PRNP: The Human Prion Gene

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Note: No AI was used during the writing process. However, it was used to help analyze and understand primary and secondary research papers.

I. Abstract

The PRNP gene, the human prion gene, codes for prions in the body. The function of a healthy human prion is still highly debated, and most of what is known about prions comes from their role as infectious agents. Primary prion diseases manifest as rapidly moving neurological diseases with no current cure or treatment, making them invariably fatal. For a time, prions were believed to be self-replicating proteins, defying the fundamental laws of biology. Prions are currently understood to be misfolded proteins produced by the host's PRNP gene. Prions are not well understood and are extremely dangerous to humans as there is no cure or treatment for prion diseases. Previous research has found that CRISPR-Cas9 techniques successfully knock out PRNP and that resulting mice are healthy and immune to prion disease. The following paper proposes research to build from the successful knockout in somatic mice cells to engineer a path toward heritable PRNP knockouts. This research could help pave the way for combating prion disease.

II. The Phenotype

The target gene of this grant proposal is the human prion gene, PRNP, which is responsible for prions in the body. I chose this gene as my research gene because my uncle died of a prion disease, Cruezfeltd-Jakobs Disease, in 2021. He died at the age of 52, leaving his wife and young daughter behind. He was an extreme endurance athlete who had run countless marathons and completed several Iron Man and long-distance bike races. Needless to say, he was in excellent health before he got ill. He was a professor of Nutrition and Metabolism at the University of Texas Medical Branch, and as a scientist, he kept careful track of his symptoms for as long as he could. He recorded that he completed his last marathon in April before beginning to feel dizzy at the start of June; he died early that August. Prion diseases are rare and vicious, taking out brilliant, kind people before their time; therefore, it is important to me that as much work is done as possible to further understand the nature of prions as disease-causing agents in the hope that a cure is found.

Prions, infectious agents in the body, have long been thought to defy the basic rules of biology. They act like bacteria and viruses yet entirely lack any genetic makeup; they cause illness, yet there is no known treatment against them. Additionally, prions have no DNA or RNA and are somehow still produced in the body. As such, it has been established that a prion most commonly fits the model of a misfolded protein. Proteins are crucial in almost all of the body's processes and mechanisms. They form channels in membranes, catalyze reactions, and provide structural support. The shape of proteins is vital to their function. Often, proteins are very selective in their actions, and their selectivity comes with a specific conformation. Any change in the conformation of a protein results in an altered function or the inactivation of the protein. There is a problem with this model: proteins do not self-replicate. If prions are misfolded proteins, they must be produced by something within the body. The idea that prions could self-replicate without genetic material would entirely rearrange how scientists approach the understanding of the Central Dogma of biology: only nucleic acids can self-replicate (Libretexts, 2020).

It has now been established that prions are produced by a gene within the host's normal genome. Most mammals, humans included, have a normally functioning PRNP gene that encodes for prions. The discovery of the PRNP gene reassured scientists that prions can not self-replicate, but it caused a new problem: if everyone has a prion gene, why are prion diseases so rare? Additionally, the PRNP is highly conserved among mammals. There is little variation in the gene sequence between mice, humans, sheep, horses, and so forth. Traditionally, it has been understood that highly conserved genes are vital to the development or survival of the organism, which makes PRNP so unique. (Libretexts, 2020).

There is no concrete definition or explanation for the role healthy prions play in the body, and scientists are still working to understand this role in hopes that it will elucidate ways to combat prion diseases. Prions are known to be expressed at different levels in different cell types in the body. Neurons and the placental cells express the highest levels of prions, while the heart and kidneys express much lower levels. Prions have also been found to bind with other molecules that play roles in essential pathways, such as those involved in the homeostasis and regulation of neural cells (Sakudo & Onodera, 2015). While the function of healthy prions is still unknown, scientists are simultaneously working to understand infectious prions. Working with prions, while essential to discovering their function, is deadly. Émilie Jaumain, a French scientist, was working with prions when she had an accidental needle stick through her layers of protection. Prion diseases are not instantaneous infections like viral and bacterial infections. Instead, it took almost eight years before Jaumain began to show symptoms of Crueztfeldt-Jakobs disease, and her death followed within a year of the diagnosis (Mole, 2021).

Crueztfeldt-Jakobs disease, CJD, is the most common prion disease in humans. It is marked primarily by a gradual decline in mental and physical capacity, as seen in dementia patients with seemingly no explanation. People with CJD begin to slowly show signs of memory loss, which rapidly progresses into loss of speech, vision, motor control, and bodily functions. There have been no documented cases of survival, and all documented cases end in mortality before a full year after symptoms begin (Mayo Clinic, 2023).

There are two primary types of CJD: classic sporadic and variant. Sporadic CJD is marked as having no moment of infection. The disease seems to come out of nowhere and attack individuals, most commonly in their 40s. Sporadic CJD is the most common and has a risk of inheritance, as some individuals may already have children since the disease manifests late in life (Mayo Clinic, 2023). Variant CJD, vCJD, occurs through a clear line of infection, such as the needle stick experienced by Jaumain. Since there is such a long latency between infection and the beginning of symptoms, it is often hard to distinguish between exposure to infectious prions and sporadic development of infection prions within the body. vCJD is generally more understood, as the concept of coming in contact with infectious material and then developing the same infection is familiar to scientists (Libretexts, 2020).

vCJD is extremely rare and most commonly transmitted through ingestion or contact with infectious animals. There have only been four documented cases of vCJD in the United States and only 200 in the United Kingdom. Bovine spongiform encephalopathy, BSE, is the prion disease people associate with 'mad cow disease.' Eating infected matter from an infected cow increases the risk of contracting vCJD. Symptoms of BSE are similar to CJD seen in humans and are also invariably fatal. Infected cows lose coordination, appetite, and control of bodily functions (Baker, 2023). Goats can also contract a prion disease called scrapie, and unlike what has been observed in humans, their susceptibility to scrapie depends on mutations in their PRNP gene. Goats with scrapie display a change in excitability and appetite and may develop a tumor. They also develop a harsh itch, which leads them to scrape their coats off—giving rise to the name scrapie. As seen in all other prion diseases, scrapie has a 100% mortality rate (Fantazi et al., 2018).

III. Molecular Function and Mouse Model

PRNP is located on the short arm of chromosome 20 in humans and has two exons. The second exon is much larger than the first, at over 2,000 nucleotides. PRNP encodes for prions, which serve an unknown purpose in the body when healthy but invariably cause fatal disease when

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misshapen. Prions are cell-surface glycoproteins hypothesized to play a role in myelin synthesis, circadian rhythm maintenance, and glucose homeostasis. Intron 1 of PRNP and sequences upstream of the transcription start site contain the binding sites for several different transcription factors, indicating that the levels of prions in the body can be regulated in response to different conditions and stimuli. PRNP expression is highest in the cells that make up the components of the central nervous system, primarily in neurons. It is also seen that expression of PRNP increases throughout childhood development, peaking in early adulthood before reducing through an individual's lifetime (Castle & Gill, 2017).

In instances of reduced or removed gene function in PRNP, some studies have shown decreased expression of almost 120 other proteins within the mouse model, indicating that prions may have a broader range of bodily functions than previously thought. None of the down-regulated proteins had off-target CRISPR sites; therefore, it is unlikely that the observation is due to an error in the CRISPR gRNA molecules or a side effect of diminished prions in the body. Included among these proteins were cytoskeletal keratins, cadherin-1, periplakin, desmoplakin, and plakoglobin. These are responsible for forming and maintaining intermediate filaments and cell junctions (Mehrabian et al., 2014).

Mutations and polymorphisms affect the age of onset and clinical manifestations of prion diseases if they arise. As with most proteins, prions only function at their standard capacities when in their native conformation. Any alterations in the gene sequence may cause a change in the conformation of the prion, rendering it useless or even an infectious agent in the host. Infectious prions can use template-directed misfolding, directly interacting with native prions, to alter the conformation of previously healthy prions in the host until all prions are in a disease-causing conformation (Appleby et al., 2022). Polymorphisms found at codon 129 have shown a range of risks in contracting sporadic CJD. A methionine-methionine polymorphism is found in less than 40% of healthy Caucasians, while it is overrepresented in cases of sporadic CJD.

Additionally, methionine homozygosity contributed to a lower mean survival time in cases of CJD. In contrast, individuals with methionine-valine polymorphism had a lower representation in CJD cases and a longer mean survival time. Homozygosity at codon 129 is associated with a shorter incubation period before clinical symptoms of the disease begin to show and progress. In the rare cases of genetic prion diseases, mutations have been found to contribute to the PRNP gene. Missense and nonsense mutations in the N-terminal domain of the protein contribute to more than half of known genetic prion disease cases (Appleby et al., 2022).

Like polymorphisms, strong linkage disequilibrium has been associated with prion disease manifestations in susceptible mammals. In cases of linkage disequilibrium, there is a non-random association between alleles at two different loci. Strong linkage disequilibrium is categorized by a higher percentage of association than expected for randomly associated alleles. In cases of weak linkage disequilibrium, the opposite is accurate, and a lower percentage of association is observed. PRND, the prion-like protein doppel gene responsible for protein metabolism, is located 20 kilobase pairs downstream of PRNP. PRNP and PRND single nucleotide polymorphisms, SNPs, have a strong linkage disequilibrium value in mammals susceptible to prion diseases, such as humans, goats, sheep, and cows. On the contrary, horses and dogs, insusceptible to prion diseases, have a weak linkage disequilibrium value between SNPs in PRNP and PRND. As such, a high linkage disequilibrium is associated with an elevated risk of prion disease (Won et al., 2020).

Historically, attempts to knock out the PRNP gene in mice were difficult due to its location in a densely packed heterochromatic region of chromosome 20. With the aid of CRISPR-Cas9 gene editing technology, attempts to make a PRNP knockout mouse model have been successful. Preliminary CRISPR trials focused on the ability to use sgRNA molecules to target the PRNP gene in embryonic stem cells in mice, which resulted in the successful knockout of the gene (Kaczmarczyk et al., 2016). Another mouse model indicates that mice with PRNP knocked out indicate no drastic change in behavior, physiology, or immune function. However, they do display minor alterations in their circadian rhythms. This model also finds that SRNP, a gene coding for the Shadoo protein, has been upregulated in prion-diseased mice cases. The overlapped expression of Shadoo proteins in prion-diseased mammals indicates that Shadoo may play a role in prion disease. Attempts were made to create an SRNP/PRNP double knockout mouse. SRNP was knocked out in wild-type and PRNP knockout mice. In PRNP knockout mice, SRNP knockout was lethal. Since mice survive the knockdown of both PRNP and SRNP, it is hypothesized that Prion and Shaboo proteins have a combined function essential to embryogenesis (Young et al., 2009).

CRISPR has also been used to introduce a null allele of PRNP into the mouse model to create a gene drive for eliminating prion diseases in a study mouse population inspired by studies on mosquito populations. A gene drive would not only allow for the introduction of a null PRNP allele, rendering that mouse immune to prion disease with almost no adverse consequences, but also for the inheritance of the null allele in future generations. This would, over time, result in a mouse population immune to prion diseases. This model could have been used to study bovine populations where Bovine spongiform encephalopathy runs rampant. The null allele consisted of a GFP reporter transgene flanked by guide RNA molecules. It would be transported to the correct location on the chromosome by the Cas9 enzyme and could transform the wild-type allele on the sister chromosome. As a result, mice with the null PRNP allele are immune to prion disease development. While CRISPR was effective in producing mice models immune to prion diseases, the result was not inherited by the next generations due to the low expression of Cas9 in the germline cells (Castle et al., 2022).

IV. Experiments For the Future

Previous research has indicated that it is possible to knockout PRNP using CRISPR-Cas9 techniques. Furthermore, research has shown that knockout mice resist prion disease infections without detrimental effects on their behavior or physiological functions. However, the knockdown is not heritable as it was unsuccessful in the germline of male mice, and any offspring of the knockout mice contained the PRNP gene sequence (Kaczmarczyk et al., 2016). Other research shows that while the knockdown of PRNP is viable, a double knockdown between PRNP and SRNP is lethal in early mouse embryos (Young et al., 2009). Studies have yet to show the effects of successful PRNP knockdown in the germline cells of mice and the effect on consequently developing embryos. The purpose of this experiment is to initiate heritable PRNP knockdown in adult control and somatic cell PRNP knockdown mice using CRISPR-Cas9 technology. The effects of heritable PRNP knockdown in mice will be observed for offspring viability and any behavioral or developmental differences.

For this study, four generations of mice will be studied, with three pairs of mice in each experimental group. The first experimental group will consist of control mice, who will not have their genomes edited and will not be experimented on, labeled as 'Control Group 1'. The second experimental group will consist of a second group of control mice injected with an empty plasmid to emulate the gene editing process and labeled as 'Control Group 2'. The third experimental group will be a pair of PRNP knockout mice labeled as the 'Experimental group.' The first generation of all mice groups will be bred and observed for viable offspring. In the second generation, viable offspring from Experimental Group mice will be tested for the presence of the PRNP gene. If PRNP is present, a knockout will be done on the oocyte and sperm cells of three mouse pairs, and they will be bred and tested for the success of generational PRNP knockout. The second generation of the two control groups will also be bred, receiving the same treatment as their respective first generations. The viability of the resulting third generation will be observed. If heritable knockout is successful, the third generation of mice will be healthy and have no PRNP gene. Granted the success and viability of third-generation mice, a fourth-generation will be bred and tested for the presence of PRNP.

Clustered-regularly interspaced short-palindrome repeats, or CRISPR, are a natural component in some bacteria and archaea and act as a version of the immune system. CRISPR sequences allow the bacteria to

activate Cas9, a restriction enzyme that recognizes and cuts viral DNA that has infected the bacteria. CRISPR DNA sequences and viral DNA are transcribed into CRISPR RNA, which guides Cas9 to where the viral DNA has been integrated into the bacterial host's genome (Lewis, 2024). CRISPR RNA, crRNA, and trans-activating crRNA complexes can be engineered to guide Cas9 to the specific site that is cut. Additionally, a protospacer adjacent motif, or PAM, is needed 3-4 base pairs downstream of the Cas9 cleavage site to prevent Cas9 from cutting the host's genome. In a scientific application, the Cas9 enzyme is purified, and the sites available for scientists to cut are limited to the regions with a PAM sequence nearby. A common Cas9 enzyme and PAM sequence is found in *Streptococcus pyogene*s and reads 5'-NGG-3', where N represents any nucleotide (Jinek et al., 2012).

For the first generation, knockout mice will be created by harvesting embryonic stem cells from a mouse embryo four days after fertilization. After gene editing, the mouse oocyte will be fertilized in vitro and transplanted back into the female mice for development through a plasmid as a vector. The first generation of Control Group 2 will be created through *in vitro* fertilization, injected with an empty vector, and implantation into the female mice. The first control group will be left to copulate naturally. In the second generation, where the heritable knockout is attempted, the oocyte and sperm of three mouse pairs are edited to remove PRNP, fertilized through IVF technology, and implanted back into the female mice.

Once embryonic stem cells are harvested, the tracrRNA:crRNA complex will be attached to the manufactured guide sequence for the PRNP gene: 5'- ccuuggcugcuggaugcugg -3', and the gRNA will be cloned into a plasmid vector to increase its abundance. The plasmid vector will contain a GFP reporter transgene flanked by the gRNA, allowing for possible success testing after the knockout is attempted (Nhgri, 2019). Next, the plasmid and Cas9 enzyme will be transfected into the cell via microinjection. Once inside the cell, the gRNA will guide Cas9 to the PRNP gene sequence, which will be cut out and replaced by the GFP reporter transgene (Chong et al., 2021). The presence of a reporter transgene that encodes for a fluorescent protein will allow for the determination of success in the gene editing process (Li et al., 2018). For the production of the germline knockout cells, PRNP will be knocked out of both the sperm and egg of the mated mouse pair using the described CRISPR-Cas9 methodology. The egg cells will be fertilized *in vitro* before implantation into the female mouse.

If the offspring of the first generation in Control Group 2 and Experimental Group are nonviable, it can be assumed that the process of gene-editing and plasmid injection are lethal to developing embryos. If the offspring of the third experimental group are nonviable, it can be rationalized that PRNP is required in both the parental mice somatic and germline cells to develop healthy offspring. If the third and fourth generations of knockout mice test positive for PRNP, then the germline knockout was unsuccessful. If the knockout is successful, PRNP is not detected, and the third and fourth generations result in viable offspring, it can be rationalized that PRNP is not critical in the germline or somatic cells of parental mice for the healthy development of embryos. This experiment hopes that the fourth-generation mice will have no CRISPR editing done at any stage of their development and 'inherit' the knockout from the third-generation mice where the oocyte and sperm underwent CRISPR knockout technology.

Fluorescence *in situ* hybridization is a technique that can be used to identify specific DNA sequences within an organism if the reporter gene is designed into the plasmid. After CRISPR-Cas9 knockout, a mouse tissue sample can be placed on a slide. The tissue is then exposed to a small, fluorescently labeled DNA sequence called a probe, which binds to its matching sequence. The DNA probe is a single-stranded DNA sequence engineered to promote binging to the PRNP sequence. The probe and sample DNA sequences are denatured and annealed to ensure binding, similar to PCR techniques. The sample can then be exposed to fluorescent light, which can be visualized if the target gene sequence is present. If the gene were successfully knocked out, there would be no evidence of the probe binding as no fluorescent color would be seen (Shakoori, 2017).

Another way to determine whether the PRNP gene knockout was successful is through Western Blot Analysis. Western Blot Analysis separates specific proteins based on their molecular size. First, the proteins are separated from the collected sample cells and then run through gel electrophoresis to separate them based on size and identity. The results are then bound onto a membrane where they are probed by a combination of antibodies, one specific to the target protein, prions in this case, and the other specific to the species where the protein is found, humans. The secondary antibody is often combined with an enzyme that produces a signal when it comes into contact with the target protein. The signal can be a very sensitive light or color change. Introducing fluorescently tagged antibodies can allow for fluorescent visualization of the protein's presence. If the knockout is successful, PRNP will no longer be present to produce prions, and there will be no signal. To ensure the technique is done correctly, a control plate must also be made where the cells are collected from a non-knockout mouse (Mahmood & Yang, 2012).

A final and sure way to test for success in the knockout mice is through PCR and DNA sequencing. Polymerase chain reactions, PCR, of a sample of DNA can be taken from the knockout mice and wild-type mice. PCR is a technique that amplifies specific sequences within the DNA through the careful design of primers. The primers are designed to attach to specific sequences in the DNA only, flanking the region in forward and reverse primers. PCR occurs in three main stages. The first is denaturation, where the DNA strands are separated in high heat. The second is the annealing stage, where the designed primers are attached to the specific target site. The final stage is elongation, where the second DNA strand after the primer attachment is synthesized. The amplification of the DNA sequence allows it to be sent for analysis, where the resulting sequence can be identified through the BLAST database for gene similarity. In the knockout mouse, the gene sequence would not be present if successful. As a result, the primers would not attach, which could be seen in a blank or messy gel if gel electrophoresis is done and in a nonconclusive result from the sequencing analysis. In the wild-type mouse, the gel would produce an amplicon at the expected weight, and the sequence analysis in the BLAST database would match the reference sequences (Nhgri, 2019b).

Potential pitfalls of this experiment include waiting until each generation of mice becomes old enough to reproduce sexually, which is between six and eight weeks of age. This will increase the time the researchers are committed to the project, with periods of inactivity as they wait for the mice to mature. Other pitfalls include the risk of including the results of an infertile mouse pair in the study data. *In vitro* fertilization has a variation of success rates and could also impact the data surrounding the viability of mouse embryos after treatment. If IVF fails in too many mouse pairs across the experimental and control groups, the number of mice left to breed for the following generations will be inadequate (Lewis, 2024). If necessary, this can be counteracted with the increase of mice within each trial. Additionally, a group of control mice must be reared to the age of reproduction to mate with the control mice of the second generation.

V. Conclusion

Initiating more research and further developing the understanding of prions is vital to the human race, as prion diseases are violent and invariably fatal. Prions are only newly understood to be misshapen proteins, and there is much left to learn about their function if a way to combat prion disease is to be found. Although prions' function is unknown, the PRNP gene is highly conserved, hinting that it may play a crucial yet undiscovered role. The proposed experiment looks for a way to understand if generating heritable PRNP knockout is possible and viable in mice embryos. If successful, PCR analysis of sample cells in the third and fourth generations of mice will reveal a null result on the sequence of PRNP. This would provide a way to study the role of PRNP in development and how mice models resistant to prion disease differ from wild-type mice vulnerable to prion disease. It would also continue to lay the groundwork for finding ways to combat human prion infections.

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