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# Generation of a new form of human $PrP^{Sc}$ in vitro by inter-species transmission from cervids prions

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**Running Title**: Conversion of human $PrP^{C}$ by cervid $PrP^{Sc}$

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**Prion diseases are infectious neurodegenerative disorders affecting humans and animals that result from the conversion of normal prion protein ($PrP^{C}$) into the misfolded prion protein ($PrP^{Sc}$). Chronic wasting disease (CWD) is a prion disorder of increasing prevalence within the United States that affects a large population of wild and captive deer and elk. Determining the risk of transmission of CWD to humans is of utmost importance, considering that people can be infected by animal prions, resulting in new fatal diseases. To study the possibility that human $PrP^{C}$ can be converted into the misfolded form by CWD $PrP^{Sc}$ we performed experiments using the Protein Misfolding Cyclic Amplification (PMCA) technique, which mimic *in vitro* the process of prion replication. Our results show that cervid $PrP^{Sc}$ can induce the conversion of human $PrP^{C}$, but only after the CWD prion strain has been stabilized by successive passages *in vitro* or *in vivo*. Interestingly, the newly generated human $PrP^{Sc}$ exhibits a distinct biochemical pattern that differs from any of the currently known forms of human $PrP^{Sc}$. Our results also have profound implications for understanding the mechanisms of prion species barrier and indicate that the transmission barrier is a dynamic process that depend on the strain and moreover the degree of adaptation of the strain. If our findings are corroborated by infectivity assays, they will imply that CWD prions have the potential to infect humans, and that this ability depends on CWD strain adaptation.**

Chronic wasting disease (CWD) is a disorder associated with infectious prions that affect deer, elk, and moose (1;2). CWD is so far the only prion disease of wild animals; it is highly contagious and the exact prevalence is currently unknown. Its origin and mechanism of transmission are also unclear. CWD-affected animals show loss of body condition, changes in behavior, ataxia, head tremors, salivation, and somnolence (1). CWD can reduce the growth and size of wild deer and elk populations in areas where the prevalence is high, and is of increasing concern in North America. The disease was thought to be limited in the wild to a relatively small endemic area in northeastern Colorado and southeastern Wyoming. However, in the last decade it has spread east and west of those areas even across natural borders, and has been reported in 14 US states, 2 Canadian provinces and South Korea (1;2). The risk of transmission of CWD to other animal species and especially to humans is unknown. Defining that risk is of utmost importance, considering that transmission of bovine prions to humans resulted in a new disease (variant Creutzfeldt-Jakob disease) and the number of cervids affected by CWD in the USA is increasing (3).

Transmission of prions between different species of mammals is often limited by the species barrier phenomenon (4). Currently the strength of the barrier between different species is unpredictable and requires bioassay studies to be assessed. These experiments are costly and time consuming, and in the case of humans cannot be measured directly, but rely on the use of animal models, such as transgenic mice or primates. It is presumed that a large number of hunters in the USA have been in contact with, or consumed, CWD-infected meat (5). Fortunately, no clinical evidence linking CWD exposed humans and CJD patients has yet been found (5;6). Experimental inoculation of CWD prions into squirrel monkeys has produced the disease (7;8), however transgenic mice expressing human PrP did not (9-11), suggesting that the

species barrier between humans and cervids is greater than between cattle and humans.

To test the possibility that human $PrP^{C}$ can be converted into the infectious form by CWD $PrP^{Sc}$ we performed experiments using the Protein Misfolding Cyclic Amplification (PMCA) technique. PMCA mimics prion replication *in vitro* at an accelerated speed (12); $PrP^{Sc}$ generated *in vitro* by PMCA has been shown to be infectious to wild type animals and maintain the strain properties (13-16). Interestingly, new prion strains can be generated, adapted, and stabilized upon crossing species barrier *in vitro* by PMCA (16;17). These findings demonstrate that PMCA is a valuable tool to investigate the strength of the barrier between diverse species, its molecular determinants, and the expected features of the new infectious material produced.

## Experimental Procedures

*Preparation of brain homogenates.* Brains from healthy transgenic mice over-expressing either human PrP (MM genotype) (18) or cervid PrP (Tg1536) (19) were extracted after animals were perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA. As inoculum for the PMCA reaction, we used brains from different naturally affected CWD mule deer, cattle BSE, sheep scrapie, humans affected by diverse forms of CJD or Tg1536 infected with CWD prions. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing NaCl 150mM, 1.0% Triton X-100, and the complete™ cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany).

*PMCA procedure.* Aliquots of 100 ul of 10% healthy brain homogenate were loaded onto 0.2-ml PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 4000, Farmingdale, NY) and programmed to perform 144 cycles of 30 min incubation at 37°C followed by a 20 s pulse of sonication set at an amplitude of 70. For serial PMCA, after each round of cycles, a 10 ul aliquot of the amplified material was diluted into 90 ul of normal brain homogenate and a new round of PMCA cycles was performed. In some experiments, 0.05% digitonin and 4mM EDTA was added to the conversion buffer. The detailed protocol for PMCA, including reagents, solutions and troubleshooting, has been published elsewhere (20;21).

*$PrP^{Sc}$ detection by western blot after PK digestion.* The standard procedure to digest $PrP^{Sc}$ consists on subjecting the samples to incubation in the presence of PK (50 µg/ml) during 60 min with shaking at 37°C. The digestion was stopped by adding electrophoresis sample buffer and protease-resistant PrP was revealed by western blotting. Proteins were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto nitrocellulose membrane and probed with 6D11 antibody (1:5,000 dilution) to recognize cervid, cattle or sheep PrP proteins or with 3F4 antibody (1:10,000 dilution) to detect exclusively human PrP. The immunoreactive bands were visualized by enhanced chemoluminesence assay (Amersham, Piscataway, NJ) using a UVP image analysis system.

## Results

Having validated the PMCA technology to assess the species barrier *in vitro* (16;17), we investigated the possibility that CWD $PrP^{Sc}$ can convert human $PrP^{C}$ to $PrP^{Sc}$. Standard PMCA using samples from three different CWD brains failed to produce a signal for human $PrP^{Sc}$ (Fig. 1A). For these studies we

took advantage of the fact that human, but not deer, PrP is detectable with the 3F4 antibody (22), so any PK-resistant signal detectable with this antibody corresponds to human $PrP^{Sc}$ material. To further attempt the generation of human $PrP^{Sc}$ from deer, we performed several rounds of PMCA, a process that has been shown to dramatically increase the amplification efficiency (23;24). As shown in figure 1B, no human $PrP^{Sc}$ signal was detectable even after 3 rounds of PMCA. Importantly, the same CWD inoculum efficiently induced the conversion of deer $PrP^{C}$ into $PrP^{Sc}$ (Fig. 1C). We used brains of transgenic mice expressing human $PrP^{C}$ as the substrate under conditions that were previously shown to efficiently propagate human $PrP^{Sc}$ from various forms of sporadic and variant CJD (14) (see also supplementary figure 1). To further attempt conversion of human $PrP^{C}$ by deer $PrP^{Sc}$, we added 0.05% digitonin and 4 mM EDTA to the conversion buffer, which we have found to increase the efficiency of PMCA amplification in human samples (Supplementary figure 1). However, these conditions were unsuccessful in generating proteinase K (PK) resistant human $PrP^{Sc}$ (Fig. 1D). In our previous experiments to cross the mouse-hamster species barrier by PMCA we found that adding a more concentrated solution of the $PrP^{Sc}$ inoculum was successful in producing inter-species conversion (17). To evaluate this possibility we used more concentrated CWD material (from 2x to 1/5x) as inoculum. Again, no human $PrP^{Sc}$ product was observed under these conditions (Fig. 1E). The conclusion from these experiments is that either the deer-human barrier is absolute or, the PMCA conditions were not optimal to cross it. A previous study from Hoover's group was also unable to replicate CWD $PrP^{Sc}$ at expenses of human $PrP^{C}$ in serial PMCA experiments (25).

Based on our previous studies, we noted that PMCA not only enables inter-species conversion, but it also allows adapting the new strain generated (17). Indeed, a recent study showed that serial rounds of PMCA produce the same adaptation of a natural CWD prion strain as did serial passages *in vivo* (26). Since the CWD prions used in our experiments are derived from naturally affected mule deer, we hypothesized that the CWD strain most likely corresponds to a not completely stable prion strain. To assess whether strain adaptation by serial PMCA rounds might influence the ability of CWD prions to cross the human species barrier, we performed several passes of CWD $PrP^{Sc}$ into deer $PrP^{C}$ by PMCA (Fig. 2A). For this experiment, 6 replicate experiments were performed in parallel. Interestingly, after 1 PMCA round, the deer $PrP^{Sc}$ produced was able to convert with low efficiency human $PrP^{C}$ (Fig. 2B, top panel). One of the 6 replicates showed a clear protease-resistant band with a molecular weight reminiscent of PrP27-30. Two or three other replicates showed faint immune-reactive bands (Fig. 2B, top panel). Moreover, after a second and third passage of CWD $PrP^{Sc}$ into deer $PrP^{C}$, a much more clear and efficient conversion of human $PrP^{C}$ to $PrP^{Sc}$ was observed (Fig. 2B, middle and bottom panels). These results suggest that CWD prions might be able to affect human beings, but only after the CWD strain has been stabilized by successive passaging. To further test this hypothesis and to rule out that the ability to convert human PrP was not an artifact produced by *in vitro* PMCA amplification, we tested samples of CWD that were serially passed in transgenic mice expressing cervid $PrP^{C}$. Samples from first and second passage *in vivo* in mice from two different inocula were used to trigger conversion of human $PrP^{C}$. As shown in Figure 3, cervid $PrP^{Sc}$ obtained from these two inocula, after either 1 or 2 passages in transgenic mice,

was able to convert human $PrP^{C}$ into PK-resistant human $PrP^{Sc}$.

Interestingly, when the Western blot profile of this newly generated form of human $PrP^{Sc}$ (termed CWD-huPrP$^{Sc}$) was compared with known strains of human prions, it was clear that CWD-huPrP$^{Sc}$ exhibited a different pattern (Fig. 4A). The electrophoretic migration of this protein after PK-digestion is similar to the type 1 strain of sCJD, but its glycosylation profile is clearly different, showing a highly predominant di-glycosylated form (Fig. 4A and B). This result suggests that CWD hu-$PrP^{Sc}$ corresponds to a new human prion strain. Interestingly, a detailed previous study from Gambetti's group comparing the biochemical characteristics of $PrP^{Sc}$ from cervids and humans showed that CWD $PrP^{Sc}$ is similar to sCJDMM1 in terms of electrophoretic mobility (6). However, the misfolded protein associated with CWD is predominantly di-glycosylated, whereas $PrP^{Sc}$ from type 1 sCJD is mostly mono-glycosylated (6). Based on the fact that transmission of BSE prions to humans resulted in a new form of $PrP^{Sc}$ very similar to the one in cattle (6;27), these authors predicted that if humans were infected by CWD it is likely that $PrP^{Sc}$ would be of type 1 and with a predominance of the di-glycosylated isoform (6). Our results agree with that prediction and suggest that the newly generated CWD-huPrP$^{Sc}$ acquires the biochemical properties of the cervid infectious material (Fig. 4A and B). We and others have shown that PMCA replication of $PrP^{Sc}$ obtained from experimental rodents, sheep, cervid and human samples faithfully maintains the prion strain characteristics (14;16;26;28-30). To further support the relevance of our results, we performed experiments in which human $PrP^{C}$ was attempted to be converted by either cattle BSE $PrP^{Sc}$ or sheep scrapie $PrP^{Sc}$. Whereas the typical vCJD type of $PrP^{Sc}$ was generated when human $PrP^{C}$ was converted by BSE $PrP^{Sc}$ (Fig. 4C), no human $PrP^{Sc}$ was generated under any condition when sheep scrapie $PrP^{Sc}$ was used as inoculum (Fig. 4C). These results further validate our PMCA assay.

## Discussion

CWD is possibly the most worrisome prion zoonosis, because it affects free-ranging animals, making it very difficult to control its spread, and because it is highly efficiently transmitted (1;2). Indeed, in dense free-ranging cervid populations, CWD prevalence can reach as high as 30%, and among captive herds, the prevalence can climb to nearly 100%. The mechanisms and routes of transmission are currently unknown, but likely involve horizontal spread through exposure to prion infected secretions, excretions, or decomposed carcasses (1;2). Moreover, it is likely that CWD prions are progressively accumulating in the environment, since $PrP^{Sc}$ binds tightly to soil and can maintain infectivity for a long time (31-33). Currently, it is unknown what proportion of natural CWD cases arises sporadically or comes from horizontal transmission among animals. Based on the available knowledge of the emergence, adaptation and stabilization of prion strains, it is likely that prions appear either spontaneously, through inter-species transmission or by genetic mutations. These "first generation" prions are unstable strains that begin a progressive and gradual process of adaptation that may take several passages and years or decades to complete. In addition natural strain stabilization may take considerable more time than the controlled adaptation done by intracerebral inoculation of brain homogenates in experimental animals. In natural cases, animals usually get infected by peripheral (most likely oral) exposure to small quantities of prions

present in peripheral tissues or secretion fluids. Recent data indicates that in some cases the strain characteristics of natural prions in peripheral organs are different than those in the brain even in the same individuals (GCT, unpublished results; MAB and CS, unpublished data). In cervids, there are at least two different strains that can be differentiated by the incubation time and neuropathological characteristics produced when inoculated into transgenic mice expressing deer PrP (34). It is currently unknown the susceptibility of these two strains to human transmission.

Our findings demonstrate that cervid $PrP^{Sc}$, upon strain adaptation by serial passages *in vitro* or in cervid transgenic mice, is capable of converting human $PrP^{C}$ to produce $PrP^{Sc}$ with unique biochemical properties, likely representing a new human prion strain. The newly generated CWD-huPrP$^{Sc}$ material has been inoculated into transgenic mice expressing human PrP to study infectivity and disease phenotype and this data will be published elsewhere. We have safely ruled out that human $PrP^{Sc}$ generated in these studies is not coming from spontaneous "de novo generation", since under the conditions used, no spontaneous PK-resistant band was ever detected in brain homogenates of humans or transgenic mice expressing human $PrP^{C}$, even after more than 20 serial rounds of PMCA (35). Furthermore, none of the many controls included in our experiments in which no $PrP^{Sc}$ was added to the reaction, showed any PK-resistant PrP band.

Various studies aimed to analyze the transmission of CWD to transgenic mice expressing human PrP have consistently given negative results (9-11), indicating a strong species barrier. This conclusion is consistent with our many failed experiments to attempt converting human $PrP^{C}$ with natural CWD, even after pushing the PMCA conditions (see figure 1). We found successful conversion only after adaptation of the CWD prion strain by successive passages *in vitro* or in cervid transgenic mice. We are not aware that in any of the transgenic mice studies the inoculum used was a previously stabilized CWD strain. Although, it has been shown that strain stabilization *in vitro* by PMCA (17;26) and *in vivo* using experimental rodents (36) has similarities with the strain adaptation process occurring in natural hosts, we cannot rule out that the type of CWD strain adaptation that is required to produce strains transmissible to humans may take much longer time in cervids or not occur at all. An important experiment will be to study transmissibility to humanized transgenic mice of CWD passed experimentally in deer several times.

Besides the importance of our results for public health in relation to the putative transmissibility of CWD to humans, our data also illustrate a very important and novel scientific concept related to the mechanism of prion transmission across species barriers. Today the view is that species barrier is mostly controlled by the degree of similarity on the sequence of the prion protein between the host and the infectious material (4). In our study we show that the strain and moreover the stabilization of the strain plays a major role in the inter-species transmission. In our system there is no change on the protein sequence, but yet strain adaptation results in a complete change on prion transmissibility with potentially dramatic consequences. Therefore, our findings lead to a new view of the species barrier that should not be seen as a static process, but rather a dynamic biological phenomenon that can change over time when prion strains mature and evolve. It remains to be investigated if other species barriers also change upon progressive strain adaptation of other prion forms (e.g. the sheep/human barrier).

Our results have far-reaching implications for human health, since they indicate that cervid $PrP^{Sc}$ can trigger the conversion of human $PrP^{C}$ into $PrP^{Sc}$, suggesting that CWD might be infectious to humans. Interestingly our findings suggest that unstable strains from CWD affected animals might not be a problem for humans, but upon strain stabilization by successive passages in the wild, this disease might become progressively more transmissible to man.

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**Figure Legends**

**Figure 1. Unsuccessful conversion of human $PrP^{C}$ by CWD $PrP^{Sc}$. A:** Three different samples of brain homogenates from mule deer naturally affected with CWD were used to attempt conversion of human $PrP^{C}$. 10% CWD brain homogenates were diluted 50, 500 or 5000-fold into 10% brain homogenates from transgenic mice expressing the human (129M) PrP gene coding segment. Samples were either frozen or subjected to 144 cycles of PMCA. Formation of $PrP^{Sc}$ was assessed by PK digestion followed by western blot using the 3F4 antibody that recognizes human, but not cervid, PrP (22). The amount of cervid $PrP^{Sc}$ in the inoculum was checked by western blot using the 6D11 antibody. **B**: To further attempt conversion of human $PrP^{C}$ with cervid prions, several serial rounds of PMCA were done using standard conditions. As previously reported, 3 serial rounds of PMCA should lead to around 100 million fold amplification of any $PrP^{Sc}$ signal present in the sample (24). **C:** The conversion of deer $PrP^{C}$ by CWD $PrP^{Sc}$ by PMCA was studied using samples of 10% CWD brain homogenate (inoculum A) that were diluted into 10% brain homogenate prepared from healthy transgenic mice expressing cervid $PrP^{C}$. The formation of cervid $PrP^{Sc}$ was assessed by western blot using the 6D11 antibody. **D**: Crossing the cervid-human species barrier was further attempted by adding detergents and EDTA to the conversion buffer, which has been shown to increase PMCA

efficiency (37), particularly for human samples (Supplementary figure 1). **E:** The CWD inoculum was concentrated by the sarkosyl procedure described in Methods, in order to add larger amounts of $PrP^{Sc}$. Additions of a 2x, 1x, 1/2x or 1/5x of $PrP^{Sc}$ relative to the 10% CWD brain homogenate were added to the human transgenic mouse brain homogenate and subjected to PMCA either in the presence or absence of digitonin and EDTA. In the experiments shown in all panels of this figure, samples were treated with PK (50 µg/ml), except for the normal brain homogenate (NBH) used as control (NBH No PK). The asterisks in panels B and E represent incomplete digestion of $PrP^{C}$, which is clearly appreciated because the electrophoretic mobility is identical to full-length $PrP^{C}$.

**Figure 2. Conversion of human $PrP^{C}$ induced by CWD $PrP^{Sc}$ after PMCA strain adaptation. A:** Six separate samples of a 1000-fold dilution of CWD brain homogenate (inoculum A) were used to convert cervid $PrP^{C}$ in three successive rounds of 144 PMCA cycles. Protease-resistant deer $PrP^{Sc}$ was measured by western blot using the 6D11 antibody. **B**: Samples from the amplified material were used to attempt conversion of human $PrP^{C}$ by PMCA and protease-resistant signal was checked using the human-specific 3F4 antibody.

**Figure 3. Conversion of human $PrP^{C}$ induced by CWD $PrP^{Sc}$ after *in vivo* strain adaptation.** Transgenic mice expressing the cervid PrP gene were used to serially passage CWD prions from two different natural inocula. Brain homogenate from sick transgenic mice after 1 or 2 *in vivo* passages was used to convert human $PrP^{C}$ by subjecting the sample to 144 PMCA cycles. The generation of human $PrP^{Sc}$ after PMCA was assessed by western blot employing the 3F4 antibody (right panel). The $PrP^{Sc}$ reactivity of the CWD inoculum from cervid transgenic mice was evaluated by western blot using the 6D11 antibody (left panel). All samples were digested with PK (50 µg/ml), except for the normal brain homogenate (NBH), used as a marker of full-length $PrP^{C}$ electropheretic migration. The asterisk represents incomplete digestion of $PrP^{C}$. As shown before, amplification of the original CWD inoculum at the expense of human $PrP^{C}$ failed to show any PK-resistant $PrP^{Sc}$ band.

**Figure 4. Western blot profile of human $PrP^{Sc}$ generated *in vitro* from CWD. A:** Aliquots of CWD-huPrP$^{Sc}$ obtained by *in vitro* conversion of huPrP$^{C}$ with CWD after 2 rounds of PMCA in deer (see figure 2) were loaded together with huPrP$^{Sc}$ associated to vCJD and sCJD Types MM1 and MM2. Samples were treated with PK and developed using the 3F4 antibody. The right side shows a schematic representation of the mobility and intensity of the different PrP glycoforms in each sample. **B**: Samples of $PrP^{Sc}$ from either cattle affected by BSE or sheep by typical scrapie were used to induce conversion of human $PrP^{C}$ by 144 PMCA cycles. Newly generated human $PrP^{Sc}$ was detected by western blot using the 3F4 antibody that recognizes human, but not cattle or sheep PrP. As a control of electrophoretical pattern of human $PrP^{Sc}$ associated with vCJD a brain sample coming from a patient affected by this disease was loaded. The asterisk represents a signal coming from incomplete digestion of $PrP^{C}$.

Figure 1

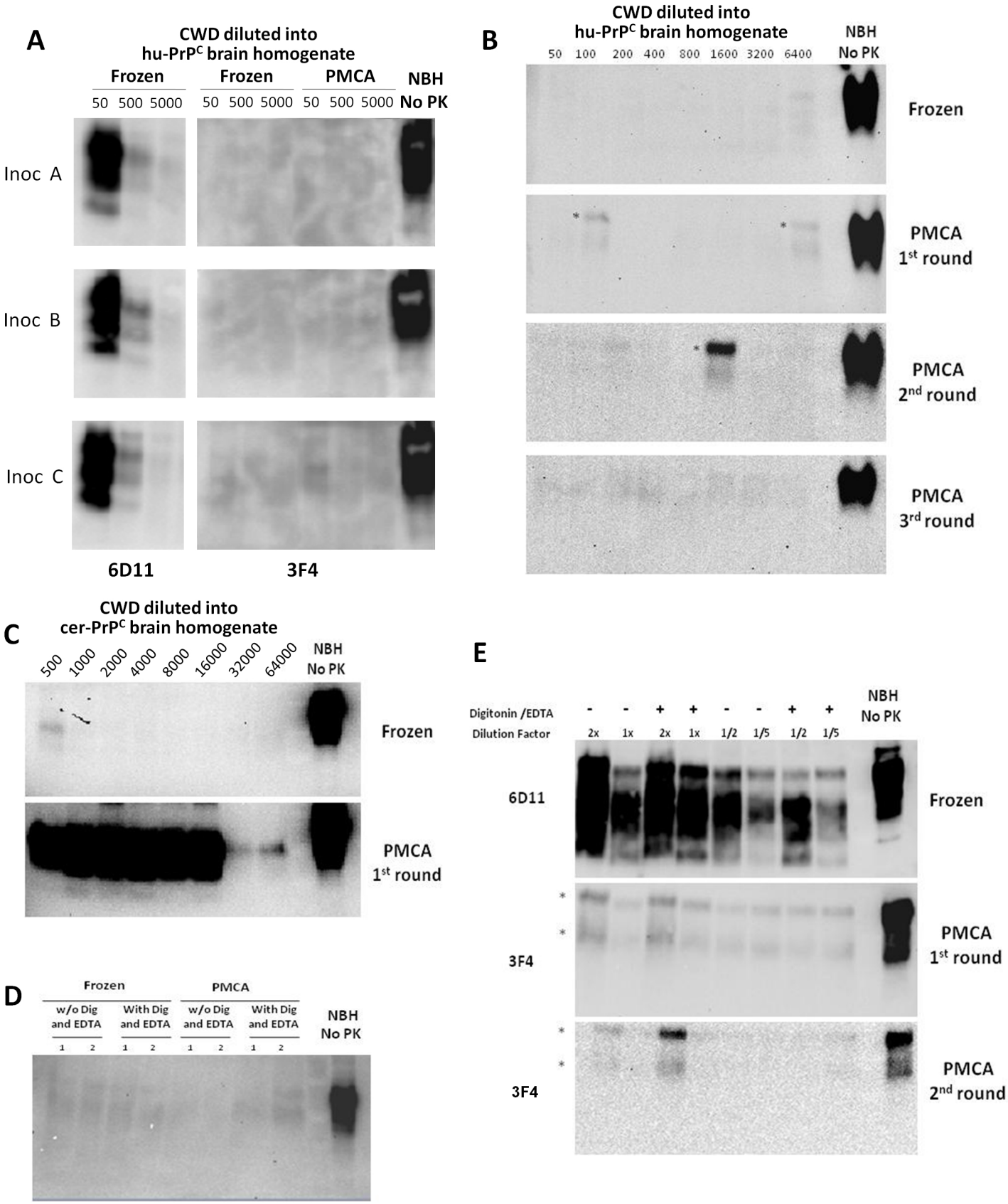
A
CWD diluted into
hu-PrP^C brain homogenate
Frozen
Frozen
PMCA
NBH
No PK
50 500 5000
50 500 5000
50 500 5000
Inoc A
Inoc B
Inoc C
6D11
3F4
B
CWD diluted into
hu-PrP^C brain homogenate
NBH
No PK
50 100 200 400 800 1600 3200 6400
Frozen
PMCA
1st round
PMCA
2nd round
PMCA
3rd round
C
CWD diluted into
cer-PrP^C brain homogenate
500 1000 2000 4000 8000 16000 32000 64000
NBH
No PK
Frozen
PMCA
1st round
D
Frozen
PMCA
w/o Dig
and EDTA
With Dig
and EDTA
w/o Dig
and EDTA
With Dig
and EDTA
1 2 1 2 1 2 1 2
NBH
No PK
E
Digitonin /EDTA
- - + + - - + +
Dilution Factor
2x 1x 2x 1x 1/2 1/5 1/2 1/5
NBH
No PK
6D11
Frozen
3F4
PMCA
1st round
3F4
PMCA
2nd round

Fig. 2

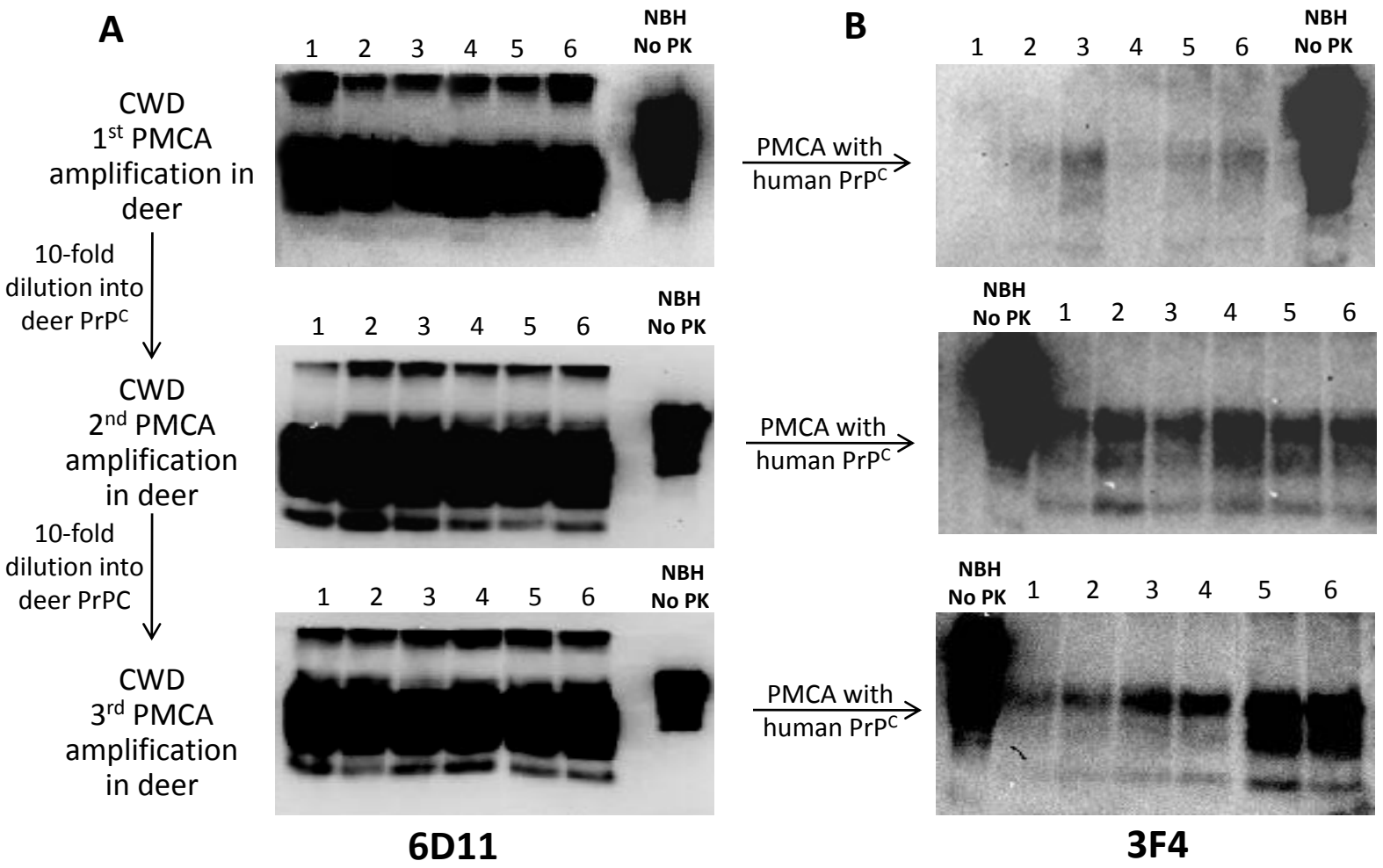

Fig. 3

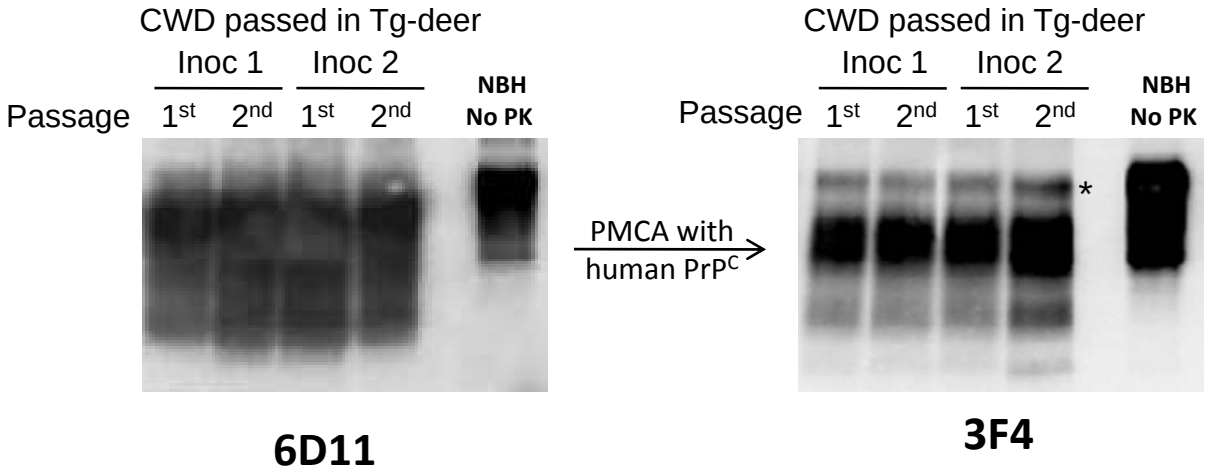

Fig. 4