Final Report – CJD Foundation Grant Program

Project Title: Generating Recombinant Human Prions in Large Scale for Structural Studies

Principle Investigators: Fei Wang, PhD.

Background and Objectives: Prion diseases are a group of inevitably fatal neurodegenerative diseases that are caused by the conversion of the normal cellular prion protein (PrP^{C}) to its misfolded pathogenic isoform, PrP^{Sc} or prion, through the PrP^{Sc}-templated, self-propagating mechanism. The different clinical symptoms associated with sCJD, the most common human prion disease, and vCJD can be attributed to different types of human PrP^{Sc} at the molecular level, i.e., prions found in these diseases represent different human prion strains that most likely have different structures. Whether different human prion strains have a similar core structure that rules the self-replication of all prions, or they can exist in fundamentally different structures is still a matter of debate. In the past couple of years, several research groups have reported the high-resolution structures of rodent prions generated in experimentally infected animals, showing for the first time what the infectious prions look like at the atomistic level. Such structural information of rodent prions will definitely help scientists understand the detailed molecular mechanism of prion propagation and develop structure-based therapeutics. However, drug candidates that were able to successfully inhibit rodent prion propagations in animal preclinical trials failed to do so in patients, which indicates that human prions might be structurally distinct from rodent prions and further structural studies of human prions are warranted. And *the objective of this proposal is to efficiently generate recombinant human prions in large scale from various native human prions, including variant Creutzfeldt–Jakob disease (vCJD) and type 1 and 2 sporadic CJD (sCJD).* Such in vitro-generated prions will be very important for structural studies that will facilitate the structure-based drug design to treat human prion diseases.

Summary of accomplishments: We are very grateful for the support from the CJD Foundation and all the families that contribute to this grant. Below is the summary of the results of the experiments proposed for each Specific Aim.

Specific Aim 1: Identifying and optimizing the conditions for large-scale production of recombinant human prions.

Methods to generate prions	Quality	Quantity
1. ***In vivo* amplified PrP^{Sc}**	Highly infectious Various strains Low purity	Time-consuming High cost
2. ***In vitro* amplified, animal brain homogenate derived PrP^{Sc}**	Highly infectious Various strains Low purity	Time efficient Low cost
3. ***In vitro* amplified recombinant PrP^{Sc}**	Specific infectivity? Strain properties? High purity	Time efficient Low cost

Table 1. The quality and quantity of prions are critical for high-resolution structural studies.

The 3rd method listed in Table 1 would be the best approach to generate prions for structural studies, provided that the specific infectivity and strain properties of recombinant prions are the same as those of prions found in brains. To prove the concept, we generated recombinant mouse prion using our proprietary Protein Misfolding Cyclic Amplification (PMCA) technology (**Figure 1A**). The PMCA amplified recombinant prion not only demonstrated its specific infectivity by inducing prion disease in animals (**Figure 1B**) but also showed the same strain properties as the native mouse RML prion (**Figures 1C**

and 1D). The structural analyses of the recombinant prion by Cryo-Electronic Microscopy (EM) are under way and the preliminary data suggests a structural match between the recombinant prion and the prion in brains.

Inspired by the proof-of-concept study of recombinant mouse prions (Figure 1), we set to generate the recombinant human prions seeded by either sCJD or vCJD prions (**Figure 2**). We first tested the condition that was used to generate the recombinant mouse prion and failed to generate the recombinant human prions under condition 1 (**Figure 2A**). We then tested another condition, namely changing one of the PMCA components from mouse liver RNA to synthetic poly A RNA, and unfortunately, condition 2 did not yield any production of recombinant human prions (**Figure 2B**). Following a series of failed attempts, including the proposed usage of total brain homogenate of PrP knockout mice as cofactors, we decide to move on from the idea of generating recombinant human prions.

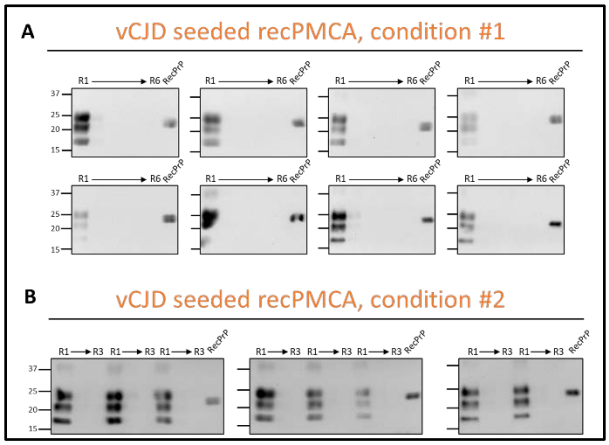

Figure 2. Attempts to generate recombinant human prions by PMCA. (A) Serial PMCA reactions seeded with vCJD prion. 10 uL of 10% vCJD BH and 90 mL of the substrate containing human recPrP and cofactors were subjected to the first round of PMCA. 10 uL of round 1 PMCA product was mixed with 90 uL of fresh substrate to carry out the second round of PMCA. Subsequent PMCA rounds were done in the same manner. After each round of PMCA, the generation of recPrP^{res} was monitored by treating 10 uL of product with 100 ug/mL PK. **(B)** The same as in (A) except that one of the cofactors was replaced by poly A RNA.

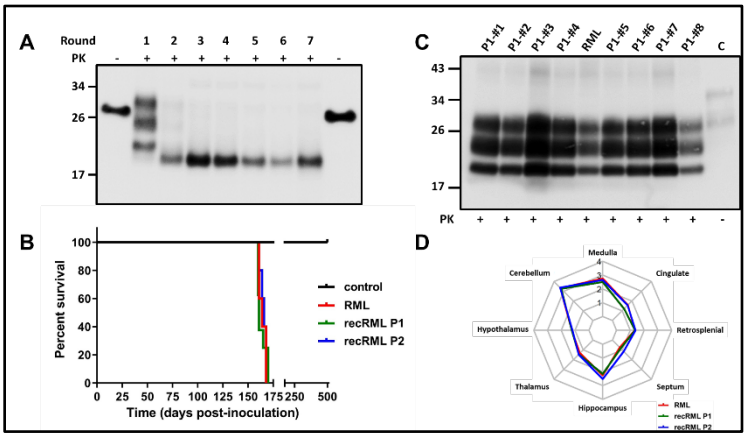

Figure 1. Generation of recombinant mouse prion by PMCA. (A) Serial PMCA reactions seeded with native murine RML prion. 10 uL of 10% RML BH and 90 uL of the substrate containing mouse recPrP and cofactors were subjected to the first round of PMCA. 10 uL of round 1 PMCA product was mixed with 90 uL of fresh substrate to carry out the second round of PMCA. Subsequent PMCA rounds were done in the same manner. After each round of PMCA, the generation of recPrP^{res} was monitored by treating 10 uL of product with 50 ug/mL PK. **(B)** Survival curves of wild-type C57B mice. Eight mice that were inoculated (i.c.) with purified recPrP^{res} succumbed to prion disease with an average survival time of 163.4 days post-infection (recRML P1). The second passage of bioassay (recRML P2) was carried out by injecting 10% BH of a diseased mouse from P1 to five mice. Seven mice were injected with 10^{-7} RML diluted in recPrP substrate. Five mice were injected with native RML as the positive control group. **(C)** Western blot analysis of the PK-resistant PrP^{Sc} in the brain of 8 mice from recRML P1. **(D)** Vacuolation profile in different brain regions of mice inoculated with recRML or native RML. Diseased animal brains were analyzed histologically for spongiform degeneration with hematoxylin-eosin staining. Eight different brain regions were selected and the values represent the average scores of vacuolation. The brain regions analyzed are medulla; cerebellum; hypothalamus; thalamus; hippocampus; septum; retrosplenial area; and cingulate area.

As we proposed in our application, if approach 3 doesn't work, we would proceed with approach 2, which is similarly time and cost-efficient as approach 3. The only difference is the PMCA-generated prions will need to be purified for the structural studies (**Table 1**). Our published results have demonstrated that the animal prions produced in vitro with approach 2 are highly infectious and maintain the strain properties of in vivo prions. By PMCA, we were able to amplify two types of sCJD prions in a highly efficient way (**Figure 3A**). The preliminary bioassay data suggests that the human prions generated with approach 2 are highly infectious. Since the PMCA-amplified human prions need to be purified from the reactions, a larger quantity of such materials is necessary for the downstream structural studies. In light of this, we implemented the Large-Scale

PMCA (LS-PMCA) to obtain a massive amount of amplified human prions in a short period of time (**Figure 3B**). Currently, we are optimizing the purification protocols for isolating human prions in high purity for future structural studies.

Specific Aim 2: Characterizing the strain properties of newly formed recombinant human prions.

As aforementioned, the PMCA conditions we tried failed to produce recombinant human prions with approach 3 and we decided to move the project forward with approach 2. Fortunately, we were able to generate infectious human prions by PMCA and further upgrade the production by implementing the LS-PMCA (**Figure 3**). Due to the delay of the experiments in Aim 1, the bioassays to test the infectivity of PMCA amplified human prions are still ongoing and the strain property characterization will be followed immediately after the conclusion of the bioassays.

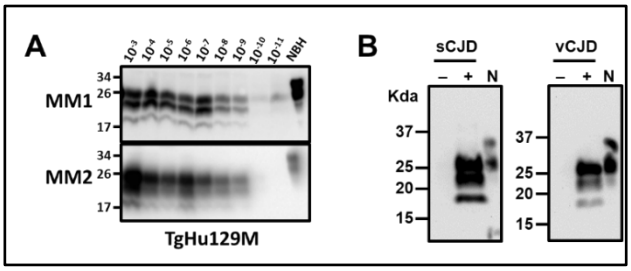

Figure 3. PMCA amplification of CJD prions in TgHu substrate. (A) PMCA reactions seeded with MM1 and MM2 sCJD prions at different dilutions. 10 uL of sCJD BH (10^{-2} to 10^{-10}) and 90 mL of the substrate containing 10% BH of transgenic mouse expressing human PrP^{C} were subjected to PMCA. **(B)** sCJD and vCJD prions were amplified by LS-PMCA using transgenic mouse BH containing human PrP^{C}. After PMCA, the generation of human PrP^{res} was monitored by treating 10 uL of product with 100 ug/mL PK.

Key findings and implications

- Although recombinant rodent prions have been generated or amplified using the PMCA technology with relatively common cofactors, i.e. lipids and RNA molecules, this recipe and various combinations of different cofactors were unable to amplify recombinant human prions, at least in our hands. Although these negative results are disappointing, they support the notion that human prions might be structurally distinct from rodent prions, the high-resolution structures of which have been obtained. Therefore, atomistic-level structures of human prions should remain one of the top priorities in the prion field.
- We and other researchers have demonstrated that PMCA-produced animal prions are highly infectious and can maintain the strain properties of native animal prions. In this study, our preliminary data suggest that PMCA-amplified human prions are also highly infectious.
- The upgraded LS-PMCA has been developed into a great platform to facilitate structural studies by producing highly infectious prions in large quantities.

Next steps: we will finish the animal bioassays and complete the strain property characterization as proposed in Aim 2. At the same time, we will continue to work on the optimization of the prion purification. The plan is to solve the structures of various human prions at the atomistic level with the ultimate goal of curing human prion diseases.

Publications supported in part by the CJD Foundation Grant

1. The LS-PMCA manuscript (Figure 3 included in this paper) has been submitted to *Molecular Neurodegeneration*.
2. The recombinant prion manuscript (Figure 1 included in this paper) is under preparation and will be submitted for publication in early 2023.