Mutating the MYF-5 gene and the analysis of mutated Myf-5 proteins

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Introduction

Skeletal Muscle Tissue is one of three major types of muscle tissue developed during myogenesis in embryonic development. The muscle is composed of striated muscle which is attached to the tendon and, in term, is directly connected to the bone (Panada 2016); therefore, skeletal muscle is voluntarily controlled and facilitates basic motor movement in humans. Skeletal muscle tissue can suffer minor damage and subsequently be repaired through hypertrophy, such as after a hard workout. However extensive damage or chronic/hereditary diseases, like muscular dystrophy, can cause the skeletal muscle to be torn from the bone or degrade, respectively, leaving those specific limbs or body parts without any means of movement.

Myf-5 is one of several Myogenic Regulatory Factors (MRF’s), which are a group of basic Helix-Loop-Helix (bHLH) transcription factors that play a key role in the differentiation of muscle tissue during myogenesis (Braun 1990). Myf-5 specifically plays a role in developing skeletal muscle and is the earliest of all MRF’s to be expressed in a developing embryo. Myf-5 is also found to express in other, non-muscles, cells such as progenitors and neurons, post myogenesis (Panda 2016). The purpose of the research was to find if one could take the Myf-5 protein and use it to grow or transform individual, undifferentiated, cells into strands of skeletal muscle cells in-vitro. The research gives an opportunity to discover if one can apply what was learned to regrow or insert skeletal muscle into humans who may have suffered a loss of skeletal muscle tissue; moreover, the research aids possible medical research with opportunities in surgery and medicine. To get Myf-5 to potentially change singular cells in to strands, the Myf-5 proteins, of Mus musculus, would be mutated at specific sites then, to see if the mutations took place, the proteins would be sequenced using an Sequencer to determine if specific amino acids where altered. The protein would then be analyzed through an SDS-Page protocol to see if the mutated proteins would overexpress and therefore potentially cause the individual cells to form muscle strands.

The mutation of Myf-5 would be carried out with a QuikChange Lightening Multi Site-Directed Mutagenesis Kit from Agilent Technologies. The overall process is like a Real Time Polymerase Chain Reaction, where primers are used to consistently create copies of DNA through heating cycles. However, a plasmid is used instead of a strand of DNA, and the primers used to mutate the plasmid are pushed into the annealed plasmid. Once the plasmid is mutated, an enzyme called Dpn I is used to digest the original, non-mutated plasmids, and the mutants are injected into cells to be cloned and further used. The Myf-5 gene would have bacterial expression and be obtained through the Addgene website. Six amino acids would be chosen to be changed based on whether the nucleotides in the acids could dimerize, therefore, six different samples of Myf-5 with their own mutations would be tested. The basis gives an idea on what could be mutated and still have the protein function. The primers used where specifically designed through the Agilent website and order through the Hampden-Sydney College Biology Laboratory.

Once the DNA is cloned, it would be sequenced in two ways for to ensure the DNA properly mutated. A set of samples would be shipped to Virginia Tech University to go through Sanger Sequencing. Another set of samples would be created to be sequenced with an Oxford Nanopore MinION Sequencer. The MinION functions by taking the DNA and using an electrode to pull DNA strand through pores composed of protein. The MinION would read the individual nucleotides based on how their individual sizes affect the flow through the pore. Finally, the plasmids would be inducted with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce over expression and then analyzed through an SDS Page protein gel where fragments of inducted, mutated, Myf-5 would be compared to fragments of induced, non-mutated, Myf-5 to see if the mutated Myf-5 still function. Moreover, to see if the mutated Myf-5 could be over express.

The research may possibly aid other studies of muscle by giving insight on how one could use Myf-5 to grow entire strands of muscle to be insert into those who have lost theirs in one way or another.

Materials and Methods

Site Directed Mutagenesis

The Myf-5 plasmids were first run through a Qiagen Spin Miniprep Kit to isolate the DNA. The kit was provided by the laboratory at Hampden Sydney Collage, and all reagents were provided by the kit. Once the DNA was isolated, the DNA was prepared to go through site directed mutagenesis. A QuikChange Lighting Multi Site-Directed Mutagenesis

Kit was provided by the Agilent website, and all reagents were provided by the kit. Six PCR tubes where gathered and each tube was labeled 1-6 to stand for the following mutations each tube would have changed:

1. Alanine 88 to Arginine
2. Threonine 89 to Leucine
3. Lysine 98 to Valine
4. Proline 119 to Glutamic acid
5. Tyrosine 130 to Lysine
6. Isoleucine 131 to Aspartic acid

Each of these reagents were pipetted into the tube:

- 2.5 µL of 10x Reaction Buffer
- 18.5 µL of ddH2O
- 2 µL of DNA template (Myf-5 plasmid)
- 1 µL of dNTP mix
- 1 µL of Enzyme blend

Once the solutions were added to the tubes, 0.5 µL of the primer that would mutate each sample to its specific mutation would be added to each tube (Tube 1 would have the primer to mutate the Alanine 88 to Arginine and so on). The tubes would then be left to go through a PCR reaction overnight. Once the PCR cycling finished, the samples are set to chill in ice for 2 minutes.

After chilling, 1 µL of Dpn I restriction enzyme is added directly to each sample and carefully mixed by pipetting each sample up and down several times. The sample are then spun down in a microcentrifuge of 1 minute, then incubated at 37˚C for 5 mins to facilitate the digest of the parental DNA.

Cloning Mutated Myf-5 Genes

Once the digestion is complete, the samples were set aside and the frozen XL 10-Gold ultracompetent cells were collected. The cells would be used to clone the mutated plasmids. The cells were gently thawed, and for each of the six samples, 45 µL of cells were aliquoted to six separate prechilled 14-ml BD Flacon polypropylene round bottom tubes. 2 µL of β-ME mix was added to each tube, and each tube was gently swirled. Each tube was then set in ice for 10 minutes and swirled every 2 minutes of the 10. After incubation, 1.5 µL of the Dpn I-treated DNA was add to its appropriately labeled tube then incubated in ice for 30 minutes. While the tubes were incubating, 3 mL of LB broth and 5 µL of ampicillin was added to a 10-mL conical tube and preheated to 42˚C in a water bath.

After the incubation, the tubes containing the cells and DNA where heat shocked in the water bath for exactly 30 seconds in the same water bath, then put back on ice for 2 mins. The heat shock is meant to open the bilayer on the ultracompetent cells to allow the DNA to enter the cell. If the heat shock went on for too long, many of the cells would have been destroyed. 0.5 mL of preheated broth was added to each sample of DNA and left to incubate for 1 hour at 37˚C shaking at 250 rpm. During the hour, 6 petri dishes were prepared and labeled with the appropriate mutations, and once the hour passed the entirety of the sample in each tube was pipetted onto each appropriate plate and evenly distributed with a spreader. Plates were left to incubate at 37˚C overnight. Remaining reagents and cells were properly stored.

Sanger Sequencing

Cells from the plates were swabbed and incubated in an overnight culture, then DNA was isolated from a Spin Kit and quantified. Instructions at the Virginia Tech Website were followed and primers where specifically designed on the Primer3 website for reading each mutant forward and reverse sequence. 10 µL of mutant Myf-5 were pipette twice into separate PCR tube strips, while 3 µL of forward primer and reverse primer where added separately for a total pf 13 µL (two PCR tubes would the same mutant Myf-5, while one tube would have forward primer, and the other reverse). One tube was mark with initials, and all tubes were marked with a number from 1to 8 for reference as to what was in the tubes. The strips are then wrap in parafilm and shipped with ice packs to Virginia Tech. The results were emailed roughly 2-3 weeks later.

MinION Sequencing

For the MinION to run, a program called MinKNOW and Metrichor had to be installed from the Oxford Nanopore website. A powerful computer is also required to properly run the MinION and a program to check for compatibility is also available on the website. MinKNOW runs the MinION and sequences the DNA, while Metrichor stores the sequencing information in a cloud to be reviewed later. Lastly, before sequencing can take place, a quality control test must be performed with the QC cell that comes with the MinION for calibration.

Once QC is performed, a lambda control test must be performed with the flow cell, the cell that contains the pores for sequencing. The test is to ensure that the pores properly work and will sequence. The protocol for lambda control is the exact same as the protocol with other strands of DNA; however, the lambda DNA is replaced with the experimental DNA.

All reagents came with the MinION when it was first ordered. In a PCR tube, the lambda control DNA was prepared with 4.0 µL of lambda DNA, 2.5

µL of FMR, and 3.5 µL of nuclease free water. Mixed with inversion, the tube is then set on a heat block at 30˚C and 70˚C for 1 minute each. 1 µL of RAD is added to the tube then left to incubate at room temperature for 5 minutes, and the overall solution is call tagmented DNA. In an Eppendorf DNA LoBind tube, a solution call prime mixing is created with 500 µL of RBF1 and nuclease water for a total of 1000 µL. The port on the flow cell is opened by sliding clockwise and 40 µL of storage buffer is pipetted into the cell. Once the storage buffer is added, pipette 500 µL of the priming mix into the cell carefully (no bubbles), and after 30 minutes, 300 µL more is added to the cell. After 10 minutes 200 µL of mix is added to the open spot port on the cell.

For loading the library (DNA into the flow cell) and running the sequence, in an Eppendorf tube a solution is created with 37.5 µL of RBF1, 31.5 µL of nuclease free water, and 6.0 µL of the tagmented DNA. 75 µL of the solution is pipetted, drop wise, into the spot port and the sequence is run on MinKNOW for roughly 6 hours. Once sequencing is finished, results could be found on Metrichor.

IPTG Induction of Mutated Myf-5

The mutated Myf-5 samples are once again swabbed and 6 mL were cultured overnight in 15-mL conical tubes. Along with the Myf-5 samples, two other samples (MyoD, another MRF that binds directly to Myf-5, and PGEM, a plasmid that holds a T7 promoter like Myf-5) were also cultured to serve as comparisons during SDS Page, since ladder wasn't used. The next day, the cells must be run through OD600 to ensure that the cells are still progressively dividing and growing, that way the cells are still expressing their genes. 1 mL of water is pipetted into a cuvette as a blank, and 1 mL of each culture is put in separate cuvettes. Once the blank is set, the cultures are set in the OD600; moreover, if the cultures are between 6.0-8.0, then the sample they were aliquoted from can be set aside for the next step. If significantly over 8.0, then the samples must be diluted at 1:100 and set in the rotator again for 3-4 hours. If under 6.0, then sample must be re-cultured. 2.5 mL of the samples can then be centrifuged at max speed for 30 seconds, and the pellets can be frozen at -20˚C, after the supernatant is removed, until needed as the uninduced samples.

For induced samples, a milter of LB broth for each sample, with 5 µL of ampicillin and 1mM of IPTG, was prewarmed in a 15-mL conical tube for 10 minutes. Once warmed, 1 mL of prewarmed IPTG solution was added to 1 mL of each culture and left to rotate for 3-4 hours at 37˚C. After the 3-4 hours, the samples were pelleted in a 1.5 mL centrifuge tube at max speed for 30 seconds. The supernatant can be removed, and the pellet can once again be frozen at -20˚C until needed.

SDS-PAGE

SDS-PAGE was run to destroy the inducted cells while preserving enough proteins to be analyzed in a protein gel. First the solutions to run the gel were created, and all reagents were provided by the lab. Gels were premade and ordered from the bio-rad website. 1 L of 10x SDS running buffer was created by taking 300 g of a pre-made Running buffer compound and mixing it with 1L of ddH2O. 4X SDS loading buffer was mixed in a fume hood with the following:

- 16 mL ddH2O
- 5 mL 0.5 M Tris, pH 6.8
- 8 mL 50% Glycerol
- 8 mL 10% SDS
- 2 mL 2-βmercaptoethanol
- • a couple of drops of bromophenol blue

Once the solutions are made, the pellets from the IPTG induction were collected, and 100 µL of loading buffer was added to each sample. Samples were then centrifuged for 1 minute at max speed, then each sample was set to boil for 5 minutes. When finished boiling, the samples were centrifuged at max speed again then set aside for later. A vertical electrode stand was set up, along with a power source, and the 10x SDS buffer was pour into the stand up to the selected point. 2 premade gels were unpacked and loaded onto the apparatus, and 10x SDS buffer was pour into the space between the gels to just over the exposed wells. The samples were loaded with each mutant having its induced and uninduced variants next to one another. The apparatus was left to run for an hour at a voltage of 50 mA, and the gels were, carefully, extracted from there casings.

The gels were set in small, used pipette containers to be stained with Coomassie blue. Gels were first washed in water for 5 minutes 3 consecutive times. The water was removed and 50 mL of blue was added to each gel, and left to rotate, gently (10-20 rpm) for an hour. Gels were then rinsed in water for 30 minutes then stored in clean water for later analysis.

Results and Discussion

Cloning the mutated Myf-5 plasmids produced results in all but two plates, with the plates being 5 and 6. The cells in the failed plates may not have cultivated due to the mutations in the plasmids possibly preventing the cells from dividing. It is unknown how the expression of the gene may have prevented the cells from dividing, but it may be possible. The cells may have also just undergone lysis, either due to the heat shock, to open the cell wall, or to the mutations.

Again, it is unknown how the mutations would cause the cells to lyse, but it is still possible.

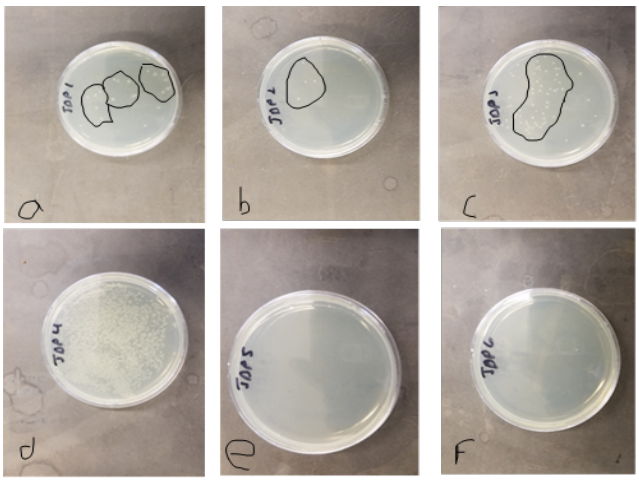

Figure 1: Transformants from site-directed mutagenesis

The Virginia Tech Sanger Sequencing had to be performed twice, and both results ended with inconclusive data. The first attempt concluded with no data due to the liquid losing volume somewhere during the shipping process. The second attempt managed to ship to Virginia Tech without a loss of volume; however, of the 8 samples sent, only 5 sequences could be analyzed, with the results that could be analyzed only being samples 1 and 2, forward and reverse, and sample 3 forward, and even then, the results are enigmatic. Figure 4a is meant to be the Myf-5 mutation for Alanine 88 to Arginine (sample 1), but the results give the codon GGC which is the amino acid for Glycine. Oppositely, the reverse primer for sample 1 gives the codon GGG which is also the amino acid for Glycine. For sample 2, which is meant to be Threonine 89 to Leucine, the Threonine, ACC, was altered to Tryptophan, TGG. Sample 3 forward, Lysine 98 to Valine, the lysine AAG, was once again changed to Glycine, GGG. It is quite possible that Sanger Sequencing may not have accurately read the plasmids due to various mishaps. One mishap may have been the shipping again, though there was never a notification from the University; moreover, after research was concluded it was discovered, from the Virginia Tech website, that the most accurate results are found from DNA suspended in ddH2O rather than the elution buffer that was provided by the Spin Kit. This claim can be further supported by fact that some of the nucleotides seem be shifted from their original positions in the graphs; unfortunately, it cannot be accurately weather appropriate mutations occurred.

The MinION sequencing couldn't even begin after the lambda control experiment. The flow cell is meant to have over nine thousand pores functioning, and last for roughly 5 more sequences. However, the new flow cell wasn't used for a year prior, and after the experiment only 1500 pores were open. After the first attempt a sequencing, only 100 pores were left, so it is quite possible that the protein pores degraded over the year it wasn't used. The first attempt at sequencing also produced inconclusive results. The MinION was left to run overnight, but even after setting the laptop to not sleep, it fell asleep. The MinION shutdown and the results could not be recovered.

Though the results for sequencing were overall inconclusive, the SDS Page gives some insight to whether the plasmids produced mutated Myf-5 proteins.

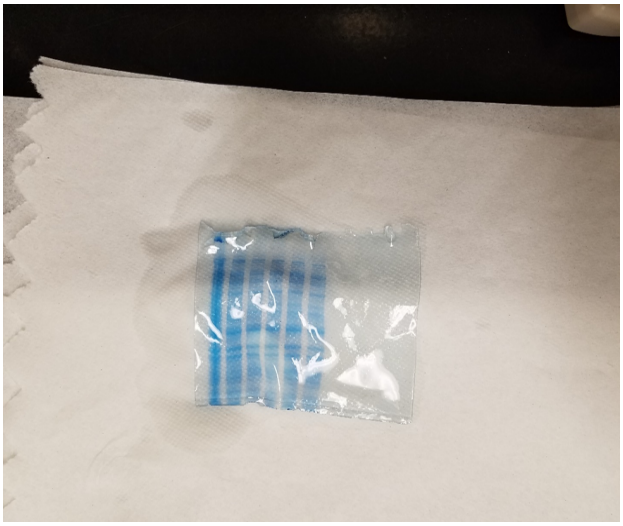

Figure 2: SDS Page and Protein Gel Sample

The first sample with the uninduced well is significantly darker than the rest due to a clumping of cells unfortunately being pipette into the well; however, the results of the well can still be seen. Across the highlights, all the bars are consecutively the same shade of dark blue meaning the IPTG cause the same genes in all samples to be overexpressed. This means that those bars be controls, and what was being observed was whether other bars formed showing some form of overexpression in the mutants. The additional bars would mean the mutants were successfully functional; unfortunately, nothing different stood out in the wells, so one could conclude that, whether the plasmids mutated, the proteins produced may not have be any different than before site directed mutagenesis.

Conclusion

The research unfortunately did not yield many results that would be helpful to use Myf-5 as a protein to incite muscle growth or development. The best option to proceed with the research would be to recreate the Virginia Tech sequencing using water to hold the

DNA rather than the elution buffer. The MinION shows the potential to sequence, and it was rather unfortunate for the flow cell to have degraded even after properly stored. The laptop sleeping also denied the ability to give results. Therefore, the best option would be to gather fresh supplies and find the time to stay with the computer and sequencer to get the best results.

SDS-PAGE went by rather smoothly, so to get bars that would imply a mutant overexpressing, one may have to mutate other areas on the Myf-5 plasmid, or one would have to run the plasmid through random mutations and chance that the protein will function and overexpress.

Moving forward, the next step would be to run the mutant's trough an affinity chromatography column.

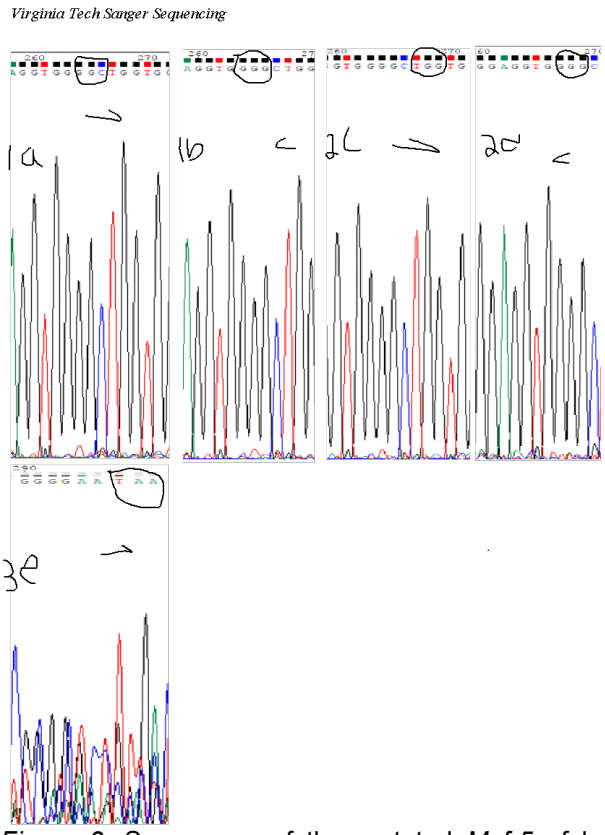

Figure 3: Sequences of the mutated *Myf-5* of both forward and reverse primers. The circles indicate the nucleic acids/amino acids that were meant to be mutated, and the arrows indicate direction of translation. Numbers indicate sample number.

The column would serve to inform if the mutant Myf-5 DNA could attach to other MRF's like it does during mutagenesis. The ligand would contain MyoD since it would already be available from the SDS Page procedure. The columns would be run with no mutated MyoD to see if the mutants could bind without change MyoD. Of course, columns would also be run with mutated MyoD to see if the Mutant Myf-5 required a mutant binder.

Though not many useful results were found, more experimentation would be required to rule out Myf-5 as a reliable protein to aid in skeletal muscle synthesis.

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