The Effects of Mutated SOD1 on Amyotrophic Lateral Sclerosis and how siRNA Treatment Could Lead to a Cure

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I pledge my word of honor that I have abided by the Washington College Honor Code while completing this assignment.

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I want to extend a gracious thanks to Dr. Lipchock for his constant support and guidance. I would also like to thank the entire Chemistry Department, as each faculty member has played an integral role in the fantastic education I have received for my future endeavors.

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Abstract

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's Disease, is an incurable muscular dystrophy disease. The degradation of motor neurons and central nervous system leads to declining physical ability until the lungs are no longer functional. Superoxide dismutase (SOD1) is an essential metalloenzyme found within motor neurons that degrades free oxygen radicals. The SOD1 A4V mutation is strongly linked to familial ALS cases. The aggregation and loss of structural integrity associated with this mutation allows for the major consequences associated with ALS. While there is currently no cure for ALS, siRNA treatment technology has shown promise in heterozygous individuals as a way to silence the *SOD1* gene with the A4V mutation. The research on siRNA demonstrates that it has been successful *in vitro* on multiple mutations of SOD1. It can bring cell viability to nearly 100% of wildtype levels due to its gene suppressing capacity. The research examined presents a strong case for the possibility of future clinical trials of siRNA that have a considerable possibility to lead to a cure for Lou Gehrig's Disease.

Introduction

History and Introduction of Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), better known as Lou Gehrig's Disease, is a fatal neurodegenerative disease that results in severe degradation of upper and lower motor neurons.[1] Despite its naming after Lou Gehrig in 1939, the first reported case of ALS was written in the 19th century by a French neurologist known as Charcot.[1] His manuscript in 1868 was the first to connect lateral sclerosis to the gray matter in the spinal cord.[2]

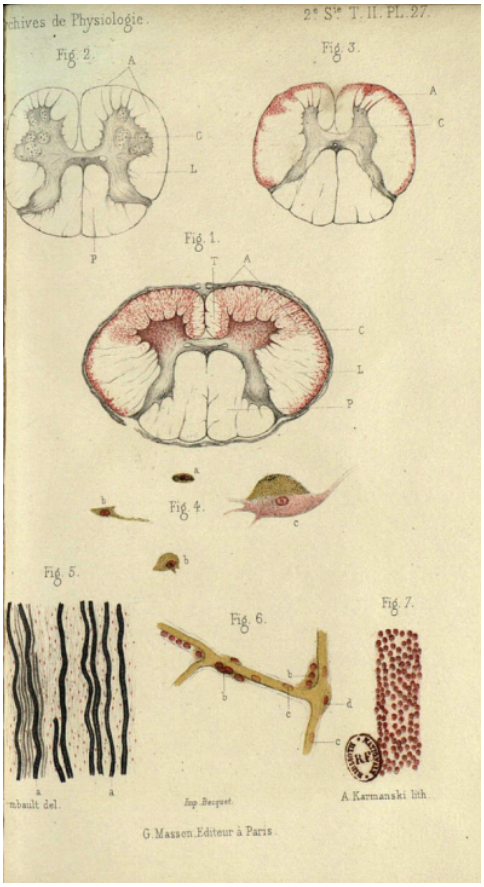

Figure 1. Charcot's sketches portraying the lesions of the nerve cells in the anterior horn.[2]

By studying two different patients diagnosed with varying forms of muscle atrophy, Charcot and his research team declared that anterior horn cells were pivotal in the development of muscle atrophy (Figure 1). This statement was significant as the role of the spinal cord was highly disputed in the scientific community, even after the manuscript was publicized. After an unexplained hiatus, Charcot formalized the diagnosis of ALS in 1874 to the Faculty of Medicine on the spinal muscular atrophies.[2] He differentiated ALS from progressive muscular atrophy by its reach into the bulbar region of the brain. The bulbar region includes critical involuntary movements of the body encompassing the pons, medulla, and cerebellum. These portions of the brain specifically control breathing, heart rate, and sleeping. The distinction Charcot shaped between progressive muscle atrophy and ALS was groundbreaking in this era of spinal muscular atrophies as it accurately conveyed the deadliness of Lou Gehrig's Disease. Even more distinguishing, Charcot entrenched that ALS is a syndrome with many causes, an accomplishment that was unprecedented at his time.[2]

Currently, there is no established cure for ALS. Unlike many forms of cancer where treatment is possible and fatality is not guaranteed, all patients with Lou Gehrig's Disease eventually pass away from complications of the disease. Although less than 15,000 people in the United States alone are currently diagnosed with ALS, it is the most common form of muscle atrophy disease.[3] Both the familial, also known as inherited, and sporadic, also known as nonhereditary, forms of Lou Gehrig's Disease appear to be equally difficult in terms of discovering a cure. The proportion of sporadic and inherited ALS is not close to equal. Familial ALS cases account for 10% of all ALS cases, while

sporadic cases comprise the remaining 90% of cases.[1] However, it is crucial not to overlook estimates that the rate of heritability of those with sporadic ALS is 61%.[4]

Although ALS is considered a rare disease, prominent figures have brought awareness to its devastating effects. Lou Gehrig was an extraordinary baseball player in 1939 when he was diagnosed with ALS. He immediately retired from his athletic career and experienced a rapid loss of voluntary movement, to the point where he could not even speak. A mere two years elapsed before ALS took Lou Gehrig's life.[5] His case is not unusual, as most patients diagnosed with ALS pass away within four years of respiratory failure. Steven Hawking is a shining example of what one can accomplish despite the debilitations of ALS. The world-renowned theoretical physicist was only 21 years old when he was diagnosed with Lou Gehrig's disease. He miraculously lived until the age of 74 while pushing the boundaries of theoretical physics as known today. However, Hawking was confined to a wheelchair with a computer to aid in text-to-speech, as he did not retain sufficient movement to move or speak without constant support. Regardless, it should be retained that Lou Gehrig's Disease has widely varying effects on those diagnosed with this disease. The one constant among all ALS patients is its inevitable destructive nature to the functionality of the human body.

Research Studies on Sources of ALS

Countless research studies have been performed to determine the direct causes of ALS. Although it is commonly accepted that the degradation of motor neurons in the central nervous system is a root source of the formation of Lou Gehrig's Disease, there are copious research findings as to the mechanisms behind the destruction of these motor neurons. Most of these findings are centered on mutated enzymes and other mutated

structures that lead to apoptosis. The majority of ALS mutations are inherited in an autosomal-dominant manner from point non-coding regions, frameshifts, and point mutations. Autosomal refers to the mutated gene coding for ALS is located on a chromosome not related to the sex of an individual. The dominant quality of the mutation is very concerning, as it means only one of the parents needs to be carrying the mutation for ALS for the offspring to receive it as well. Frameshift mutations are particularly dangerous because they insert or delete a certain number of nucleotides that are not equal to three. Since proteins are synthesized using the three-nucleotide groupings for each amino acid, known as codons, any insertion or deletion that is not a multiple of three can completely alter the proteins that would have been synthesized from the gene. Lastly, a missense mutation, or point mutation, occurs when a single nucleotide is altered to a different nucleotide, resulting in the synthesis of a different amino acid.

The mutated enzymes commonly found in ALS that will be discussed are GLT8D1, TDP-43, and SOD1, although the focus of this paper will be on mutations to SOD1. The goal of my research is to study the superoxide dismutase enzyme and prove that a therapeutic treatment involving siRNA is a viable path to potentially discover a cure for Lou Gehrig's Disease. Both the function and structure of SOD1 are studied to develop a full biochemical understanding of its underpinnings. Furthermore, the literature on various SOD1 mutations is to be reviewed to best discover the linkage between its mutations and the advancement of ALS. Although my research is centered upon SOD1, the treatment involving siRNA, it likely can be applied to numerous other mutations of different mutated enzymes and structures associated with sporadic and familial ALS.

Chapter 1: Causes of ALS

Glycosyltransferase 8 domain-containing protein 1 (GLT8D1)

Glycosyltransferase 8 domain-containing protein 1 (GLT8D1) is a widely expressed protein of unknown function that has been linked to ALS.[4] As a glycosyltransferase, GLT8D1 is believed to be involved in the transfer saccharides to form glycosidic linkages. Its link to ALS was established through exome sequencing in autosomal dominant fALS cases to identify heterozygous p.R92C mutations in the glycosyltransferase 8 domain that codes for substrate binding.[4] From a pool of 103 familial and sporadic ALS patients, five patients revealed missense mutations in GLT8D1.[4] The gene GLT8D1 had not been previously related to neurodegeneration, despite it being considered a risk gene for schizophrenia that shares a common genetic risk with ALS.[4] To determine if there is a correlation between the GLT8D1 mutant and Lou Gehrig's Disease, multiple experiments were performed to observe the functional impacts of mutation on GLT8D1.[4]

One *in vitro* study of GLT8D1 provides a clear portrayal of how missense mutations lead to considerably decreased enzymatic activity (Figure 2).[4] Both the G78W and R92C mutations demonstrated decreased enzymatic activity compared to their wild-type counterpart. Specifically, the V_{max} was reduced by approximately 35% with a wildtype value of 67.5 µmol/min, G78W value of 58.6 µmol/min, and a R92C value of 51.4 µmol/min. The K_m was decreased by approximately 50% for the mutated forms compared to the wildtype, with a wildtype value of 3.5 mmol/l, G78W value of 2.3 mmol/l, and a R92C value of 2.5 mmol/l.[4] The V_{max} proportionally refers to the maximum catalytic turnover rate for the enzyme, whereas the K_m value inversely indicates the

enzyme affinity for the substrate. Although the lower K_m value suggests higher substrate affinity, it negatively affects substrate cycling, thus reducing overall velocity.[4] As such, the effectiveness for the mutated enzymes is considerably diminished relative to the wildtype. This is consistent with previous research studies suggesting that approximately 20 amino acids adjacent to the substrate-binding site impact enzyme activity.[4] Strikingly, the R92C's degradation in enzymatic activity compared to G78W aligns with clinical severity as well.[4] Given this experiment and others performed in this research article, it concluded that therapeutic targeting of mutated GLT8D1 could likely lead to subsided ALS contraction.[4]

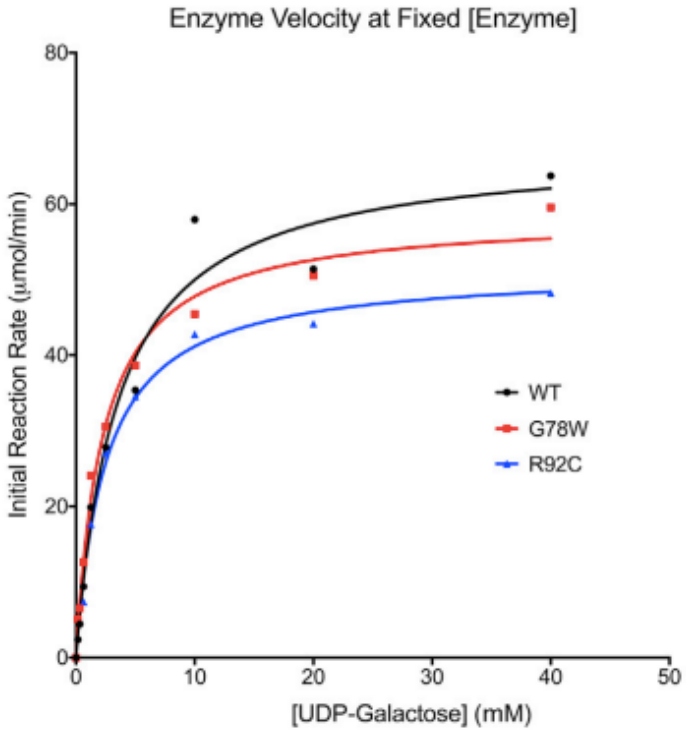

Figure 2. Michaelis-Menten curves of enzyme velocity of mutated and wild-type forms of GLT8D1, fitted with nonlinear regression. The initial reaction rate is plotted with respect to uridine diphosphate galactose as the substrate. The reaction entails a galactose being cleaved and transferred to a protein substrate. Red represents the G78W mutation of GLT8D1, blue represents R92C mutation, and black trendline represents wildtype counterpart.[4]

TAR DNA-binding protein 43 (TDP-43)

Another enzyme that has been linked to Lou Gehrig's disease is TDP-43, an RNA binding protein that plays a role in RNA splicing. When mutations occur in TDP-43, the contraction of familial ALS is a likely outcome as muscle weakness and motor neuron loss are conceived.[6] It is debated within the scientific community whether the decreased TDP-43 function after nuclear clearance or the neurotoxicity of TDP-43 is truly responsible for ALS.[6] One recent study attempted to bring more clarity to this subject.[6] By targeting the two orthologs of the human TDP-43 protein of the zebrafish genome, mutant lines were developed to result in deletions of premature stop codons coding for TDP-43.[6] The resulting mutant zebrafish were analyzed for impaired spinal motor neuron axon outgrowth. Although the motor neuron axons were shorter, they did not portray any abnormalities in functional properties. However, the double-homozygous mutants, *tardp* and *tardpl*, were evaluated for muscle performance with profound results.[6] In contrast to wild-type muscles that contain a proper arrangement of the sarcoplasmic reticulum, the mutant muscles displayed a stark disarrangement of their sarcoplasmic reticulum (Figure 3).[6] Specifically, the reticulum consisted of misplaced mitochondria between muscle fibers and parts of the sarcoplasmic reticulum were misaligned.

Additionally, the TDP-43 mutations studied are located in the C-terminal glycine-rich domain of the protein.[6] This domain impacts the process of alternative splicing and autoregulation, thus minor disruptions to this domain can detrimental to the functionality of TDP-43 by causing the inclusion or exclusion of exons important to the function of the protein. Moreover, the C-terminal domain is crucial for protein-protein interactions and autoregulation.[6] The research found that a relatively subtle loss of glycine in this domain

leads to ALS pathology, and a complete loss is equivalent to death of the protein.[6] The main conclusion of this research article is that the neurotoxicity associated with mutated TDP-43 provides strong evidence of its connection with ALS.[6] Specifically, the mutations of TDP-43 result in its insolubility and pathology of ALS.[6] This research article proves that future treatments focused on mutations of TDP-43 are likely to be advantageous to the cure of ALS.

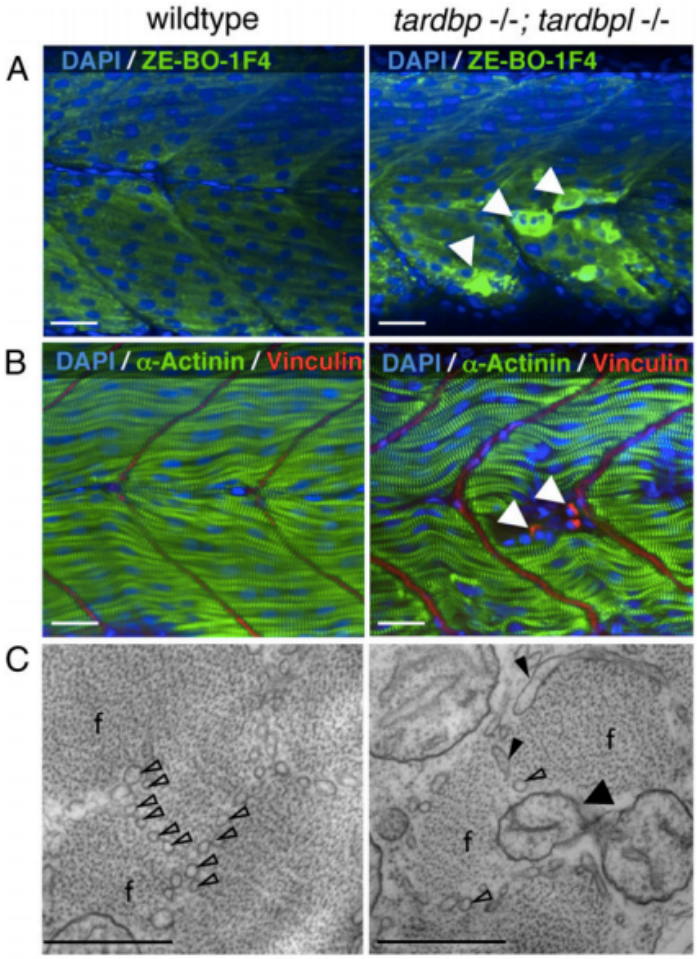

Figure 3. Antibody staining of mutant and wild-type forms of TDP-43 with DAPI and ZE-BO-1F4 in row A and DAPI, **α**-Actinin, and Vinculin in row B. Left column is wildtype TDP-43 and the right column is the mutated TDP-43. White arrows indicate degraded myocytes. Black arrows indicate a disorganized network of myofibrils. Open arrows indicate a degraded pattern of the sarcoplasmic reticulum.[6]

Chapter 2: Superoxide Dismutase (SOD1)

One enzyme that may be the most significant to the pathogenesis of Lou Gehrig's Disease is superoxide dismutase. The *SOD1* gene was the first ALS gene discovered in 1993 and since then at least 25 other genes have been connected to fALS and sALS.[7] The *SOD1* gene is especially crucial because over 180 mutations have been linked to the five exons of the *SOD1* gene.[7] Mutations of SOD1 are most commonly discovered in the familial version of ALS, which accounts for a relatively small portion of all ALS cases. However, the mutations associated with SOD1 comprise 20% of all fALS cases.[1] The A4V mutation is commonly considered the most pivotal as it comprises half of all ALS-causing mutations in the United States.[7] Any discovery toward mitigating the A4V mutation on the SOD1 gene would be a momentous step on the pathway to cure ALS.

The goal of my research is to study the superoxide dismutase enzyme and prove that a therapeutic treatment involving siRNA is a viable path toward discovering a cure for Lou Gehrig's Disease. Both the function and structure of SOD1 are analyzed to develop a full biochemical understanding of its underpinnings. Furthermore, the literature on various SOD1 mutations is reviewed to best discover the linkage between its mutations and the advancement of ALS. Although my research is centered upon SOD1, the treatment involving siRNA, this approach can be applied to numerous other mutations of different mutated enzymes associated with sporadic and familial ALS.

SOD1 Structure and Function

It is imperative to thoroughly examine the structure of superoxide dismutase to gain a complete understanding of its functionality. SOD1 is a dimeric, 153 residue metalloenzyme that is responsible for degrading free radical oxygen species (ROS).[8]

Superoxide dismutase is a key enzyme that serves as a crucial antioxidant in many organisms. More specifically, it degrades harmful free radical species that can be present in the body and converts it to hydrogen peroxide, which can be degraded to water and molecular oxygen by the enzyme catalase.[9] If ROS levels are not maintained at low levels, they can irreversibly bind to hemoglobin molecules, resulting in a lack of oxygen delivery to vital tissues. Due to the polar and reducing nature of the intracellular space, SOD1 can be commonly found in the cytosol, peroxisomes, nucleus, and intermembrane spaces of eukaryotic cells.[8]

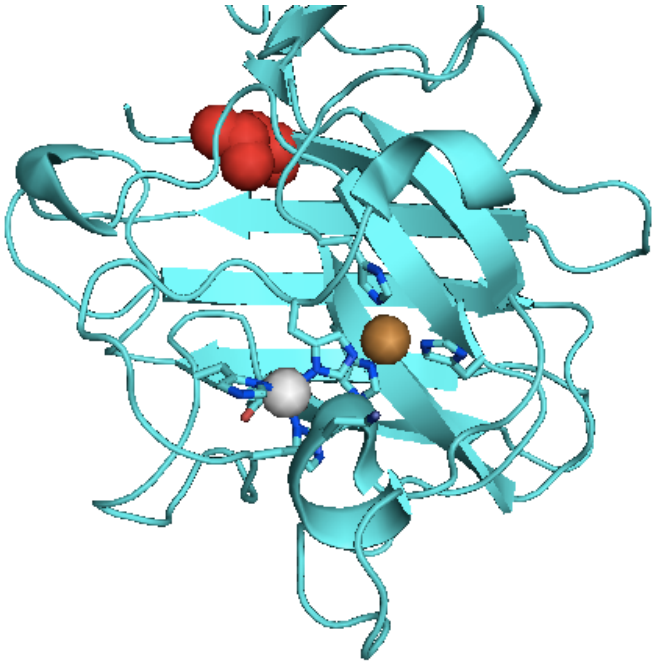

Figure 6. One monomer of a β-barrel of SOD1. Red spheres near the dimer interface represent the residues bound to the A4V mutation. Gray and brown spheres in the center of the β-barrel indicate the locations of zinc and copper, respectively.[10] (Pymol) PDB ID: 1UXM

SOD1 is a globular protein comprised primarily of eight antiparallel β-sheets (Figure 6). Each monomer binds two divalent metal cations, typically copper and zinc, which provide structural stability and are involved in the catalytic mechanism through

oxidation-reduction chemistry. These ions are represented by brown and gray spheres in Figure 6. Represented by the pentagonal rings stabilizing each of the metal residues, histidine plays a major role in the bonding of zinc and copper and the lone pair on the imidazole nitrogen is capable of forming a coordinate covalent bond with the divalent metal ions (Figure 6).[8] The alanine residue which is commonly mutated to valine (A4V) in ALS patients is located at the dimer interfaced and is highlighted in red spheres in Figure 6.

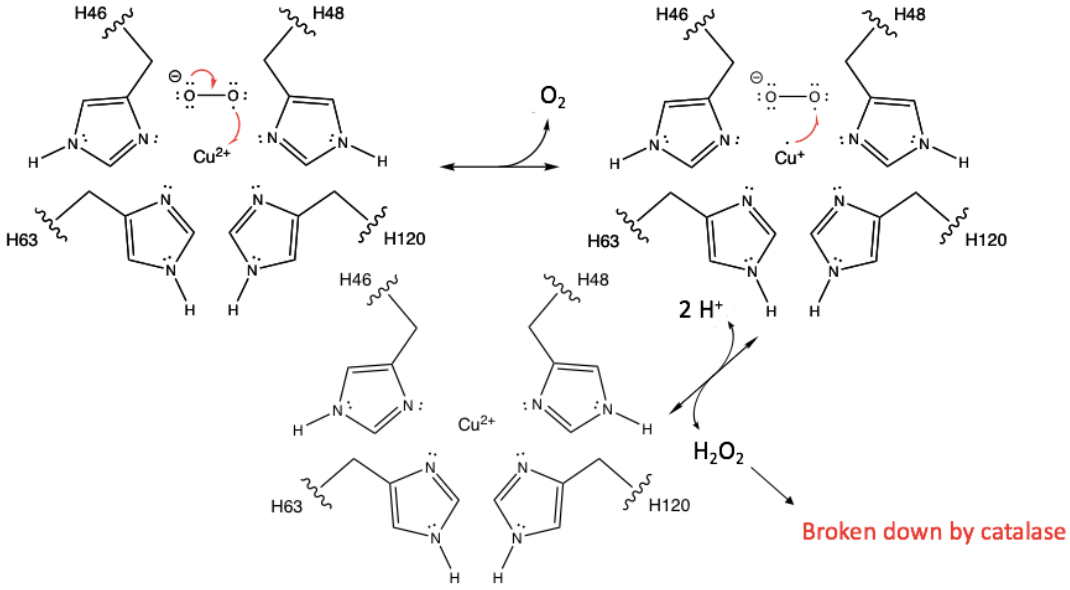

Figure 7. Mechanism of SOD1 converting free oxygen radical species to hydrogen peroxide. (Lipchock, J.)

In the above mechanism, SOD1 undergoes two round of catalysis and converts O_2^- to its neutral state. The copper in the center of the enzyme accepts a single electron to become reduced and is oxidized to its original form by a second superoxide ion to form hydrogen peroxide. The enzyme catalase then breaks down the hydrogen peroxide, effectively saving the cell from any reactive oxygen species harm.

Aggregation of SOD1

Although the subunits have the necessary components to function independently, dimerization is necessary for a properly functioning enzyme. Mutations that disrupt dimerization cause an overall reduction in protein stability, ultimately resulting in protein denaturation and aggregation.[11] As mentioned previously, the SOD1 A4V mutation comprises half of all ALS-causing mutations in the United States.[7] As the mutation implies, the fourth residue of SOD1's monomeric subunit is substituted from alanine to valine amino acid. Both of these amino acids are nonpolar and relatively modest in size when compared to the majority of amino acids. The only difference is the addition of two methyl groups off the Cβ carbon (Figure 7). Yet, the metal binding and dimeric interface is remarkably hindered by the A4V mutation. One research study utilized differential scanning calorimetry to test the thermal stability of the A4V mutant of SOD1. In comparison to the wildtype, the A4V mutant consistently demonstrates a melting temperature that is 8 ℃ lower.[12] Furthermore, the wildtype SOD1 contains an equivalence of 0.57 to 1.74 of copper to zinc, whereas the A4V mutant has an equivalence of 0.21 to 1.69.[12] These data demonstrated that both the thermal stability and metal binding are adversely affected by the A4V mutation. These alterations to the structure of SOD1 make aggregation likely.

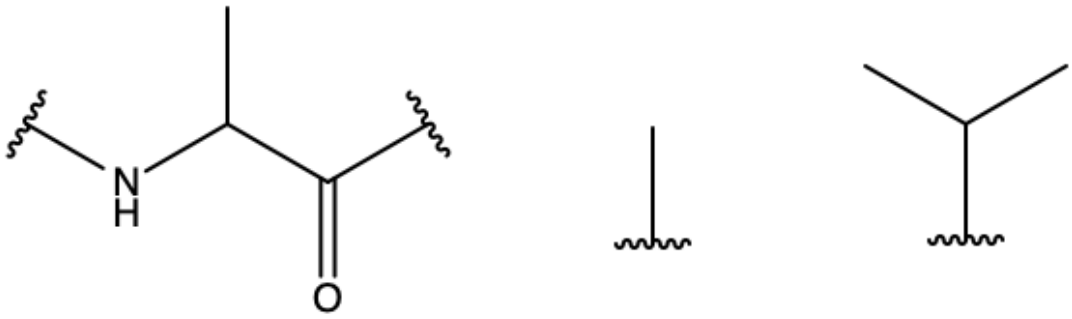

Figure 8. Amino acid structures of the peptide backbone and side chains alanine, and valine, respectively.

To gain insight into how known mutations in SOD1 that lead to ALS result in structural instability, one research study performed computational molecular dynamics simulations on the wildtype and A4V mutation.[13] For these calculations, the protein was solvated in water at a temperature of 310 K and a density of 0.993 g/mL. Functions of potential energy were employed to calculate MD trajectories, resulting in approximately 180,000 structures of both wildtype and A4V apoproteins. Analysis of these simulations revealed that the A4V mutation results in a reduction in the binding interactions between interface amino acids (Figure 8), especially among the residues of the dimer residues and the other residues that compose the enzyme. Namely, isoleucine and phenylalanine on residue positions 149 and 20, respectively. Although not in direct contact with the mutated residue, a 51% decrease in contact residue time between residues I and F was measured in the computational simulation.[13] The decrease in binding leads to a tighter N and C-termini association, potentially impairing the dimerization of the mutant enzyme.[13]

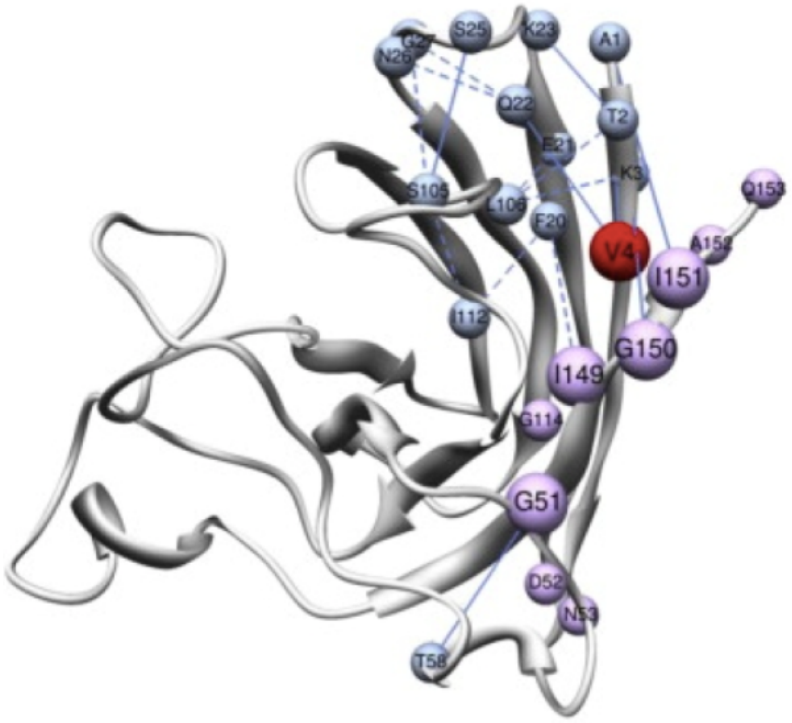

Figure 10. Computational modeling of the monomeric subunit of SOD1. Purple residues represent dimer amino acids, red represents A4V mutation, and blue residues are residues not involved in the dimer interface but may be bonded to residues that compose the dimer.[13]

In addition, copper and zinc experienced a sharp decline in contact bonding with surrounding residues after the development of the A4V mutation. In the simulation of the A4V mutant, zinc sustains considerable binding integrity loss with the residues histidine 71 and aspartate 83.[13] Consequently, the zinc metal begins to move outward, toward the copper as it loses its binding interactions with surrounding residues. In total, the mutation led to a more than 50% decrease in total contact time for both residues to zinc during the simluation.[13] The alteration in contact time also permits the residues to be in closer proximity, leaving less room to bind zinc, which helps to explain the 30-fold decrease in affinity for zinc to the A4V mutant.[13] Interestingly, the residues binding copper do not experience a variation in residue distance and less of a difference in contact time relative to zinc.[13] However, the mutant form contains half as much copper as the wildtype.[13] The

proposed mechanism for this drop in copper is that the destabilization of the electrostatic loop hinders the ability of copper to conform to its binding site.[13] Since zinc and copper are integral to the stability of the enzyme, the consequential loss of metal binding likely leads to protein aggregation.[13] The loss in dimerization, metal binding, and overall structural integrity are all main contributors to protein aggregation. As the rate of aggregation in SOD1 increases, so does the likelihood of Lou Gehrig's Disease onset.

Chapter 3: siRNA-based Treatment

Mechanism of siRNA

ALS is currently considered an incurable disease. The complexities of its origin in the body and the difficulty in combating aggregation are two major roadblocks in the way of discovering a cure. However, there is one developing therapeutic treatment that shows significant promise: single interfering ribonucleic acid (siRNA), which allows for the silencing of mutated genes to halt aggregation before it results in Lou Gehrig's Disease.[14] Similar to miRNA, but more selective, siRNA can be designed to target mutated genes, while leaving wildtype unaffected.[14] This approach is advantageous in heterozygous individuals with ALS, as siRNA can be used to target the chromosome with the mutated gene. siRNA would be especially beneficial in the *SOD1* gene that contains the A4V mutation, as it would prevent the translation of mutated SOD1 enzymes and aggregation.

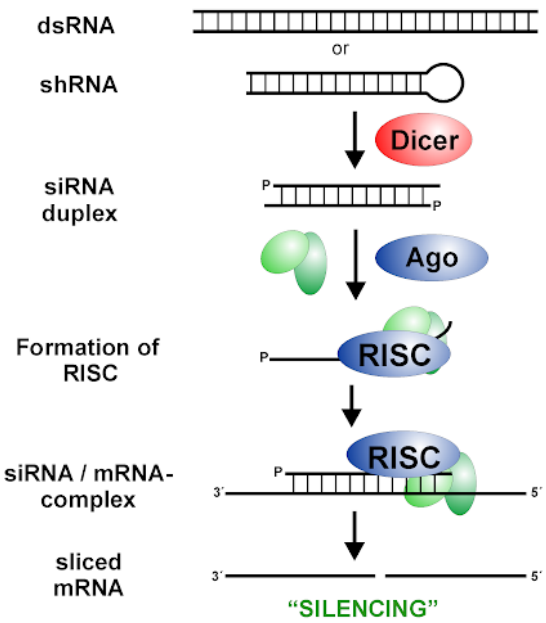

Figure 11. Mechanism of siRNA preventing the translation of the transcription of a DNA gene. (Miami.edu)

All of the enzymes depicted in Figure 11 are naturally occurring enzymes in the cytosol.[15] The only addition to the cell in the siRNA silencing process is the RNA that is complementary to the target mRNA sequence that encodes for the protein you want to silence. The siRNA process begins when a double-stranded RNA (dsRNA) or a single hairpin RNA (shRNA) is cleaved by Dicer, a riboendonuclease.[14] shRNA is another means of introducing siRNA into the cell, with a hairpin turn at its end. The RNA then becomes a siRNA duplex, which is recognized by the enzyme Argonaute, and is then brought into association with its complementary mRNA strand.[14] This forms the RNA-inducing silencing complex (RISC), which subsequently cleaves the mRNA.[14] Once the mRNA is cleaved, its pathway to translation is effectively canceled. The entire siRNA process transpires naturally because the enzymes involved are simply performing their normal functions. Dicer is involved in the RNA interference pathway and ordinarily cleaves dsRNA molecules.[15] Argonaute commonly facilitates the repression of RNAs by obstructing transcription and translation.[16] The family of ribonucleoproteins that make up the RISC is arranged to target any nucleic acid with the objective of silencing.[17] By allowing these enzymes to function naturally, the siRNA pathway to gene silencing takes advantage of mechanisms already present in the cell.

The delivery of siRNA into the cell has proven to be difficult due to its negative charge and size that makes it difficult to cross the cell membrane.[18] Usually, siRNA crosses the cell membrane with the use of viral vectors such as lentivirus (LV), adeno-associated virus (AAV), and rabies-glycoprotein-psuedotyped lentivirus (RGP-LV.[18] AAV has been the most popular virus for introduction to the cell as it can effectively target astrocytes and neurons.[18] Despite its success in targeting the correct structures

involved in ALS, there are concerns about its ability to provoke an immune response.[18] Nanoparticles and liposomes have been suggested as substitutes for AAV to bypass the possibility of an immune response.[18] As more research is performed on the stabilization of siRNA, it could circumvent the need for viral vectors entirely.[18]

Research Studies of siRNA

Whether *in vitro* or *in vivo*, siRNA-based treatment has proven to be successful. One study tested the viability of mammalian cells with wildtype and mutated forms of SOD1.[14] The study primarily focused on the G93A and A4V mutations in SOD1 within mouse Neuro2a cells. siRNA with varying degrees of specificity were synthesized to target these mutations and observe the effects in the cell. Polymerase chain reaction (PCR) was implemented to synthesize copies of the sense and antisense siRNA sequences necessary to hybridize to the mutated SOD1 genes.

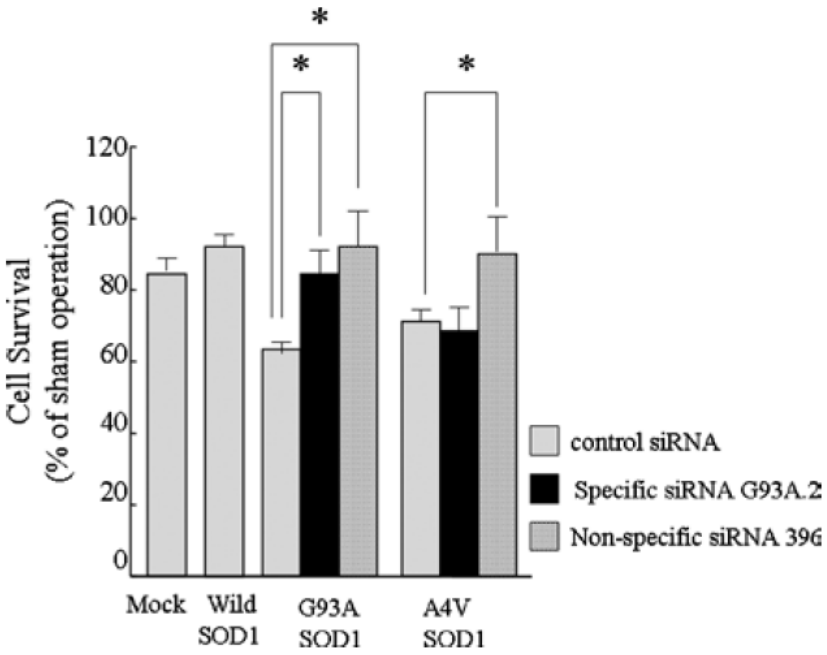

Figure 12. Relative viability of SOD1-induced toxicity of mammalian cells. Each transfectant is treated with 20 μm of lactacystin to enhance the toxicity of Neuro2a cells. Non-specific siRNA 396 increased the cell survivability of the A4V and G93A mutant SOD1 to nearly wildtype levels.[14]

In this study, cell survivability was used as an indicator of protein aggregation within a cell (Figure 12). The non-specific siRNA 396 was the most effective at not only bringing the A4V mutant to wildtype levels of cell survivability, but also the G93A mutant. The A4V mutant went from about 65% cell survivability without siRNA 396, to nearly 90%.[14] The G93A mutant went from about 60% cell survivability to 90% as well.[14] These results suggest that siRNA is effective at preventing protein aggregation without harming the cell in multiple ALS-linked mutations.

Although there are not many published studies of the effects of siRNA on the A4V mutation, its effects on similar mutations are encouraging. A protein known as Homer1b/c, is expressed frequently in the central nervous system and is a crucial regulator of dendritic spine structure and synaptic function.[19] Despite its benefits within the nervous system, overexpression of the protein leads to aggregation and onset of ALS.

Overexpression leads to intracellular calcium overload, calcium-dependent production of reactive oxygen species, and glutamate-mediated excitotoxicity.[19] Transgenic mice with the G93A mutation in SOD1 are associated with overexpression of Homer1b/c at the mRNA and protein levels compared to wildtype human cells.[19] One research study used *in vitro* studies of cells with overexpression of Homer1b/c to test the effectiveness of siRNA in reducing the expression of Homer1b/c.[19]

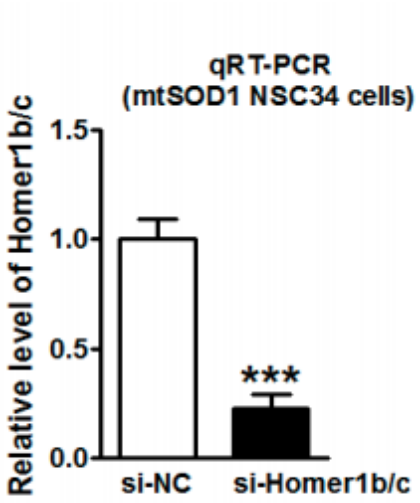

Figure 13. NSC34 cells with mutated SOD1 treated with siRNA specific to knockdown of Homer1b/c genes decreased level of Homer1b/c proteins significantly. si-NC refers to cells treated with negative control, and si-Homer1b/c refers to cells treated with siRNA. Level of Homer1b/c was detected by qRT-PCR.[19]

In the study by Jiang and co-workers, the level of Homer1b/c decreased from a relative level of 1.0 to approximately 0.3 (Figure 13).[19] The cells treated with siRNA were then found to have reduced levels of induced neuronal apoptosis relative to the negative control due to the normal expression of Homer1b/c.[19] The relative level of Homber1b/c decreased to approximately 25% of its negative control counterpart.[19] These results further prove that siRNA is an effective strategy in mediating the toxic effects of other SOD1 mutations and could be applied to the A4V mutation as well to combat the onset of ALS.

Other methods of gene suppression have been proposed and tested alongside siRNA. Ribozymes are synthesized naturally in the cell but can be made artificially to target cis or trans sequences to cleave a specific target motif.[14] In contrast, DNA enzymes (deoxyriboenzymes), can only be found *in vitro*, meaning that they are not naturally found in cells of organisms. The DNA enzymes and ribozymes can be engineered to selectively cleave a target sequence purine-pyrimidine that is cleaved. While this is frequently done *in vitro*, it can also be completed *in vivo* by transfecting cells with a gene that encodes for the desired DNA enzyme or ribozyme. As a testament to the specificity of ribozyme and DNA enzyme, both can target a single nucleotide mismatch as well as degrade specific RNAs.[14]

To test the suppression effectiveness of siRNA, DNA enzyme, and ribozyme, each gene silencer targeted mutated G93A SOD1 enzyme in 293T cells.[14] Each of these gene silencers were transfected together with 0.05 μg GTP (Guanine-5'-triphosphate) expression plasmid.[14] The control transfection was performed of the same volume of siRNA and DNA enzyme for an unrelated MachadoJoseph disease gene or an empty pcDNA3.[14] The effectiveness of these gene silencing mechanisms was compared to each other using Western blot analysis.

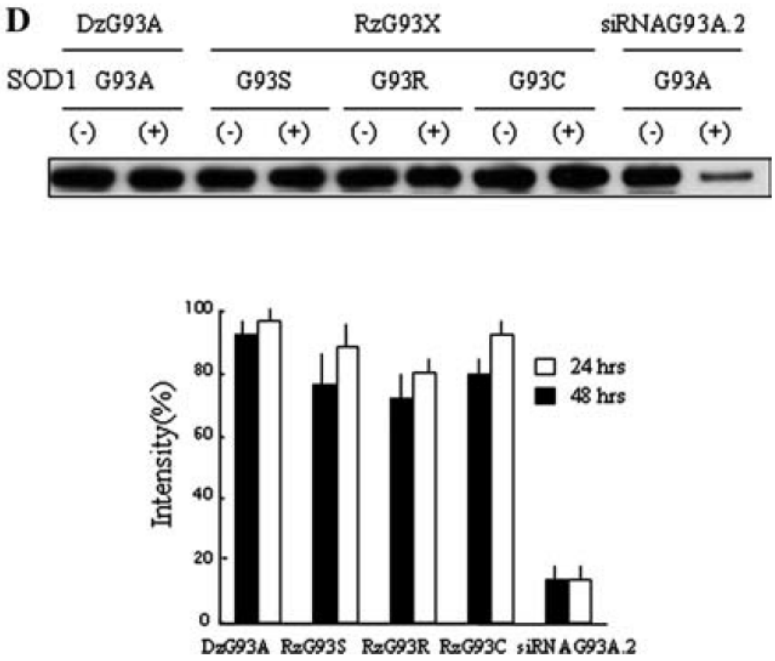

Figure 14. Suppression effects of DNA enzyme, ribozyme, and siRNA on G93A SOD1 mutation in 293T cells. The design of ribozyme did not allow for G93A to be targeted, but other mutations on the same codon; G93S, G93R, and G93C were targeted instead. Assay at the top of Figure 14 is Western blot analysis that displays the expression of G93A mutant SOD1 48h after transfection. The graph represents the band intensity of ribozyme, DNA enzyme, and siRNA G93A relative to those with mock transfection.[14]

The Western blot analysis measures the relative protein content of SOD1 G93A with and without DNA enzyme, ribozyme, and siRNA.[14] The dark bands for every trial appear similar, except for the one with siRNA present.[14] As opposed to the dark thick bands demonstrated for every other assay, the siRNA assay presents a thin line, which indicates that the siRNA method is effective at suppressing G93A expression.[14] The graph in Figure 14 illustrates that this corresponded to a 90% reduction in G93A expression after only 24 hours.[14] This is in stark contrast to the results obtained for the DNA enzyme and ribozyme for which the DNA enzyme had virtually no effect after 24 hours, and approximately a 10% decrease after 48 hours. Out of the three ribozyme variations, the one targeting G93R had the most success, but it was a mere 25% reduction

after 48 hours.[14] These results prove that siRNA can be more than three times as effective as other gene silencing strategies as well as quicker to take effect.[14] The article also mentions that siRNA exhibited comparable levels of reduced mutant expression for the A4V SOD1 mutation.[14] The research presented in this article adds to the mounting evidence that siRNA can mitigate the effects of ALS.

Conclusions

There are many possible causes of ALS, but the aggregation of proteins is a prevailing theme. Whether it is GLTD8D1, TDP-43, or SOD1, each protein is vital to the proper functioning of the body and their aggregation is linked to the onset of Lou Gehrig's Disease. SOD1 is of particular importance, as it accounts for 20% of all fALS cases alone. The A4V mutation in SOD1 is also of great interest as it is the source of half of all ALS-causing mutations, including sALS cases. The prominence of mutated SOD1 in the general population's contraction of ALS justifies its further examination.

The structure and functionality of SOD1 make it clear why mutations to this protein can be devastating to the body's motor functions. The metalloenzyme is expressed at high levels within motor neurons and other structures of the central nervous system and is responsible for degrading oxygen free radical species. Mutations, especially the A4V mutation, have been proven to impair the structure of SOD1. These impediments lead to reduced thermal stability and decreased integrity in the binding of the copper and zinc ions that are crucial to the protein's functionality. Without properly functioning SOD1, the body is at great risk for irreversible binding of reactive oxygen species to hemoglobin, which can result in improper delivery of oxygen to tissues. This symptom of ALS is what permits for the decline of muscles in the body until fundamental functions of the body are no longer viable.

There is currently no conclusive path to a cure for Lou Gehrig's Disease. Given the prevalence of mutations prone to aggregation, gene silencing mechanisms are promising approaches to mitigate the symptoms of ALS. Each mechanism is standard in that they degrade the mRNA synthesized from the gene of interest. Among the options to

suppress genes effectively, siRNA has proven to be the most attractive possibility for gene suppression *in vitro*. siRNA takes advantage of enzymes and RNA already present in the cell to promote gene knockdown. Ribozyme and DNA enzyme are well-recognized methods of gene silencing, but both are insufficient when compared to the time efficiency and effectiveness of siRNA. miRNA is the most comparable to siRNA, but it lacks the effectiveness of gene silencing capabilities of siRNA. Research studies *in vitro* of siRNA have proven its effectiveness against well-established mutations of ALS, including G93A and A4V. A shortcoming of this approach, however, is that it can only be used on individuals who are heterozygous and only have one mutated *SOD1* gene in their chromosomes.

There are numerous published researched studies detailing the success of siRNA in its ability to suppress mutated DNA. Most of these research studies are demonstrated *in vivo* with the use of cell lines, but others present positive results to mouse models as well. Undoubtedly, additional research studies are required before moving on to mass clinical trials on humans, but early results are promising. The research studies presented in this literature review justify the continued experimentation of siRNA and future clinical trials. Gene suppression by siRNA is a feasible avenue to the long-awaited cure for Lou Gehrig's Disease.

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