TEP6 yeast. When compared to the growth of the FY86 yeast, it appeared as if the yeast grew the same. This result occurred in both the dual growth in the same test tube as well as the dual growth that occurred when mixing the two on the media. As for the growth that occurred on different media, it grew as predicted. Media that favored the bacteria showed a stronger presence of bacteria while media that favored yeast showed a stronger growth of yeast. Even under a microscope, it was determined that the presence of the bacteria did not discourage the growth of the yeast. The first method to determine whether or not the bacteria deterred the growth of the yeast was tested by developing two separate liquid samples and then growing them on the same plate, which was decided to be too ineffective. Thusly, the second method of growing the two in the same test tube, as well as on the same plate was developed. This method, though it yielded the same results, was chosen for the reminder of the research because of its high efficiency. Since the data collected did not show an impediment upon yeast growth, further research could include different forms of bacteria that could commonly cause infection. Also, studying how exactly the bacterial infection causes the presence of cancerous cells would constitute further research. From the pH range that was selected, it was determined that the yeast was able to grow at the same rate, regardless of changes within this range. From this result, further research could include a wider range of pH.

The creation of the bacterial plasmid proved to be a difficult task. Originally, a bad batch of genomic DNA provided several bad samples of PCR. After creating a new, and more saturated batch of genomic DNA, several PCR attempts proved to be bad due to electrical failures. Once a PCR product yielded a high enough concentration of DNA a bacteria plasmid was created. However, due to a lack of time, this plasmid was unable to be confirmed if it was in fact the desired product.

CONCLUSION

Ultimately, there were two main aspects to this experiment. The first aspect of this experiment was the growth-related aspect. By growing FY86, *TEP1*, and TEP6 on different media, a study of the effect of bacteria deterrence on the yeast was

conducted. It was determined that the bacteria and yeast were able to grow at the same rate, even in the presence of one another. A bacterial plasmid was created; however, due to time constraints, it was unable to be tested to confirm if it was the desired product. Future research on this particular project would be to confirm if the produced plasmid is the desired product.

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