The Longevity and Aging Mediated by FOXO Proteins and The Methuselah Gene

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Abstract

Forkhead box (FOXO) transcription factors have been shown to influence longevity and ageing in various species. FOXO has shown to be involved in the regulation of cancer through the ageing process. The gene methuselah in Drosophila melanogaster is involved in longevity due to its connection to FOXO transcription factors. This study aims to discover the effects of FOXO on the gene methuselah through gene knockout. D. melanogaster cells were infused with FOXO transcription factors to alter the DNA strand. The methuselah gene within this DNA was knocked out by inhibition of the FOXO transcription factors. Knockout of this gene should negatively affect the longevity of the organism. This is due to methuselah's role in increased longevity when FOXO is present. Discovering the effects of gene knockout with FOXO can increase longevity in various species.

Introduction

Science is all around us, whether it's how things are created or what we consume on and everyday basis. The natural world we inhabit is an epic spawning ground for many scientific discoveries such as the beginning of life. The natural world and life all began with cells, which are the smallest building blocks that compose all living and non-living organisms and structures all around us. On an organismal level, cells are essentially life and opens a vast world of unknown. Billions of cells compose numerous organisms and have a variety of functions. As scientist study and learn more about cells and life, it allows for a clearer understanding and comprehension of the various organisms that inhabit the planet. As life is study more in depth, this allows for molecular biology to blossom and further the knowledge in understanding the cellular level of life (Molecular 2021). By comprehending the nucleic acids and protein structures that compose cells, this plants opportunities for science and future studies to occur and

dive deeper into the complexity of cellular functioning (Molecular 2021). One of the most important structures within cells is deoxyribonucleic acid (DNA) and it forms a double helix structure which is composed of hundreds of nucleic acids that carry genetic information within the nucleus of cells. Biotechnology and molecular biological techniques allow DNA molecules to be altered, manipulated, and explored genetically.

This experimental study investigates the vast field of using molecular biological techniques and biotechnology to genetical modify genes in *Drosophila melanogaster* cells, commonly known as the fruit fly. The gene chosen to manipulate is the methuselah gene and its correlation with Forkhead box O (FOXO) transcription factors. *D. melanogaster* cells that derive from an invertebrate, which only have one FOXO gene. In this scientific experiment observed ageing effects and growth factors of the FOXO protein and what will happen if this protein is removed from the methuselah gene. It's known that many studies have shown that FOXO transcriptional factors serve as a very important determinant for aging and longevity (Martins et al, 2016). However, some studies have shown that with deregulating FOXO can lead to genetic disorders, increased ageing, and potentially cancer. With the information known and unknown about FOXO and its aging and longevity influences, it was hypothesized that with the FOXO transcriptional factors removed from the methuselah gene of *D. melanogaster* cells, there will be rapid cell growth as a result. With FOXO heavily associated with ageing and when deregulated, known to have one of several effects such as cancer, clumps of cells are expected to be present.

Materials and Methods

Transformation

The chemically competent cells were thaw on ice initially. Then, 2 ul of plasmid was added to the cells which were then incubated on ice for 30 minutes. After which the cells underwent heat shock for 30 seconds exactly at 42 degrees Celsius. Then the cells were incubated for 2 minutes. Then 1 mL of LB broth was added to the cells and then incubated at 37 degrees Celsius where they were shaken for 1 hour to recover. After the 1 hour, there were two LB-agar plates with AMP created. One plate with 100 ul and another with 900 ul. The cells were transferred with a sterile technique onto the plates by a spreader that distributed the cells along each agar plate surface. The plates were set agar side down which allowed for the liquid to be absorbed into the plates. The plates were then incubated at 37 degrees Celsius with the agar side up to prevent condensation accumulation on the surface of the agar.

Plasmid Formation

The liquid culture was retrieved from prior incubation. We then transferred 1.5ml of the cultured bacteria cells to a microcentrifuge tube. The microcentrifuge tube was then centrifuged one minute. Then, 200 ul of buffer was added. Then, 200 ul of buffer was added and mixed thoroughly and set aside for 1-2 minutes. Then, 300 ul of buffer was incorporated and mixed quickly. Then, the tube was centrifuged for 3 minutes. A column was placed in a 3 ml collection tube. The supernatant that was formed after the addition of the PD3 buffer was added to the collection tube. The collection tube was then centrifuged for 30 seconds. The column was put back in the 2 ml tube. Then, the cells were washed and added into the column and then centrifuged for 30 seconds. The column was put back in the collection tube, the tube was the centrifuged for 3 minutes. The column was the placed into a new 1.5 ml microcentrifuge tube. Then 50 ul of Buffer was added to the center of the PD column matrix. The column was placed

aside to stand for 2 minutes to allow the Elution Buffer to be absorbed. The PD column matric was the centrifuged for 2 minutes to elute the purified DNA. The flow-through was then transferred back into the center of the PD column matrix and centrifuged again for 2 minutes.

PCR DNA clean-up & Agarose Gel Electrophoresis

The PCR DNA sample was diluted with DNA cleanup Binding Buffer of a 5:1 ratio which contained 750 ul of Binding Buffer to 150 ul of sample PCR DNA. The sample ratio was then mixed by pipetting up and down. Then a column was inserted into a collection tube and the sample was added and spun for one minute with the flow-through discarded after. After the flow-through was discarded, the column was reinserted into the collection tube and 200 ul of DNA Wash Buffer was added and the spun again for 1 minute. This step was repeated twice. Following, the column was transferred to a clean 1.5 ml microfuge tube. Less than too equal to 6 ul of DNA Buffer was added to matrix and set aside for 1 minute and then spun for 1 minute.

As the gel was prepared to be loaded, 1 ul drops of gel loading dye, purple (6X) was aliquoted out on a strip of parafilm. Then, 5 ul of the sample was added to the loading dye drop. Then, 6 ul of Quick-Load Purple 1 kb Plus DNA Ladder was added into the furthest left lane. In the next lane, the sample was loaded carefully with the load dye from the parafilm.

The cover was then placed on the gel box and plugged into the corresponding electrodes into the power supply. The gel was run at 150V for 1 hour. After 1 hour, the gel tray was removed from the gel box with the use of paper towels to catch buffer drips. The gel doc imaging glass was wiped down with water and then ethanol. The gel was the slid of the gel tray onto the gel doc glass and the door to the gel doc was secured. The UV light was turned on and the image of the gel was captured.

In vitro transcription-1 reaction each for the T3 RNAP and T7 RNAP

Reagents were thawed on ice and vortexed until they were completely in solution. NTPs were kept on ice and 100X reaction buffer was kept at room temperature. The reaction was assembled at room temperature. Water was added first and then the NTPS and then the 10X reaction buffer.

The reaction was mixed thoroughly and then was microfuged to get the mixture at the bottom of the tube and then incubated overnight at 37 degrees Celsius. The reaction was stopped, and the RNA was precipitated. The reaction was set aside to chill for at least 30 minutes at -20 degrees Celsius and the centrifuged at 40 degrees Celsius for 15 minutes at maximum speed to pellet the RNA. The supernatant was carefully removed, and the pellet was washed once with 1 mL 70% ethanol and recentrifuged to maximize removal of unincorporated nucleotides. Then, 70% ethanol was allowed to air dry and then the RNA was resuspended in 100 ul 1X TE 7.5 after which the RNA was stored frozen at -20 degrees Celsius to -80 degrees Celsius.

Anneal dsRNA

Equal amounts of both strands were added to 1 tube and heated to 95 degrees Celsius for 2 minutes. Then, transferred to 65 degrees Celsius block for 30 minutes. Then the tube was cooled to room temperature by putting heat block on the bench and allowed it to gradually cool down to room temperature.

Prepare Cells

S2C1 cells were grown in Schneider's media with pen/strep and 2 mM glutamine 2 days before RNAi experiment (for T25 flask). The cells were split 1:3 into fresh media by carefully aspirating the old media and any floating cells. The semi-adherent cells were resuspended in 6 mL complete media by washing them of the plate via pipette and then transferred to 2 mL to 3 x 25 cm^2 flasks which contained 4 mL media.

RNAi

The media and any floating cells were aspirated, and the adherent cells were resuspended in 5 mL of fresh media. The cells were counted and diluted to 1.5 x 10^6 cells/mL. Then, 8 mL of the cells were plated into each T25 flask. There were 10 flasks for 1 dFOXO dsRNA + 1 mock (no dsRNA) per group. The cells were placed aside to adhere for 1 hour.

The media and any floating cells were aspirated. The cells were gently washed twice with serum-free media. After the final wash, there was 2.5 mL of serum-free media added. Then, 20-40 ug/mL final concentration dsRNA was added.

Volume of dsRNA to add = (40 ug/mL final concentration) x (2.5 mL final volume) / 25ug.mL dsRNA stock)

The cells were then set aside to incubate for 1 hour and room temperature. After the cells had incubated for 1 hour, 5 mL of complete media was added.

Results

Once data was collected and analyzed carefully, it was shown that there was an increase in the specified target gene due to FOXO being knocked down. It's been understood that the methuselah gene codes for family BG protein-coupled receptor (GPCR), that is widely known to be closely associated with longevity. Due to this increase in transcription of FOXO proteins, it has been assumed that the *D. melanogaster* cells will live longer than the estimated normal life span. We can see that relative gene expression of the targeted gene and dFOXO is represented as a fraction of mock (Graph 1). For the mock cells, both the target and dFOXO gene were both equal as 1.

Discussion

The increase in the target gene due to FOXO being knocked down successfully, it can be assumed and interpreted that the hypothesis can be partially supported. We hypothesized that cell proliferation would be present with FOXO transcription factors being knocked down, so those results were highly anticipated. The unexpected finding was the partial support of the hypothesis due to cell proliferation being present, however, it's not directly known if this is due the procedure taken to retrieve the collected results of this study.

The experimental design was carried out successfully but does not include the limitation of not being able to perform more research and conduct more intensive studies to determine if the cell growth that was present is directly due to FOXO transcriptional factors being knocked down and what this could lead to besides cancerous cell growth and tumors. More research on if this increased cell longevity as well would need to be further studied since this factor was not the focus within the study or able to be tested. Therefore, the hypothesis is partially supported and with such limitations leads to the question on whether this study adequately provided the expected results.

What could've been done differently was to include steps within this study to test these limitations and potentially perform or extend the level of research performed through this study. More time in intense background research regarding the methuselah gene and how this gene is expressed in more studies could allow for the appropriate critique of this study to collect better results that could support the hypothesis fully.

Figures and Tables

Figure 1: SnapGene Viewer image showing how the primers were created form the DNA sequences retrieved from the methuselah gene.

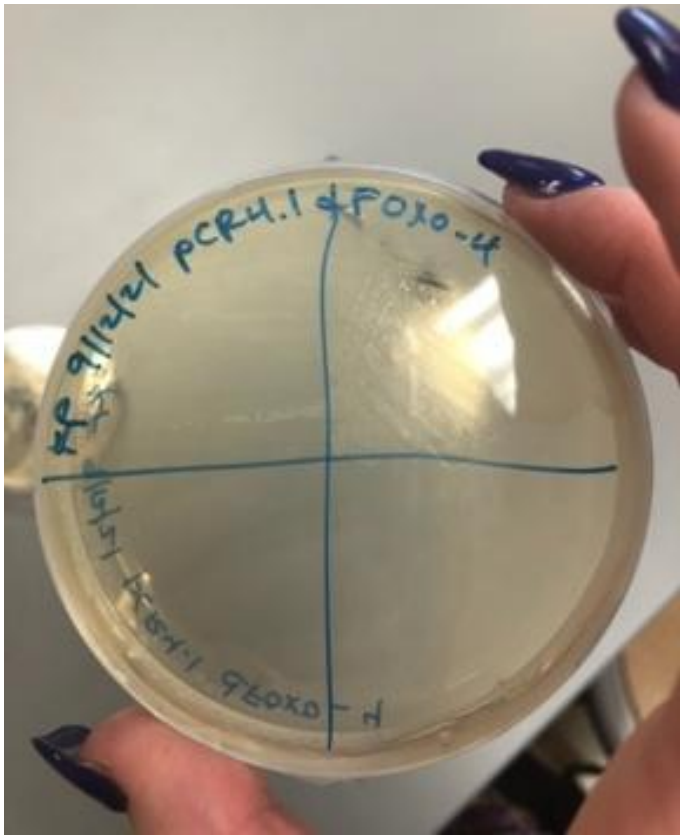


Figure 2: The results from the transformation process of the cells on the Agar plates. The left image shows the agar plate of the mock cell and the right image show the agar plate of the dFOXO cells.

Figure 3: The image above shows the sample (circled in orange) in the agarose gel electrophoresis image that was captured through UV light. The sample sits at about 700 bp.

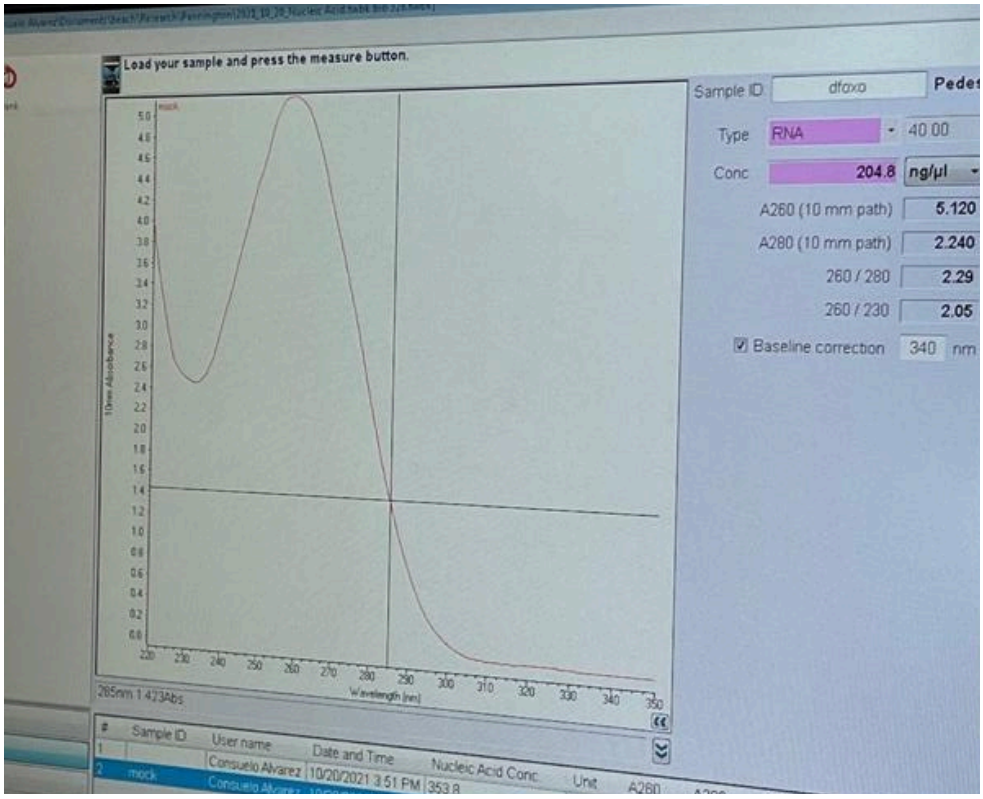


Figure 4: Results after RNA extraction of dFOXO cells. The concentration via spec was 204.8 ng/ul. The peak sits at A260 as shown to be 5.12 and a dip at A280 which ended at 2.24.

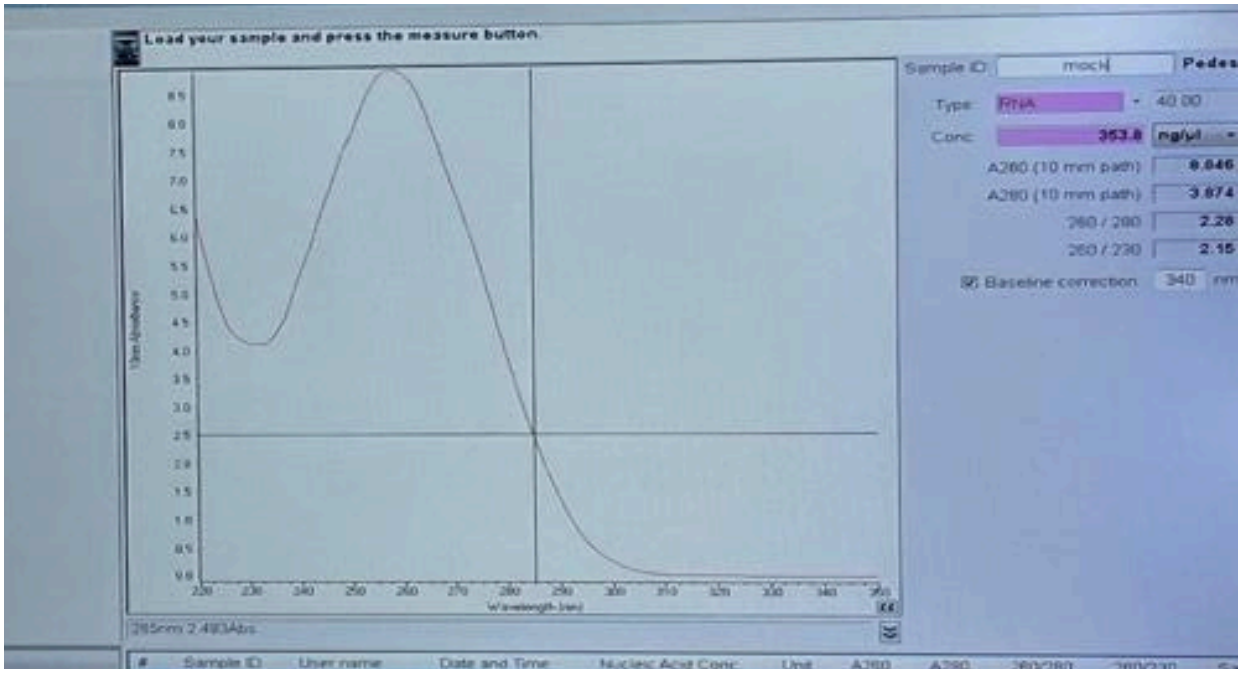
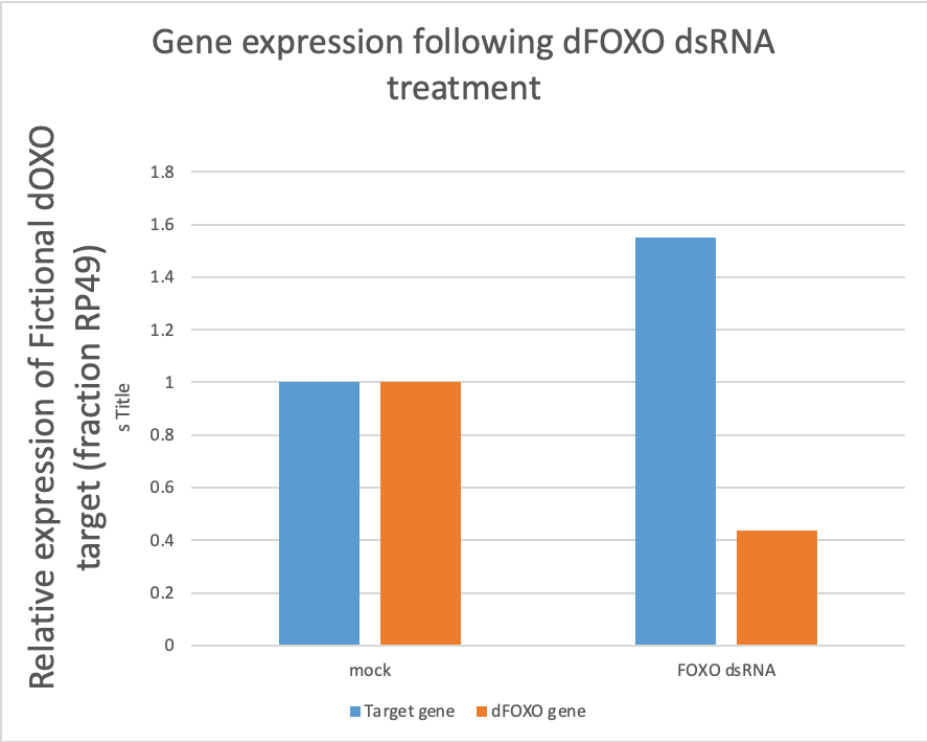


Figure 5: Results after RNA extraction from Mock cells.



Graph 1: Results of the RT-qPCR of mock and FOXO.

Literature Cited

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