Aptamer Selection RNA Pool N71 Against Amytorphic Lateral Sclerosis Related Mutant SOD-1 for Targeted Delivery of Functional Proteins

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RNA Pool: N71

Cobalt Bead Based Selection

Aptamer Target: SOD-1

Overview:

Amytorphic lateral scelerosis (ALS) is a disease that when diagnosed, an individual will lose voluntary muscle movements, ability to speak and ultimately face respiratory failure. Approximately 20% of ALS cases are caused by Superoxide-dismutase 1 (SOD1) mutations (Cashman, 2003). SOD1 is a homodimer protein linked to a gene on chromosome 21 (Gene Cards, n.d., Roulou, 2011). The mutation in the SOD-1 gene results in death of motor neurons due to an accumulation of superoxide radicals (Figure 1) due to misfolding of the protein by altering the amino acid sequence, and in most cases replacing alanine with valine (U.S. National Library of Medicine, n.d.). The alteration of the amino acid sequence leading to breakdown of muscle movements are known to be ALS.

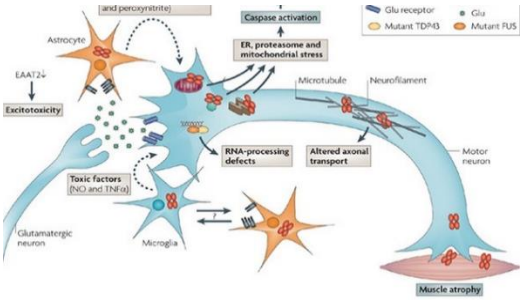

Figure 1: Mutation in SOD-1 (Bosco, 2015)

The intention of this project is to provide enough functional SOD-1 protein by an aptamer delivering it to the affected individuals. An aptamer is an oligonucleotide that binds to a specific target. In this case an aptamer will be designed for an *in vitro* targeted drug delivery of the functional SOD-1 protein. The aptamer will bind to the functional protein as a means of to introduce functional SOD1 proteins to aid to motor neurons lacking sufficient protection against oxgyen radicals. Aptamer selection may be a more efficient and cost-effective way to deliver an appropriate amount of functional proteins to eliminate the accumulation of oxygen radicals, preventing further damage to motor neurons by binding tightly with the protein.

This drug delivery aptamer will be found by cobalt bead-based selection and used for drug delivery. SOD1 features a poly-histidine tag at the N-terminus that can be utilized in cobalt bead based selection (Biosystems Acro, n.d.) Several rounds of systematic evolution of ligand by exponential enrichment (SELEX), are required to narrow down the RNA strand that functions as an aptamer.

The first round of selection is underway for a 1:1 pmol target SOD1 with RNA pool N71. To current date binding and selection of N71 RNA pool to the target SOD1 and reverse transcription has been completed to attempt having more concentrated washes. Washes W0, W3 and E1 participated in reverse transcription in PCR to produce single-stranded DNA. Originally, for ccPCR, where the optimum number of amplification cycles are necessary,

bands were produced from the W0 wash but W3 and E1 had none. W0, the most concentrated wash, with bands from cycle six to twenty. However, the bands for each cycle appeared faint. It is suspected though that there is simply a low concentration of target in the washes. For a more accurate determination of the appropriate number of cycles to amplify the DNA, binding and selection should be repeated.

Introduction and Background:

ALS is a neurological disease that involves the deterioration of motor neurons (Cashman, 2003). Currently, there is no cure for ALS. Those individuals affected lose voluntary muscle movements, ability to speak and ultimately face respiratory failure. Approximately 20% of ALS cases are caused by Superoxide-Dismutase 1 (SOD1) mutations (Cashman, 2003). SOD1 is a homodimer gene of two identical proteins located in the cytoplasm on chromosome 21 that has a molecular mass of 16.8 kDa (Gene Cards, n.d., Roulou, 2011). It is important to note that homodimers are proteins that have a much higher mean of hydrogen-bonding than heterodimers, meaning they have an emphasis of hydrophobic interactions (Zhanhua, 2005). The hydrophobic interaction improves the binding affinity of aptamers (Hasegawa, 2016).

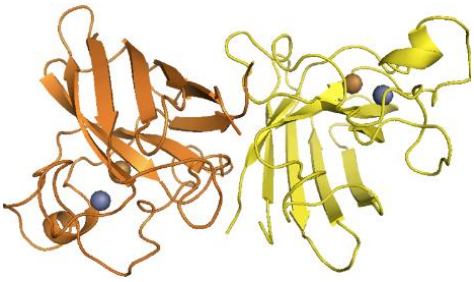

Figure 2: SOD1 homodimer (Minikel, 2015)

The mutation in the SOD-1 gene results an aggregation of misfolded non-functional proteins resulting in the death of motor neurons due to an accumulation of superoxide radicals. A superoxide radical is a charged oxygen molecule that is toxic to animals. In normal conditions the gene codes for SOD-1 which binds to copper and zinc to break down superoxide radicals (U.S. National Library of Medicine, n.d.). The mutation results of the misfolding of the protein by altering the amino acid sequence (U.S. National Library of Medicine, n.d.).

Current attempts to reverse the mutation include Cluster Regularly Interspaced Short Palindromic Repeats to genetically replace SOD1 (Gene Cards, n.d.). However, this is an expensive kit that costs about $1300 per knockout box. CRISPR incorporates plasmid DNA into the system to produce the corrected gene in the new plasmid DNA. While this could be beneficial to patients at a young age before ALS does too much irreversible damage, for adults the help this would offer would be minimal. For adults who have suffered from the side effects of the disease will have already deteriorated their body which CRISPR cannot even undo. Aptamer selection may be a more

efficient and cost-effective way for drug delivery to the 154 amino acid containing SOD1 to reverse the misfolding of the mutated gene.

An aptamer is an oligonucleotide that binds to a specific target. They are beneficial because there are several applications to them including diagnostics, therapeutics, and drug delivery. Benefits of aptamers compared to other treatments such as CRISPR is that they are less expensive and not immunogenic. An aptamer has been discovered to diagnose and provide therapy for colorectal cancer (Chen, 2017). Their success provides that aptamers are worthy of treating serious diseases. In this case an aptamer will be designed as targeted drug delivery applied to SOD-1. An aptamer will bind to a functional SOD-1 protein where it will deliver additional wild type SOD-1 to make up for the accumulation of superoxide radicals that the misfolded SOD-1 cannot fixate. This cannot remove the mutated proteins but will help convert the toxic molecules that inhibit cell functioning. It has been found in recent studies that the primary location of chemical radicals is the spinal cord and the motor cortex in both sporadic and familial cases of ALS (Barber, 2006). For an aptamer to reach the targeted motor neuron site, it must be able to pass the blood brain barrier. The blood brain barrier (Figure 3) protects the brain from changing plasma concentration. Only small molecules can pass through this barrier of endothelial cells. Most drugs are unable to pass these limitations, making it difficult to deliver treatments to neurodegenerative diseases. Several methods are being tested as the best way to pass the blood brain barrier. The most promising for delivering a functional protein through the blood brain barrier is to attach an enhancer with it. A permeability enhancer could be co-administered to increase permeability, allowing the aptamer and functional SOD-1 to pass (Dong, 2018). Recent studies show the most success with a combination of puerarin and borneol to increase permeability in humans (Dong, 2018).

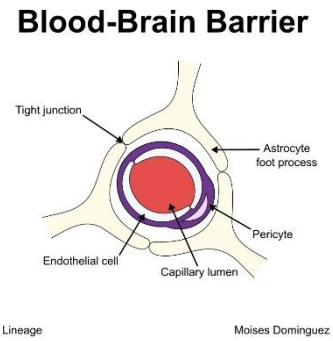

Figure 3: Blood Brain Barrier: Endothelial Cell

In recent years an aptamer has been proposed to diagnose SOD-1 associated with ALS (Cashman, 2003). In this study a peptide aptamer is suggested to be used as an aptamer targeting SOD-1 because they have unique affinities for small differences such as the change in one amino acid and varying folding conformations, like SOD-1's mutation (Cashman, 2003). This is a proposed application of an aptamer and has not yet been discovered. The lab currently assigned to develop this is Promis Neuroscience Inc.

To current date binding and selection of N71 RNA pool to the target SOD1 and ccPCR to determine the optimal numbers of PCR cycles have been completed. N71 is an RNA pool that in dsDNA form has 132 base pairs and as RNA 112 base pairs. If an aptamer can be discovered, functional SOD-1 proteins can be delivered to prevent further damage to motor neurons by binding tightly with the protein. This would affect those diagnosed with familial ALS allowing their symptoms to be alleviated.

Materials and Methods:

To begin to identify an aptamer SELEX is required, beginning with a binding reaction. A selection buffer of 200 mM HEPES of pH 7.5 (will be used to immobilize cobalt beads and the target protein SOD-1. The immobilized protein and RNA pool N71 were incubated for thirty minutes at 37C, same as the conditions it will be under in the human body. Three washes were collected of RNA to separate the unbound and bound target. The RNA was ethanol precipitated and then a reverse transcription reaction underwent denaturation at 65C for five minutes to purify the RNA. To the denatured RNA added and incubated at 42C for fifty minutes followed by being heat inactivated at 70C for fifteen minutes. From this process, ssDNA is acquired.

Following this process, cycle course polymerase chain reaction of N71 reverse and forward primer with T7 promoter and Taq DNA polymerase was performed on the ssDNA to amplify the ssDNA and determine the best number of PCR cycles for the RNA pool. The annealing temperature set to 55C during PCR for the N71 primers to attach to the DNA in preparation for elongation. Agarose gel electrophoresis concluded that selection was successful by products producing bands under UV light. Following, large scale PCR was performed to amplify ssDNA from the most concentrated E1 wash of the RNA N71 pool in large amounts. EtOH precipitate was performed and then a transcription reaction was completed to reverse the dsDNA to RNA again. Polyacrylamide gel electrophoresis purified the full-length RNA and tested that it was produced from the reaction under UV shadowing. This concludes one round of in vitro selection. Upon finishing one round a binding assay must be completed. Multiple rounds are required to identify aptamers.

Target Information:

Superoxide dismutase-1, SOD1, has an affinity His-tag. This target can be purchased from Sino Biological at https://www.thermofisher.com/antibody/product/Human-SOD1-Recombinant-Protein/LF-P0010 , their phone

number is 1-800-955-6288. Its catalogue number is LF-P0010. The molecular weight is 15,936 g/mol. The target costs $227 for 500 ug. For each round 200 pmol is required, therefore each round will cost $1.45.

Results and Discussion:

The first round of aptamer selection is using 1:1 pmol target SOD-1 target to RNA pool N71 ratio. This selection occurred with HEPES buffer and cobalt bead-based binding and selection. In every mL of bead solution, 95,238 pmol of protein can be used. For binding and selection, the binding affinity was 2.1 μL of beads were used because 200 pmol of protein was being used. In this selection an incubation time of twenty-five minutes and 37C was set. At this point target immobilization, binding and selection, RNA elution, EtOH precipitation, reverse transcription, and cycle course PCR have been completed. Washes W0, W3 and E1 participated in reverse transcription to produce single-stranded DNA that is isolated for the ccPCR reaction. The results from ccPCR did not allow for the experiment to continue the next step in SELEX.

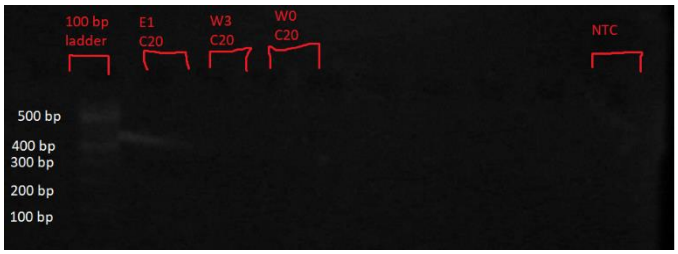

Figure 4: ccPCR 20 (20 cycles of ccPCR) of round one of selection against SOD-1 with washes E1, W3, and W0 dsDNA product. Washes E1, W3, and W0 are eluted following reverse transcription used as template DNA in ccPCR. A NTC (no template control) excluding any DNA product (replaced with diH2O) is included ccPCR 20 of round one of selection against SOD-1 with washes E1, W3, and W0.

The initial performance of ccPCR to 20 cycles of washes E1, W3, and W0 produced a gel (Figure 4) that suggests a failed ccPCR reaction. The 100 base pair ladder appeared, suggesting that gel electrophoresis was successful. This prompted for an efficacy check.

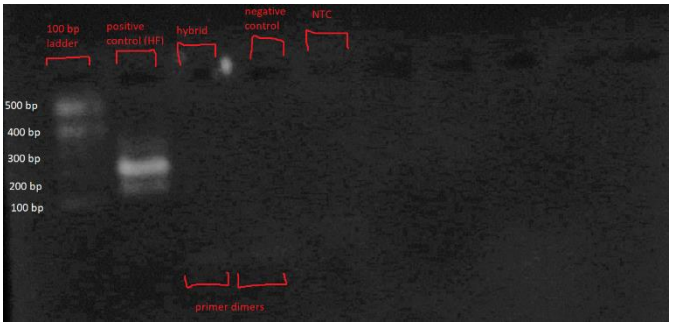

Figure 5: ccPCR 20 efficacy check with Hailey Ferrel's reagents as a positive control, a hybrid replacing forward and reverse primers, a negative control of original reagents with wash W0.

An efficacy check was performed with a positive control from Hailey Ferrell's reagents. The hybrid replaced the negative control's reverse and forward primers. The efficacy check provided useful information to the future of the experiment. The positive control exhibited bands as well as the 100 bp ladder. If the primers were the reagent causing the original reaction to fail, then bands would have been produced in the hybrid reaction.

However, the primer dimers labeled in Figure 5 appeared. This illustration leads to the conclusion that the primers were amplified, and another component of PCR failed. It came to the attention of the research lab that the Taq DNA polymerase made on a particular date was dysfunctional. An additional ccPCR reaction with the newly made Taq DNA polymerase would have bands if that were the cause of original failure.

An additional PCR reaction of round one of selection against SOD-1 target protein was completed with newer Taq DNA polymerase. Gel electrophoresis was successful, as indicated by bands from the 100 bp ladder in Figure 6, but ccPCR failed. This suggests that an earlier step in SELEX had failed, explaining as to why no bands could be produced with functional reagents. Reverse transcription should be repeated at this point to examine if it is the cause of failure in PCR.

Figure 6: Second attempt of ccPCR 20 of round one of selection against SOD-1 with washes E1, W0, and W3.

Reverse transcription was repeated to ensure that the reaction had been previously performed correctly. Following reverse transcription ccPCR followed. This ccPCR failed as well. Due to technology failure, a gel couldn't be recovered for this reaction. The gel from this reaction only produced bands for the ladder. At this point in efficacy check testing the primers and another for Taq DNA Polymerase had been performed. The results from these experiments strongly suggest that binding and selection had not produced a concentrated enough product.

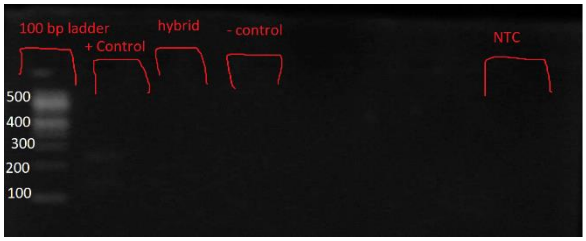

*Figure 7: An efficacy check using Anaelle's positive control reagents and W0 product*In this efficacy check, Anaelle's reagents and successful W0 were used for the positive control. The hybrid consisted of my reagents and Anaelle's W0 product. The image quality of the gel (Figure 7) doesn't clearly demonstrate what was observed in the UV light visualizer. The 100 base pair ladder and the positive control produced bands, but the hybrid did not. This confirms there was an issue with binding and selection. At the point, it is necessary to restart round one of selection.

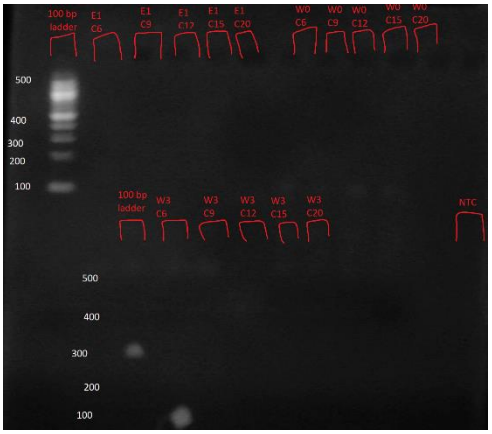

Figure 8: Round one of SELEX has been restarted. This is the first attempt of ccPCR 20 with the new RT product, including W0, W3, and E1 and an NTC.

The first steps of SELEX were repeated. A binding and selection reaction with cobalt beads was performed, followed by reverse transcription. The results of ccPCR20 are shown in Figure 8. Again, only the ladder provided bands. Due to this, an efficacy check must be performed. It is suspected that the 10X PCR buffer crashed. In a future experiment, the success of the 10X PCR buffer will be tested in an efficacy check. The hybrid will consist of my reagents and a positive 10X PCR buffer.

Conclusion and Future Works:

The purpose of this experiment is to identify an aptamer against SOD-1 to deliver functional proteins to the body to fixate super oxide radicals. The aggregation of radicals and mutated SOD-1 proteins lead to the symptoms of ALS, all of which lead to eventual death. The experiment is currently on round one of the SELEX method of discovering an aptamer. Issues arose with ccPCR and measures have been taken to find as to why the PCR reaction failed. Following the failed efficacy checks, it was concluded that binding and selection failed. Round one had to be repeated. A new binding and selection reaction was performed by cobalt bead based selection, followed by reverse transcription. However, the repeated round one ccPCR20 failed. It is suspect 10X PCR buffer crashed. The next step is to complete an efficacy check of ccPCR of 20 cycles with the a positive control 10X PCR buffer and W0 to test the success of the buffer.

If bands are produced from the hybrid in this efficacy check, then it will mean that ccPCR failed due to the 10X PCR buffer. At that point ccPCR can be repeated with a working buffer and it would be expected that bands would be produced. With the optimal number of cycles known, the experiment can move on to large scale PCR of the E1 wash. Assuming successful lsPCR, transcription can be performed followed by polyacrylamide gel electrophoresis. Those are the final steps required in round one of SELEX. In round two conditions such as stringency may be altered depending on the results from round one. Multiple rounds will follow until an aptamer is identified.

Round	Pool:Target (pmol:pmol)	Washes (# x volume)	Number of PCR cycles necessary	Amount of Recovered pmol
1	200:200	5 x 200 uL	Unknown	Unknown

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