Suppression of Gene Expression Following Knockdown of Transcription Factor dFoxo

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Abstract: Looking at the expression of genes, this experiment was aimed at observing how divergence from normal gene expression could lead to either an increase, decrease or inhibition of expression. The goal of this experiment was to knockdown (KD) the transcription factor dFOXO by the addition of double stranded RNA to see how it impacted Insulin. FOXO transcription factors are regulators and mediators of insulin signaling. It was believed that Insulin expression would decrease due to the KD of . It was hypothesized that if the foxo transcription factor was knocked down with an RNAi, then the gene expression of INS (insulin) would decrease. Results showed both a decrease in the FOXO and the dsRNA expression compared to the mock. This indicated that the FOXO transcription factor was successfully knocked down and as a result inhibited the expression of the insulin gene.

Introduction

Expression of genes can be influenced by a number of factors, including both internal and external environmental factors, such as temperature, chemicals and hormones. The divergence from normal gene expression can result in a decrease or increase in expression or it can be inhibited. If a gene is unable to be expressed or over expressed, it can lead to disorder in a system and can cause issues to arise. One way for the expression of a gene to be impacted is to employ the use of RNAi, which regulates the expression of protein coding genes (Paddison et al, 2002). RNAi can inhibit the translation of a protein through the binding to the messenger RNAs of the protein being coded. The purpose of this paper is to explore how knocking down a transcription factor with the use of an RNAi can impact the expression of a target gene of choice. Insulin gene expression is being observed following the knock down of an activator transcription factor that is a sequence-specific DNA binding protein.

In this case, the activator transcription factor, FOXO, was targeted. FOXO transcription factors are mediators and regulators of insulin signaling and belong to the large Forkhead family of proteins. Along with insulin regulation, FOXO can determine human longevity and can regulate stress response, nutrient signaling and DNA repair (Brown, 2018). dFOXO function is necessary for growth inhibition associated with reduced insulin signaling, with the loss of it resulting in suppressing the reduction in cell number (Martins et al, 2016). Since dFOXO regulates insulin, suppressing this pathway could be detrimental if insulin is needed. Insulin is a natural hormone produced in the pancreas and helps control the storage and use of blood sugar in one's body. Insulin also plays a key part in metabolism, since the glucose that enters the cells is then used as

energy and helps maintain normal function. If insulin production were to be suppressed then function of the body would go awry. An example of this is diabetes type 2, where there is an impairment in the ability of the body to regulate and use glucose. This impairment can lead to too much sugar in the bloodstream, which can eventually cause issues dealing in the nervous and immune system (Porte, 2001).

It was hypothesized that if the foxo transcription factor was knocked down with an RNAi, then the gene expression of INS (insulin) would decrease. This is due to FOXO having the ability to increase the insulin-like growth binding protein-1 expression (Gross et al., 2008). If the transcription factor were to be knocked down, then expression of these proteins could decrease, meaning a decrease in the insulin gene expression.

Materials and Methods

Transcription Factor and Primer Selection

First the transcription factor, FOXO activator, was chosen for this experiment. Next, a gene that was to be observed was chosen with insulin being selected.Then, a hypothesis was formulated on how the transcription factor would influence gene expression once it was knocked down by the RNAi. Genome browser was then used to find the mRNA of choice, where the forward and reverse primer and exon were pulled from. Once there was a strand of mRNA, it was put through the Oligo analyzer tool to ensure that it met the requirements of a primer, such as ~40-60% GC content, ended with a GC clamp, and that the melting temp was between 50-60 degrees Celsius. Once the primer met all requirements, and an exon was found, it was submitted into the input form. The primers used during this experiment includes T3/T7 PCR primers, qPCR primers and insulin receptor (INR) primers as seen in table 1.

Plasmid Prep

To prep the plasmid, the chemically competent cells were thawed on ice. Then, 2 ul of the plasmid was added to the cells, making sure there was not more than 10% of the volume of the cells. Once plasmid was added, the cellsflicked gently to mix and then incubated on ice for 30 minutes. After incubation, the cells were heat shocked for exactly 30 seconds at 42^{o}C. Following the heat shock, the cells were placed back on the ice and incubated for 2 minutes. Using a sterile technique of having a Bunsen burner next to the cells when pipetting, 1mL of LB broth was added to the cells. Next, the cells were incubated at 37^{o}C with shaking for 1 hour to recover. Following incubation, 100 ul were plated on one LB agar plate (with Amp) and 900 ul onto another LB agar plate (with Amp), using a sterile technique. The cells were then transferred onto the plate and then used a plate spreader to distribute the cells along the surface of the plate evenly. After allowing the plates to sit agar side down to allow the liquid be absorbed into plates, they were incubated at 37^{o}C overnight with the agar side up.

Picking Colonies

One medium colony from the agar plate was selected to be used to inoculate a liquid culture. After culturing overnight, this liquid culture was used for the plasmid prep. To pick up the chosen colony, Bunsen burner was lit and a culture tube with 5 mL of LB broth was prepared. The cap was loosed on the culture tube and picked up a tip with a P200 pipette, using the tip to pick up the selected colony. The tip with the colony was inserted into the 5 mL of broth into the culture tube and pipetted up and down to distribute the cells in the broth. The tube was then discarded. The Bunsen burner was turned off and the culture tube was placed in the 37^{o}C shaker to incubate overnight.

Plasmid Prep

First, the cultured bacterial cells had to be harvested. Transfer 1.5 mL of the cultured bacterial cells to a microcentrifuge tube and centrifuge at 14-16,000 x g for 1 minute then discard the supernatant, repeating until all cells are used. Next, the cells needed to be resuspended by adding 200 µl of PD1 buffer to the tube and resuspend the cell pellet by pipetting. Next add 200 µl of PD2 buffer and mix gently by inverting the tube 10 times. Let the tube sit at room temperature for at least 2 minutes before adding 300 µl of PD3 buffer and mix immediately by inverting the tube 10 times. Next is the DNA binding. Centrifuge at 14-16,000 x g for 3 minutes and then place a PD column in a 2 ml collection tube. Next add the supernatant from the previous step and centrifuge again for 30 seconds at the same setting, discarding the flow-through once done. Then place the PD column back in the 2 mL collection tube. The next step is washing, start this by adding 600 µl of the wash buffer into the PD column then centrifuge for 30 seconds with the same settings as before. Discard the flow through and place the PD column back in the 2 ml collection tube. Centrifuge for 3 minutes to dry the column matrix. Then place the PD column in a new 1.5 ml microcentrifuge tube. Finally, the step of DNA elution begins with adding 50 ml of elution buffer into the center of the PD column matrix. Let stand for at least 2 minutes to allow the elution buffer to be completely absorbed. Centrifuge for 2 minutes at 14-16,00 x g to elute the purified DNA. Transfer the flow-through back into the center of the PD column matrix and centrifuge at the same settings for 2 minutes.

PCR

For this experiment, stock plasmid was added and EB was added. 3 x 50 ul PCR reactions will be needed, so a master mix that is 165 µl (10% excess for handling) is needed. To do this, 62.7 µl of

Nuclease-free water was first added to the microfuge tube containing the diluted stock solution. Next the T7 primer and the T3 primer was added to the diluted stock solution. Then the template DNA and the Taq 2X Master Mix was added. The reaction was gently mixed and all liquid was collected to the bottom of the tube through a quick spin in the centrifuge. 50 µl of the master mix was pipetted into 3 separate reaction tubes. Tubes were spun to collect the mix at the bottom of the tubes and kept on ice until thermocycling began. The PCR tubes were transferred to the machine with the block preheated to 95°C and began thermocycling. Thermocycle for initial denaturation at 95°C for 1 minutes, then at 95°C for 15 seconds, 54°C for 30 seconds and 68°C for 20 seconds, repeating for 15 cycles. Next, they were thermocycled at 95°C for 15 seconds, 54°C for 30 seconds, and 68°C for 20 seconds, adding a second for each cycle, for 25 cycles. Last step of thermocycling is the Hold, thermocycle at 4°C for a determined time.

Anneal dsRNA

The first step in the DNA cleanup and concentration is to dilute the sample with a DNA cleanup binding buffer. A 5:1 ratio of binding buffer to sample is needed, in this case that means 750 µL:150µL was used. The sample and buffer solution was mixed well by flicking the tube. A column was then inserted in a collection tube and the sample was loaded into the column. The tube was spun for one minute and the flow-through was discarded. The column was then reinserted into the collection tube and 200 µL of the DNA wash buffer was added and then the tube was spun for a minute. Discard the flow-through. The previous step is repeated once more. Next, transfer the column to a clean 1.5 ml microfuge tube. Ensure that the tip of the column does not come into contact with the flow-through. Add 10 µL of DNA elution buffer to the center of the matrix. After waiting for a minute, spin for one minute to elute DNA. For loading the gel, on a strip of Parafilm, aliquot out 1 ul drops of Gel Loading Dye, Purple (6X), one for

each of your samples (negative controls, positive controls, and experimental samples). Add 5 ul of each sample to the corresponding loading dye drop. Load 6 ul of quick-loading purple 1kb plus DNA ladder to the furthest left lane. In the next lane, to the right, add the first sample with the load dye from the Parafilm, making sure to mix the sample in the load dye before loading. Repeat for the positive control and experimental samples in the subsequent lanes. For running the gel, place the cover on the gel box and ensure that the electrodes are aligned. Plug the electrodes into the power supply and run the gel at 150V for roughly 1 hour or until the red dye is about 2 inches from the end of the gel. The gel was imaged on a gel doc to visualize the PCR product.

In Vitro Transcription

First, thaw the frozen reagents on ice. Vortex 10x reaction buffer and NTP solutions until they are complete in solution, keeping the NTPs on ice and 100x reaction buffer at room temperature. Assemble the reaction at room temperature, adding water first, then the NTPs and then the 10x reaction buffer. Since it was a 20 ul reaction,it was scaled up to 100 ul reaction for each RNAP.

Mix thoroughly by gently flicking the tube, microfuge the tube briefly to collect the reaction mixture at the bottom of the tube. Incubate the reaction at 37 degrees for 2 hours to overnight. Stop the reaction and precipitate the RNA by adding 30 ul nuclease-free water and 30 ul LiCl. Mix thoroughly, chill for at least 30 minutes at -20 degrees. Centrifuge at 4 degrees C for 15 minutes at maximum speed to pelt the RNA. Carefully remove the supernatant and wash the pellet once with 1 mL 70% ethanol and re-centrifuge to maximize the removal of unincorporated nucleotides. Carefully remove the 7-% ethanol, allow it to air dry briefly and resuspend the RNA in 100 ul 1X TE 7.5. Determine the RNA concentration and store frozen RNSA at -20 to -80

degrees Celsius. Make sure to complete steps above for one reaction each for the T3 RNAP and T7 RNAP.

Anneal dsRNA

Add equal amounts of both strands to 1 tube and heat to 95 degrees Celsius for two minutes. Transfer to a 65ºCelcius block for 30 minutes, then cool to room temperature by heating the heat block on the bench and allowing it to gradually cool to room temperature.

Prepare Cells

Grow the S2C1 cells in Schneider's media with the pen/strep and 2 mM glutamine 2 days before the RNAi portion of the methods. Split the cells 1:3 into fresh media by carefully aspirating the old media and any floating cells. Resuspend the semi-adherent cells in 6 mL complete media by washing them off the plate by repeated pipetting. Transfer 2 mL to 3 x 25 cm^2 flasks each containing 4 mL media.

RNAi

First, aspirate media and any floating cells, and then resuspend the adherent cells in 5 mL of fresh media. Count the cells and then dilute them to $1.5x10^6$ cells/mL. Plate 8 mL of these cells into each T25 flask. Allow cells to adhere to the flask for 1 hour. Then aspirate the media and any floating cells. Gently wash the cells 2 times with serum-free media, and after washing, add 2.5 mL of serum free media. Add 25 mL of dsRNA. Incubate 30 minutes to 1 hour at room temperature and then add 5 mL of complete media. Cells were harvested and assayed 4 days later.

Anneal and Harvest Cells

First, proper amount of starting material needed was determined. In this case, 700 µL of the TRK Lysis buffer was used. The cells were harvested and disrupted with TKR Lysis Buffer, pipetting up and down to mix thoroughly. The cells were homogenized by loading the lysate into a Homogenizer Mini Column inserted into a 2 mL Collection Tube. They were then centrifuged at maximum speed for two minutes to collect the homogenized lysate. The cleared supernatant was transferred to a clean 1.5 mL microcentrifuge tube, ensuring that the fatty upper layer was not transferred, as it may reduce RNA yield or clog the column. 1 volume 70% ethanol was added and vortexed to mix thoroughly. Then, a HiBind® RNA Mini Column was into a 2 mL Collection Tube. The 700 µL sample (including any precipitate that may have formed) was transferred to the HiBind® RNA Mini Column and centrifuged at 10,000 x g for 1 minute. The filtrate was discarded and reused in the Collection Tube. Repeat transfer of sample and centrifugation steps until all of the sample has been transferred to the column. 500 µL RNA Wash Buffer I was added and Centrifuged at 10,000 x g for 30 seconds. Then the filtarte was discarded and reused in the collection tube.500 µL RNA Wash Buffer II diluted with 100% was added and centrifuged at 10,000 x g for 1 minute. Filtrate was discarded and reused in the collection tube. Last step was repeated for a second RNA Wash Buffer II wash step. Next, centrifuge the empty HiBind® RNA Mini Column at maximum speed for 2 minutes to dry the column. This step is critical for removal of trace ethanol that may interfere with downstream applications. Transfer the HiBind® RNA Mini Column to a clean 1.5 mL microcentrifuge tube and add 40-70 µL Nuclease-free Water. Centrifuge at maximum speed for 2 minutes. Store eluted RNA at -70°C. Spec sample to determine if RNA was properly extracted and purified.

PCR

First the RNA samples were thawed and diluted to 100 ng/ul in RNase-free 1X TE 7.5. Then the total volume for the appropriate number of reactions, plus 10% overage was determined and the assay mixes of all components except RNA template were prepared accordingly. These were mixed thoroughly by gentle pipetting and the liquid was collected to the bottom by brief centrifugation.

Results

The primers used during this experiment includes T3/T7 PCR primers, qPCR primers and insulin receptor (INR) primers as seen in table 1. The insulin receptor primers were used to locate the insulin gene that would be impacted from the dFOXO transcription factor being knocked down. Figure 1 displays the spec of the plasmid yield, with the absorbance levels falling between A260-A280. The agar plate (Figure 2) displays the successful growth of colonies for plasmid prep that would follow. In Figure 3, it shows the gel electrophoresis of the agarose, with the well to the furthest left (#1) containing the ladder and well #5 containing the sample. The bands indicate the DNA traveled roughly 700, matching up with the ladder and meeting with what was expected. Figure 4 displays the RNA spec data, with the 260/280 ratio being 2.47 and the 260/230 ratio being 4.01.Given the spec data, it appears the extraction and purification of the RNA sample was only partially successful. Figure 5 shows the RT-qPRC data analysis, showing the relative expression as a fraction of RP49 of the double stranded RNA target (FOXO) and INR**.** It displays the impact the knockdown had on the RNA target and if it worked. Figure 5 shows that both the FOXO and dsRNA were reduced about ⅛ compared to the mock data.

Discussion

Figure 1 displays the spec of the plasmid yield, with the absorbance levels falling between A260-A280, meeting expected parameters, meaning we could continue on with the experiment. If it had been closer to A230 then there would have been salt present and we would have had to filter the sample again. In figure 3, the bands indicate the DNA traveled roughly 700, matching up with the ladder and meeting with what was expected. If the DNA had traveled elsewhere it would mean the sample hadn't been filtered completely. In figure 4 it is seen that the 260/280 ratio is 2.47 and the 260/230 ratio is 4.01. A pure RNA sample is supposed to have a ratio of 260/280 and 260 /230 of roughly 2. While the 260/280 ratio is relatively close to 2, the 260/230 ratio is double that. This could be due to unwanted organic compounds or contamination.

Results as indicated in figure 5 show a decrease in the FOXO and the INR expression as compared to the mock sample. While not a huge decrease, this still indicates that the FOXO transcription factor was successfully knocked down and as result inhibited the expression of the insulin gene expression. These results support the hypothesis that if the foxo transcription factor was knocked down with an RNAi then expression of the INS gene would decrease.

Limitations of this study included some slight human error and having to use insulin receptors instead of the insulin gene itself. An error that occured was that when centrifuging the cells, there appeared to be a backup in the filter, meaning too many cells had probably been scrapped off. This did not appear to affect the results though.

Citations

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Figures and Graphs

Table 1. Primer Sequences used during Experiment. Includes T3/T7 PCR and qPCR primers

Oligo Sequence	Description
AATTAACCCTCACTAAAGG	T3 Promoter
TAATACGACTCACTATAGG	T7 Promoter
CCACCAGTCGGATCGATATGC	dRP49 F
CTCTTGAGAACGCAGGCGACC	dRP49 R
TCGAAACGTTGAGATCGTTG	dINR F
CGCACTTGTATTTCGTGGAA	dINR R

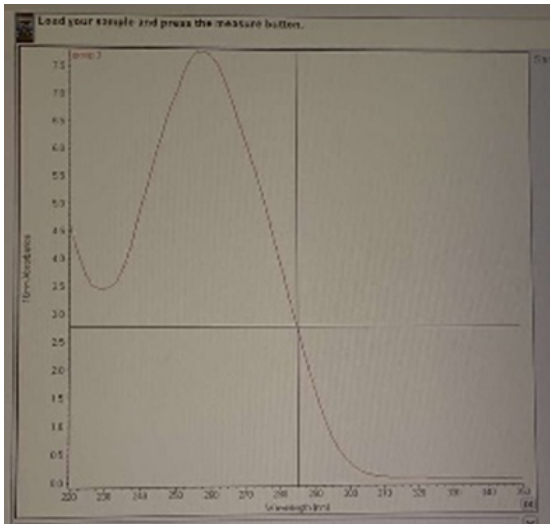

Figure 1. Spec of Plasmid Yield. The absorbance levels fell between A260-A280.

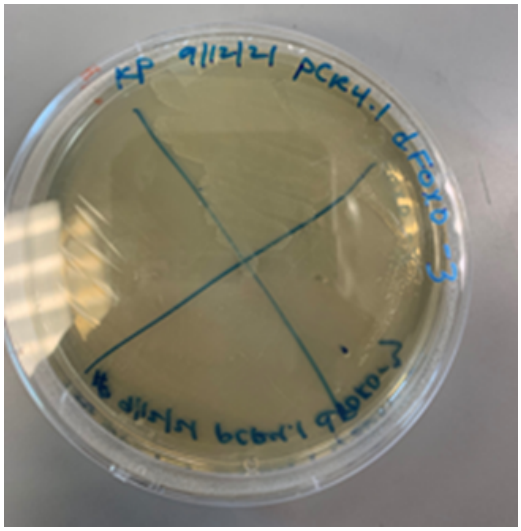

Figure 2. Agar Plate displaying growth of colonies for plasmid prep.

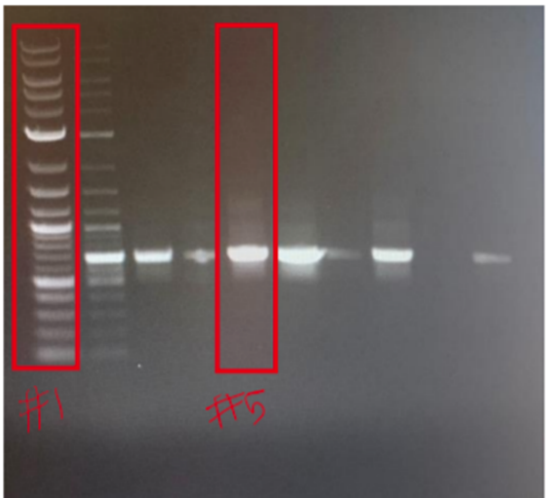

Figure 3. Agarose Gel Electrophoresis. The well to the furthest left (#1) contains the ladder and well #5 contains the sample. The bands indicate the DNA traveled roughly 700 bp.

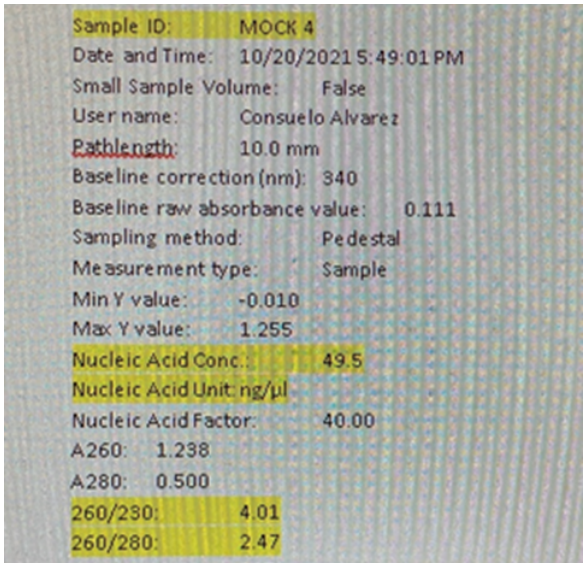

Figure 4. RNA Spec Data. The 260/280 ratio is 2.47 and the 260/230 ratio is 4.01. The RNA concentration is 49.5 ng/ul.

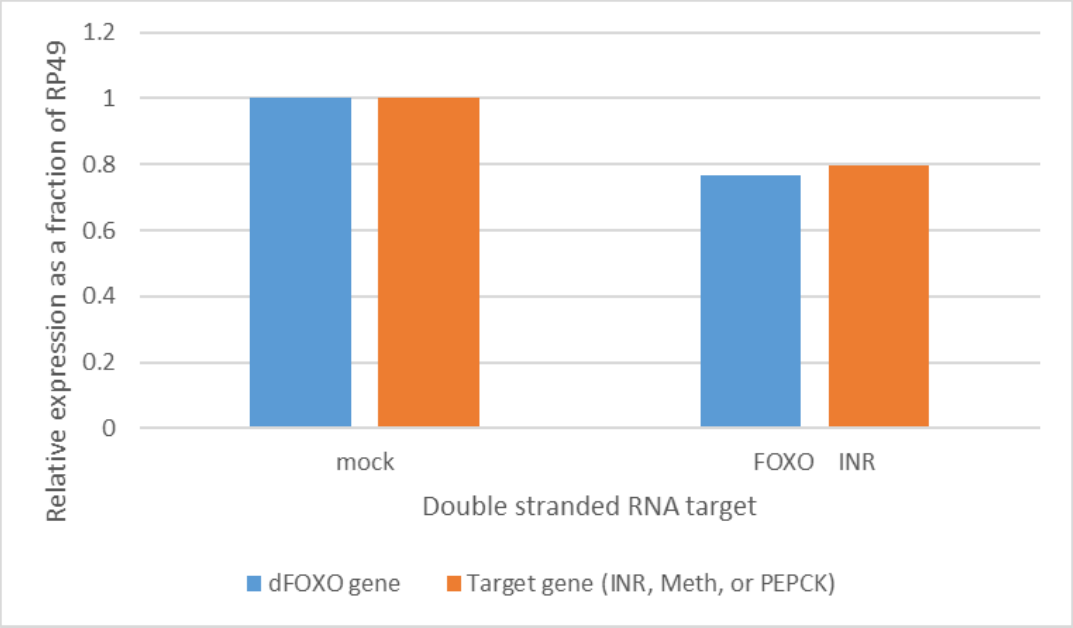

Figure 5. Relative expression as a fraction of RP49 of the double stranded RNA target (FOXO) and INR. Displays the impact the knockdown had on the RNA target and if it worked.