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Early Host Responses to Prion Infection: Development of in Vivo and in Vitro Assays

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The first goal was to identify changes in mRNA expression and in plasma glycoproteins that are induced by prion infection in mice. The unusual nature of prion disease prompted a systems approach to identify networks specifically perturbed by prion infections and to determine which perturbations are essential for various aspects of the disease. We tracked changes in gene expression in brain and spleen for two different prion strains and five different lines of mice over their entire incubation periods. A novel approach identified shared differentially expressed genes (DEGs) that were integrated with the kinetics of PrPSc accumulation, pathology, and protein-protein interaction databases to construct prion disease-specific, dynamic protein networks. A Prion Disease Database web site will share these data. The second goal was to determine whether CNS stem cells could provide an in vitro assay for prion infection. We have shown that CNS-stem cell lines mirror the genetic susceptibility of the mice from which they were derived. Cell lines from PrP over expressing mice can detect high dilutions (10^-8) of RML prions much more rapidly and economically than mouse bioassays.
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Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

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INTRODUCTION

Prions are unique among transmissible, disease-causing agents in being composed of a misfolded form of a normally benign, host-encoded protein. Prion replication, which results in fatal neurodegenerative disease, involves posttranslational conformational conversion of the normal prion protein (PrP\textsuperscript{C}) to disease-specific PrP\textsuperscript{Sc} isoforms. Although devoid of nucleic acids, the existence of distinct prion strains indicates prion-specified heritable information is passed from the innoculum to newly formed prions. PrP\textsuperscript{Sc} can exist in several different conformations as indicated by differences in susceptibility and/or cleavage site following limited proteinase K digestion and by variation in the concentration of denaturant required to reveal antibody epitopes that are buried in the misfolded protein. Thus, PrP\textsuperscript{Sc} may act as a template for misfolding of PrP\textsuperscript{C}. Studies involving transgenic mice indicate involvement of unknown molecules, designated protein X, in this templating. Although protein-mediated cyclic amplification can be obtained \textit{in vitro} using brain homogenates, it appears likely that accessory molecules facilitate the autocatalytic propagation of PrP\textsuperscript{Sc}. To find such molecules and to identify a molecular signature that could be used as a marker for identifying infected individuals before the appearance of clinical signs, we applied a systems approach that combines advances in genomics and proteomics. We have also refined CNS stem cell-containing neurosphere cultures as a potential prion bioassay and for analysis of pathways involved in or perturbed by prion replication.

BODY

Progress towards completing each specific sub-task in the Statement of Work are indicated in \textbf{bold type} below:

\textbf{Task 1.} Determine whether there are specific changes in mRNA and protein expression profiles in the blood of prion-infected mice.

\begin{itemize}
  \item a. Expand our colonies of mice to provide sufficient numbers of C57BL/6J (B6), B6.1-1 (B6.I), FVB/Ncr (FVB), FVB.129-Prnp\textsuperscript{tm1Zrch}(Prnp\textsuperscript{0/0}), (FVB x Prnp\textsuperscript{0/0})F1, and Tg(Mo-PrP-A)B4053 (Tg4053) mice for the experiment. Fourteen groups of mice with 20 mice per group were needed. \textbf{Successfully completed FY 01.}
  \item b. Establish reproducibility of mRNA isolation. \textbf{Successfully completed FY 01.}
  \item c. Inoculate these genetically defined mice with one of two different prion strains harvest plasma, brain and spleen mRNA at regular intervals (7, 14, or 28 days) throughout the pre-clinical incubation period and after clinical signs appear. \textbf{Successfully completed FY 02 & FY 03.}
  \item d. Perform DNA microarray and serum glycoprotein analyses, analyze the data, and determine whether there are expression profiles unique to each host-agent combination and/or whether a genotype-independent and agent-independent
profile specific to prion exposure can be detected. Each timepoint will consist of 4 replicates per group. Use of Affymetrix chips rather than custom arrays necessitated using 3, rather than 4, mice per timepoint. Chip hybridization studies were successfully completed in FY 03. Final data analyses are in progress and we anticipate publication and release of the data on a publicly accessible and searchable web site by July 2006.

e. Changes in mRNA expression suggestive of specificity for prion infection will be re-evaluated using larger volumes of blood pooled from relevant groups of mice. An alternative approach was undertaken in FY03. Nervous system-specific genes identified by massively parallel signature sequencing that were detected as DEGs in our microarray analyses and predicted to encode secreted proteins were explored as potential biomarkers. These experiments are continuing.

Task 2. Using existing mouse neurosphere lines, determine whether CNS stem cells can provide an in vitro model for prion infection.

a) Import and establish cultures of mouse CNS neurosphere lines provided by our collaborators at StemCells, Inc. and the Salk Institute. Successfully completed, FY 01.

b) Assess levels of PrP^C expression by immunoblotting and immunofluorescence in the neurosphere lines in comparison to N2a cells that can be infected with prions. Successfully completed, FY 01.

c) Infect PrP^C-expressing neurosphere lines in culture with RML scrapie isolate. Assess, viability, proliferation and differentiation at each passage. Harvest cultures, prepare protein isolates and determine whether proteinase K-resistant PrP^Sc increases over the amount present immediately after infection. Successfully completed, FY 02. This aspect of the project has been greatly expanded in FY 03 to include additional neurosphere lines, development of neurospheres as an in vitro assay for prions, and use of neurosphere cultures to dissect the genetics of prion susceptibility. See progress report below and Giri et al. 2006. Prion infection of mouse neurospheres. Proc Natl Acad Sci 103: 3875-80.

d) Determine whether prions are propagated in infected neurosphere cultures by incubation time analysis in mice. Successfully completed, FY 02.

e) Using mRNA expression profiling and quantitation of secreted or released glycoproteins determine whether a subset of prion-specific changes are shared by neurospheres and mice. In progress. These expanded studies are currently funded by a USAMRMC Institutional Award.

Task 1. An essential feature in our systems approach to prion disease is comprehensive time-course transcriptome analysis on different mouse-prion groups, and integration of mRNA measurements from such different groups to generate coherent hypotheses about mechanisms of pathogenesis and identification of disease markers. We used Affymetrix GeneChip mouse array 430 2.0 which contains about 45,000 probesets corresponding to ~25,000 annotated genes. To investigate the dynamics over the entire incubation time, we collected brain and spleen tissues from three mice from each group and each time point. Total RNA was isolated from each sample and then hybridized individually. The total dataset represents more than 450 chips and ~20 million data points. The array intensities in each group were normalized using gcRMA algorithm. The DEGs with consistent temporal patterns across multiple mouse-prion groups were considered likely
to be associated with fundamental prion disease processes. All DEG data has been assembled in a searchable web site (home page in Figure 1 below) that will be available on publication of these results; this web site also will include the networks described below and associated analytical tools.

Figure 1. All microarray data from this project will be available soon on the Prion Disease Database web site.

The DEGs were grouped into Gene Ontology (GO) functional clusters that included lipid metabolism, inflammatory responses, synaptic transmission, and programmed cell death, among others. These GO clusters were regrouped into four major pathological functional groups: a) microglial/astrocyte activation; b) synaptic degeneration; c) neuronal cell death; and d) protein transport and degradation. The DEGS fell into temporal patterns of gene activation reflecting sequential activation of genes in these clusters. Groups a-c are activated in sequence with genes reflecting microglial activation differentially expressed as soon as 6 to 8 weeks after inoculation, well before clinical signs of disease appear. Some group d DEGs also appear early and may include responses to intracellular events related to PrPSc accumulation.

The proteins encoded by DEGs within each functional were assembled into prion disease-specific, dynamic networks reflecting known biological pathways and protein-protein interactions. Transcriptional regulatory networks also were assembled based on known regulatory sequence of differentially expressed proteins and transcription factors. As an example, results for one network are summarized in Figure 2.

Comparison of dynamic network behaviors among the various prion strain-mouse strain combinations, and with perturbations in other neurodegenerative disorders, provides a novel perspective to evaluate involvement of pathways specific to prion replication and prion pathogenesis. Our results also provide new insights into how systems approaches will enable new strategies for drug target identification and ultimately disease prevention. The dynamic network models were integrated with the brain-specific secreted proteins predicted by massively parallel signature sequencing of the transcriptomes of many different mouse organs and tissues and used to predict early changes in blood proteins during the course of disease. Preliminary proteomic analysis of some serum proteins
identified several early blood marker candidates for mouse prion disease. A manuscript
describing our results, along with the methods developed for the analyses will be
provided soon, as will instructions for accessing the Prion Disease Database.

Task 2. As detailed in the publication included with this annual report (Giri et al., 2006),
the susceptibility to prion infection of CNS stem cell-containing neurosphere cultures
from FVB, FVB-Tg4053, and \textit{Prnp}^{0/0} mirrors that of the mice from which they were
derived. The following is a brief overview of these studies and their extension to
additional strains of mice and strains of prions. Neurosphere lines now have been
established from the same strains and transgenic lines of mice used in the microarray
analyses described in Task 1. This will allow us to compare differential gene expression
in uniform populations of cells that replicate prions, but that do not elicit the pathological
responses seen in the brain.

\textbf{PrP}^{\text{C}} \textit{is expressed in neurosphere cultures}. Susceptibility to prion disease requires the
expression of host \textit{PrP}^{\text{C}} levels of which were analyzed by immunoblotting. Cell blot
analysis of FVB, \textit{Prnp}^{0/0}, and Tg4053 neurospheres clearly demonstrated the higher level
of \textit{PrP}^{\text{C}} expression in Tg4053 neurospheres compared to FVB neurospheres. No \textit{PrP}
immunostaining was observed in \textit{Prnp}^{0/0} neurospheres, making them an ideal negative
control for persistence of \textit{PrP}^{\text{Sc}} in subsequent infection studies (See Figure 1 in Giri et
al., 2006).

\textbf{Replication of \textit{PrP}^{\text{Sc}} in neurospheres correlates with level of \textit{PrP}^{\text{C}} expression.}
Having established the expression of \textit{PrP}^{\text{Sc}} in neurosphere cultures, we tested their
susceptibility to prion infection. FVB, Tg4053, and \textit{Prnp}^{0/0} cultures at passage 3 were
incubated with a 50-fold dilution of RML prions for 4 days. At the end of 4 days, the neurospheres were washed and split 1:4. To detect the persistence and de novo production of proteinase K (PK)-resistant PrPSc in infected cultures, cells growing on plastic cover slips were blotted and tested for the presence of PK-resistant PrPSc. All three neurosphere lines showed PrPSc at 12 days postinfection (dpi), whereas control neurospheres that were not exposed to RML prions had no PrPSc. By 24 dpi, little PrPSc remained in Prnp<sup>−/−</sup> neurospheres while PrP<sup>Sc</sup> was readily detected in FVB and Tg4053 cultures. By 36 dpi and 2 passages, no PK-resistant PrP<sup>Sc</sup> was seen in Prnp<sup>−/−</sup> neurospheres, but substantial amounts of PK-resistant PrP<sup>Sc</sup> were found in Tg4053 neurospheres; fewer PrP<sup>Sc</sup>-positive neurospheres were found in FVB cultures, but PrPSc was clearly present. Independently isolated cultures from the three mouse lines gave similar results. Western immunoblots show that PrPSc increased from passage 2 to 3 and was maintained at high levels thereafter in two Tg4053 neurosphere isolates.

Infected FVB neurospheres also produced PrP<sup>Sc</sup> but to a lesser extent than Tg4053 cultures (see Fig. 2, Giri et al. 2006). At passages 2–4 postexposure, FVB neurospheres replicated PrP<sup>Sc</sup> more slowly than Tg4053 cultures. FVB cultures were allowed to grow to a density similar to that of Tg4053 neurospheres, and, at passage 5, a dramatic increase in PrP<sup>Sc</sup> level was observed. Infected Tg4053 neurospheres continue to produce high levels of PrP<sup>Sc</sup> for more than 20 passages postexposure; this represents a ~10<sup>8</sup>-fold dilution of the original RML-infected brain homogenate. Infected Tg4053 neurospheres can be cryopreserved and produce PrP<sup>Sc</sup> upon thawing. Tg4053 cultures are highly susceptible to infection and replicate PrP<sup>Sc</sup> from passage 3 to passage 14. There were no obvious differences in cell morphology or growth rates between infected and uninfected cultures. In summary, prion replication takes longer to become established in FVB than in Tg4053 neurospheres.

**Neurosphere cultures produce infectious prions.** To determine whether PrP<sup>Sc</sup> replicating in Tg4053 neurospheres is infectious, we inoculated cell lysates containing 4 µg of protein into Tg4053 and FVB mice. All inoculated Tg4053 and FVB developed disease. In contrast, all mice injected with lysates (40 µg protein) from Prnp<sup>−/−</sup> neurosphere cultures exposed to RML prions remained healthy and showed no pathological changes in their brains. Again, Prnp<sup>−/−</sup> neurospheres provide an important control to distinguish prions produced in culture from residual inoculum. Western blot analysis demonstrated the presence of PK-resistant PrP<sup>Sc</sup> with a glycoform profile similar to that of the original RML inoculum. As expected, formation of PK-resistant PrP isoforms in Tg4053 neurospheres was accompanied by the production of infectious prions.

**Cells in infected cultures contain intracellular aggregates of PrP<sup>Sc</sup>.** Previous results from several laboratories indicate that PrP<sup>Sc</sup> accumulates intracellularly. Immunofluorescent detection of PrP on fixed, permeabilized single cell preparations and on cells grown on poly-L-lysine–coated coverslips (Figure 3) from infected and uninfected Tg4053 neurospheres was performed with or without denaturation by guanidine thiocyanate (GdnSCN). The epitopes detected by D18 and D13 Fabs are buried in PrP<sup>Sc</sup> so denaturation is required for PrP<sup>Sc</sup> detection. Cells from infected cultures treated with GdnSCN prior to anti-PrP staining appeared different from uninfected cultures and from nondenatured, infected cells. Fluorescence was more intense and granular in GdnSCN-treated infected cells than in the other samples. Single cell preparations were used to quantify differences in fluorescence intensity in individual cells among the groups (see Figure 3 in Giri et al., 2006). In uninfected cells, no significant difference was seen in PrP immunostaining intensity between denatured and nondenatured samples. In contrast, the
intensity of PrP immunostaining was significantly greater in denatured, infected cells than in non-denatured, infected samples and uninfected cultures. This increased staining and punctate distribution in infected, denatured cells indicate accumulation of PrP$^{\text{Sc}}$ since the epitopes detected by the D13 and D18 Fabs are buried in undenatured PrP$^{\text{Sc}}$.

Importantly, the bright punctate staining provides a marker for infected cells; as shown in Figure 3, other markers, in this case nestin, can be used to identify the cell types that are infected.

**The majority of cells in neurosphere cultures are infected.** In contrast to ScN2a cultures, where only a minority of the cells produce PrP$^{\text{Sc}}$, more than 95% of the cells in infected Tg4053 neurosphere cultures are positive for PrP$^{\text{Sc}}$ as indicated by the denaturation-dependent, bright, punctate intracellular staining (see Giri et al., 2006 and Fig 3). The high proportion of infected cells can also be seen using less labor-intensive cell blots (see Figure 4 in Giri et al., 2006 and Figure 4 below).

**Neurosphere cultures as a prion bioassay.** As shown in Figure 5 of Giri et al. 2006, we were able to detect RML prions in mouse brain homogenate diluted 50,000-fold. We have recently extended these findings and are able to detect RML prions diluted $10^{-8}$ as shown in Figure 4. At passage 1, only rare PK-resistant PrP$^{\text{Sc}}$-positive colonies were detected in cell lines infected with high dilutions of RML prions. On subsequent passages, the level of PK-resistant PrP$^{\text{Sc}}$ produced by cultures exposed to high dilutions of prions increased. Importantly, cultures exposed to RML diluted $10^{-8}$, which showed little PrP$^{\text{Sc}}$ at passages 1 and 2, showed many positive colonies by passage 3. These findings demonstrate that prions in Tg4053 neurosphere cultures replicate and spread from cell to cell.
**In vitro model for genetic control of prion incubation time.** The effect of Prnp on prion incubation time in mice is dramatic. Alleles of the prion protein gene that differ at codons 108 and 189 control incubation time for experimental prion disease. For RML prions, incubation time in mice homozygous for the Prnp<sup>a</sup> allele is much shorter (100 to 170 days) than for Prnp<sup>b</sup> homozygous mouse strains (225 to 350 days). Neurosphere lines from B6 (Prnp<sup>a</sup>) and B6.I-Prnp<sup>b</sup>, which express normal levels of PrP, and from Tg(MoPrP-A)4053 and Tg(MoPrP-B)C2091, which overexpress similar levels of PrP, were challenged with RML prions. Results from one experiment in Tg4053 and TgC2091 are shown in Figure 4. By passage 3, PrP<sub>Sc</sub>-positive colonies are evident in Tg4053 cells incubated with isolate diluted 10<sup>-8</sup>. The PrP-B producing neurospheres were far less sensitive to infection, with de novo PrP<sub>Sc</sub> production achieved only by dilutions of 10<sup>-4</sup> to 10<sup>-5</sup>. To our knowledge, this is the first demonstration of genetic differences in prion susceptibility in mice modeled in tissue culture.

In summary, neurospheres may not only provide a novel system for the bioassay of prion infectivity but they offer new approaches to studying the replication of prions, the spread of prions from one cell to another, and a tool for the study of genes modulating prion susceptibility. We hope to develop cell lines from several strains of mice infected with various strains of prions as a resource.

**KEY RESEARCH ACCOMPLISHMENTS**

- Identified DEGs based on temporal patterns of expression that are shared in five mouse strain-prion strain combinations. Many of these are differentially expressed well before clinical signs are apparent.

- Used DEGs to construct dynamic protein interaction and gene regulatory networks describing pathological events occurring during the course of disease.

- Developed a searchable Prion Disease DataBase web site as a public resource. This will be completed and available within one or two months.

- Used DEGs to identify candidate nervous system specific proteins as signature blood markers. These studies are continuing.

- Demonstrated that CNS stem cell cultures grown as neurospheres can be infected with prions.

- Demonstrated that CNS stem cell cultures can be used to model the genetics of prion susceptibility in vitro for the first time.

- Developed neurosphere cultures as a sensitive bioassay for mouse prions. Work to extend this work to detection of human and bovine prions is in progress.
REPORTABLE OUTCOMES, YEAR 3
Prion Disease DataBase Web Site.


Abstracts of our presentations at the National Prion Research Program meeting held in Chantilly, Virginia in December 2005 also are appended to this report.

CONCLUSIONS

A systems view of disease attempts to understand the initiation and progression of disease in terms of their initial disease-perturbations and their dynamic transitions as disease progresses. Shared DEGs, kinetics of PrPSc accumulation and pathogenesis, biological pathways, and protein-protein interaction databases were used to construct prion disease-specific, dynamic protein networks. Comparison of dynamic network behaviors among the various prion strain-mouse strain combinations, and with perturbations in other neurodegenerative disorders, provides a novel perspective to evaluate involvement of pathways specific to prion replication and prion pathogenesis. Our results also provide new insights into how systems approaches will enable new strategies for drug target identification and ultimately disease prevention. The dynamic network models were integrated with the brain-specific secreted proteins predicted by massively parallel signature sequencing of the transcriptomes of many different mouse organs and tissues and used to predict early changes in blood proteins during the course of disease. Preliminary proteomic analysis of some serum proteins identified several early blood marker candidates for mouse prion disease.

We have infected CNS stem cell containing mouse neurosphere cultures with prions. The ability to readily produce prion-infectable cell lines from any strain or transgenic line of mice offers the unprecedented opportunity to explore the genetics of disease susceptibility in culture. Work to extend these studies to include prion strains in addition to RML and to develop sensitive bioassays for prions from other species is in progress. If successful in establishing infected lines with various prion strains, these could serve a reference cultures that could be shared among laboratories. Application of systems biology to these cultures provides an additional tool to identify differentially expressed genes whose encoded proteins may be necessary for prion replication.
Molecular signatures of prion infection

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Currently, prion infection in humans and livestock is diagnosed by clinical signs, the presence of misfolded, proteinase K-resistant PrP\textsuperscript{Sc} in the brain, and histopathology. Development of improved methods for detection of the disease-specific PrP\textsuperscript{Sc} isoform is one approach towards development of an ante-mortem blood test to identify prion-infected individuals. Using the mouse as a model, we have applied global genomic and proteomic analyses to develop a network-based framework integrating molecular signatures of mRNA, proteins, and protein-protein interactions in brain, spleen, and blood. Identification of differentially expressed genes (DEGs) used the Affymetrix Mouse GeneChip 430A 2.0; differentially expressed proteins (DEPs) in plasma were profiled using liquid chromatography and mass spectroscopy (LC/MS) and LC/MS/MS. To focus on networks specifically perturbed by prion infection, two distinct prions strains and seven mouse strains were analyzed. Analysis to date (07/25/05) has revealed 57 DEGs in brain and spleen common to four agent-host combinations. Of these, 25 are expressed before clinical signs or pathological changes develop, making them good candidates for early diagnostic markers. Four of these genes encode secreted, glycosylated proteins present in plasma. We have recently infected neurosphere cultures with prions with little apparent cytopathic effect; this new model will permit discrimination of genes altered due to prion replication from genes related to the pathological response of the host. Neurosphere lines have been isolated from the same prion strain:mouse strain combinations used in our global analyses to identify prion-specific signatures. A network model to understand relationships among differentially expressed genes and proteins and their biological significance is under development.

National Prion Research Program Meeting, Chantilly, VA.
Neurosphere cultures from transgenic and non-transgenic mice can be infected with prions

Ranjit K. Giri, Rebecca Young, Rose Pitstick, and George A. Carlson

Few cell lines have proven susceptible to infection with prions, precluding \textit{in vitro} analysis of the mechanisms underlying genetic differences in susceptibility to infection. Similarly, cell lines, mouse N2a for example, are resistant to many prion strains that are readily transmissible to mice. With one exception, sensitive bioassay of prions requires inoculation of mice with incubation times ranging from months to over a year. Neurosphere lines grow as non-adherent aggregates and contain CNS stem cell activity; we now report that these cultures can be infected with prions. Using a defined, serum-free medium, cell lines were isolated from brains dissected from embryonic day 12 to 15 day fetuses. In addition to expressing the stem cell-associated marker nestin, most cells from PrP transgenic or from wild-type mice express the normal isoform of PrP (PrP\textsuperscript{C}), which is essential for prion replication. RML scrapie brain homogenate was added to neurosphere cultures from FVB, FVB transgenic mice that overexpress mouse PrP (Tg4053), and FVB mice with a targeted null mutation in the PrP gene (Prnp). Presence of the proteinase K-resistant, misfolded PrP\textsuperscript{Sc} isoform was measured at each passage by Western, dot, or cell blots. A dramatic rise in PrP\textsuperscript{Sc} with time was observed in the Tg4053 cells while the level PrP\textsuperscript{Sc} decayed to undetectable levels in the cultures of cells lacking PrP; levels of PrP\textsuperscript{Sc} in FVB cultures increased gradually over several passages. Prions produced in culture were transmissible to mice and produced the pathology typical for this scrapie strain. Intracellular aggregates of PrP were seen in infected cultures. To date, infection of Tg4053 neurospheres by prion isolate dilute 1 to 50,000 has been achieved. Neurosphere lines from transgenic mice overexpressing PrP may provide a sensitive \textit{in vitro} bioassay not only for mouse prions but for those from other species, including humans.

\textit{National Prion Research Program Meeting, Chantilly, VA}
Prion infection of mouse neurospheres

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Contribution by Stanley B. Prusiner, December 20, 2005

Only a few cell lines have been infected with prions, offering limited genetic diversity and sensitivity to several strains. Here we report that cultured neurospheres expressing cellular prion protein (PrP\(\text{C}\)) can be infected with prions. Neurosphere lines isolated from the brains of mice at embryonic day 13-15 grow as aggregates and contain CNS stem cells. We produced neurosphere cultures from FVB/NCr (FVB) mice, from transgenic (Tg) FVB mice that overexpress mouse PrP-A (Tg4053), and from congenic FVB mice with a targeted null mutation in the PrP gene (Prnp\(0/0\)) and incubated them with the Rocky Mountain Laboratory prion strain. While monitoring the levels of disease-causing PrP (PrP\(\text{Sc}\)) at each passage, we observed a dramatic rise in PrP\(\text{Sc}\) levels with time in the Tg4053 neurosphere cells, whereas the level of PrP\(\text{Sc}\) decayed to undetectable levels in cell cultures lacking PrP. PrP\(\text{Sc}\) levels in cultures from FVB mice initially declined but then increased with passage. Prions produced in culture were transmissible to mice and produced disease pathology. Intracellular aggregates of PrP\(\text{Sc}\) were present in cells from infected cultures. The susceptibility of neurosphere cultures to prions mirrored that of the mice from which they were derived. Neurosphere lines from Tg4053 mice provide a sensitive in vitro bioassay for mouse prions; neurosphere lines from other Tg mice overexpressing PrP might be used to assay prions from other species, including humans.

Results

PrP\(\text{C}\) Is Expressed in Neurosphere Cultures. Susceptibility to prion disease requires the expression of host PrP\(\text{C}\) (8), levels of which were analyzed by immunoblotting. Cell blot analysis of FVB, Prnp\(0/\text{NCr}\), and Tg4053 neurospheres clearly demonstrates the higher level of PrP\(\text{C}\) expression in Tg4053 neurospheres compared with FVB neurospheres (Fig. 1A). PrP\(\text{C}\) immunostaining was observed in Prnp\(0/0\) neurospheres, making them an ideal negative control for persistence of PrP\(\text{Sc}\) in subsequent infection studies. Dot blot analysis indicates that Tg4053 neurospheres express 4- to 8-fold more PrP\(\text{C}\) than FVB neurospheres (Fig. 1B). Western blot analysis confirmed the higher PrP\(\text{C}\) levels in Tg4053 than in FVB neurospheres and showed that most PrP\(\text{C}\) is expressed in FVB and Tg4053 neurospheres is diglycosylated, as is the case in the mice from which they were derived (Fig. 1C).

Neurosphere Cultures Likely Contain CNS Stem Cells. Immunofluorescence showed coexpression of PrP\(\text{C}\) and nestin, a commonly used marker for CNS stem cells (26), in our neurosphere cultures (see Fig. 6, which is published as supporting information on the

G.A.C., S.J.D., and S.B.P. have financial interest in InPro Biotechnology, Inc.

Abbreviations: PrP, prion protein; Prnp\(0/0\), FVB.129-Prnp\(0/0\); Tg, transgenic; FVB, FVB/NCr; Tg4053, FVB-Tg(MoPrP-A)4053; RML, Rocky Mountain Laboratory; PK, proteinase K; dpi, days postinfection; GdnSCN, guanidine thiocyanate; En, embryonic day.n.

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PNAS web site). Most nestin-stained cells are also positive for PrPc in FVB neurosphere cultures at passage 3. Similar results were obtained with Tg4053 neurosphere lines but with more intense staining for PrP. As expected, neurospheres isolated from Prnp0/0 mice do not stain with anti-PrPc antibodies but express nestin, similar to FVB cultures. Most cells in the neurosphere cultures also expressed vimentin, another commonly used marker for CNS stem cells. Some of the cells adherent to the coverslips expressed glial fibrillary acidic protein, a glial cell marker, or microtubule-associated protein-2, a marker for the neuronal pathway. These observations suggest that our neurosphere cultures contain CNS stem cells, as expected from the results of others (22, 23, 27). However, stem-cell activity was not formally tested.

Neurospheres Expressing PrPc Can Replicate Prions. Having established the expression of PrPc in neurosphere cultures, we tested their susceptibility to prion infection. FVB, Tg4053, and Prnp0/0 cultures at passage 3 were incubated with a 50-fold dilution of Rocky Mountain Laboratory (RML) prions for 4 days. At the end of 4 days, the neurospheres were washed and split 1:4. Every 3–4 days, one-half of the medium was replaced with fresh medium. To detect the persistence and de novo production of prion protein (PK)-resistant PrPSc in infected cultures, cells growing on plastic coverslips were blotted and tested for the presence of PK-resistant PrPSc. All three neurosphere lines showed PrPSc at 12 days postinfection (dpi) (Fig. 2A Left), whereas control neurospheres that were not exposed to RML prions had no PrPSc. By 24 dpi, little PrPSc remained in Prnp0/0 neurospheres, whereas PrPSc was readily detected in FVB and Tg4053 cultures (Fig. 2A Center). By 36 dpi and two passages, no PK-resistant PrPSc was seen in Prnp0/0 neurospheres, but substantial amounts of PK-resistant PrPSc were found in Tg4053 neurospheres; fewer PrPSc-positive neurospheres were found in FVB cultures, but PrPSc was clearly present.

Independently isolated cultures from the three mouse lines gave similar results. Western immunoblots show that PrPSc increased from passage 2 to passage 3 after exposure and was maintained at high levels thereafter in two Tg4053 neurosphere isolates (Fig. 2B Left and Center).

Replication of PrPSc in Neurospheres Correlates with PrPc Expression. Infected FVB neurosphere lines also produced PrPSc but to a lesser extent than Tg4053 cultures (Fig. 2B Right). At passages 2–4 after exposure, FVB neurosphere cultures replicated PrPSc more slowly than Tg4053 cultures. FVB cultures were allowed to grow to a density similar to that of Tg4053 neurospheres, and, at passage 5, a dramatic increase in PrPSc level was observed. Infected Tg4053 neurospheres continue to produce high levels of PrPSc for at least 12 passages after exposure (data not shown); this passage history represents an ~103-fold dilution of the original RML-infected mouse brain homogenate (i.e., RML), washed, and passed as described in Results. Controls incubated with isolate diluent (−RML) were treated similarly. (A) PrPSc is normally glycosylated. Lysates of neurospheres containing 40 μg of protein were electrophoresed, Western blotted, and stained with anti-PrP. The blot was stripped and reprobed with anti-GAPDH to normalize for loading or transfer differences. Fab D18 was used to detect PrP in all cases.

Cells in Infected Cultures Contain Intracellular Aggregates of PrPSc. Previous results from several laboratories indicate that PrPSc accumulates intracellularly (15, 18, 28). Immunofluorescent detection of PrP on fixed, permeabilized single-cell preparations (Fig. 3A) and on cells grown on poly-L-lysine-coated coverslips (Fig. 3B) from infected and uninfected Tg4053 neurospheres was performed with or without denaturation by guanidine thiocyanate (GdnSCN). Cells from infected cultures treated with GdnSCN before anti-PrP staining appeared different from uninfected cultures and from nonadenated, infected cells. Fluorescence was more intense and granular in GdnSCN-treated infected cells (Fig. 3A Lower Right and B Lower) than in the other samples. The single-cell preparations were used to quantify differences in fluorescence intensity in individual cells among the groups. In uninfected cells, no significant difference was seen in PrP immunostaining intensity between denatured and nondenatured samples (Fig. 3C). In contrast, the intensity of PrP immunostaining was significantly (ANOVA; F = 37.896; P < 0.0001) greater in denatured, infected cells compared with...
The D18 epitope is poorly accessible in nondenatured PrPSc. Exposure fluorescent staining using anti-PrP Fab D18. Nuclei were stained with DAPI GdnSCN. The cells were applied to albuminized slides for indirect immuno-

Cells were fixed, permeabilized, and denatured (Lower) or not (Upper) with GdnSCN. The cells were applied to albuminized slides for indirect immuno-fluorescent staining using anti-PrP Fab D18. Nuclei were stained with DAPI (blue). The D18 epitope is poorly accessible in nondenatured PrPSc. Exposure of all panels is equivalent. (Scale bars, 10 μm.) (B) To assess better the intracellular localization of PrPSc, infected Tg4053 neurospheres were dissociated and grown on poly-L-lysine-treated slides for 4 days, denatured or not with GdnSCN. PrP was stained with D18 (green); nuclei were stained with DAPI (blue). (Scale bars, 10 μm.) (C) Quantitative analysis of the fluorescence intensity of individual cells, some of which are shown in A, as described in Materials and Methods. Histograms show the number of cells plotted against the intensity in arbitrary units; n indicates the number of cells analyzed. PrP immunofluorescence was significantly greater in infected, GdnSCN-treated Tg4053 neurosphere cells than in infected cells without denaturation or in uninfected cultures, indicating the accumulation of PrPSc aggregates in infected cells (ANOVA; F = 37.896; P < 0.0001).

nondenatured, infected samples and with uninfected cultures (Fig. 3C). This increased staining and punctate distribution from infected, denatured cells indicate accumulation of PrPSc because the epitopes detected by the D13 and D18 Fabs are buried in nondenatured PrPSc (29). The bright punctate staining provides a marker for infected cells. In contrast to ScN2a cells, where only a minority of the cells produce PrPSc, >95% of the cells in infected Tg4053 neurosphere cultures are positive for PrPSc (see Fig. 7, which is published as supporting information on the PNAS web site). The high proportion of infected cells can also be seen in cell blots, where nearly all Ponceau-staining entities transferred to nitrocellulose are positive for PK-resistant PrPSc (Fig. 4).

**PrPSc Replicating in Tg4053 Neurosphere Cultures Is Infectious.** To determine whether PrPSc replicating in Tg4053 neurospheres is infectious, we inoculated cell lysates containing 4 μg of protein into Tg4053 and FVB mice. All inoculated Tg4053 and FVB mice developed disease with mean incubation times of 75.4 ± 3.8 and 171 ± 0 days, respectively (Table 1); 40 μg of the brain homogenate containing RML prions produced disease in 50 ± 2 and 127 ± 2 days in Tg4053 and FVB mice, respectively (4, 5). Mice inoculated with Tg4053 neurosphere lysates developed neuropathology typical of mouse RML prions (see Fig. S4, which is published as supporting information on the PNAS web site). In contrast, all mice injected with lysates (40 μg of protein) of PrPSc Neurosphere cultures exposed to RML prions remained healthy and showed no pathological changes in their brains. Western blot analysis demonstrated the presence of PK-resistant PrPSc with a glycoform profile similar to that of the original RML inoculum (see Fig. S8). As expected, formation of PK-resistant PrP isoforms in Tg4053 neurospheres was accompanied by the production of infectious prions.

**Neurosphere Cultures as a Prion Bioassay.** RML prions in mouse brain homogenate (18 μg of protein per μl) diluted 50-, 500-, 5,000-, or 50,000-fold were incubated in triplicate with two independent isolates of Tg4053 neurospheres. Dot blot analysis was conducted on neurosphere lysates starting at passage 2 (36 dpi) and continuing through passage 5 (76 dpi) (Fig. 5). At passage 2, PK-resistant PrPSc was detected in 20 μg of lysate from both cell lines infected with lower dilutions (1:50 and 1:500) of RML prions. Little, if any, PK-resistant PrPSc was detected in 80 μg of protein from neurospheres incubated with RML prions diluted 50,000-fold at passage 2, although comparable amounts of PrPSc were present in all samples. On subsequent passages, the level of PK-resistant PrPSc produced by cultures exposed to high dilutions of prions increased (Fig. 5, passages 3–5). Importantly, most cultures exposed to RML diluted 50,000-fold, which showed very little PrPSc at passages 2 and 3, had detectable PrPSc in 20 μg of protein at passage 4, which increased with additional...
passages. These findings demonstrate that prions in Tg4053 neurosphere cultures replicate and spread from cell to cell.

Discussion

Neurosphere cultures offer the advantages of both primary cultures and established cell lines. Several cell lines, N2a or GT1 for example, can be infected and maintain prion replication over many passages. Much of our knowledge of the cell biology of prion replication comes from experiments using such cell lines (9, 11, 12, 15), but the number of independently isolated lines is limited and thus impedes analysis of the mechanisms underlying genetic differences in susceptibility to infection. Prion replication in the neurosphere lines described in this report reflects the prion susceptibility of the mice from which they were derived. Using well-established procedures to derive neurosphere stem-cell cultures, we have produced neurosphere lines from 10 mouse strains and Tg lines as well as the three lines reported here. Neurospheres provide the choice of genotypes offered by primary cultures and stable replication of prions over many passages, as indicated by our results. Both FVB and Tg4053 neurospheres were able to be infected soon after they were established (passage 3), when growth was slower, and remained infectible over at least nine additional passages.

Prion replication takes longer to become established in FVB than in Tg4053 neurospheres. In mice, the length of the incubation time is inversely proportional to the level of PrPC expression (5, 6). It is not known whether the decreased incubation time (and increased rate of prion replication) in Tg mice overexpressing PrP is due to increased efficiency of infection resulting in more cells initially infected, to faster replication in each infected cell, or both. After incubation with RML-infected brain homogenates, neurospheres from FVB, Tg4053, and Prnp<sup>0/0</sup> mice showed different time courses for PrP<sup>Sc</sup> immunostaining (Fig. 2). In cell blots, PrP<sup>Sc</sup>-positive neurospheres/cell clumps persist in the Prnp<sup>0/0</sup> line for 24 days but decline to undetectable levels with passage. Similar results also were reported with use of primary cultures of Prnp<sup>0/0</sup> cerebellar neurons in which infectivity persisted at substantial levels for ~28 days postinoculation; however, these cultures could be maintained for little more than a month (17). In contrast, cell blots of Tg4053 neurospheres show much more PrP<sup>Sc</sup> immunostaining at 36 dpi than can be explained by residual inoculum, and high levels of PrP<sup>Sc</sup> continue to be produced with repeated passage. Cell blots of FVB cultures also show production of PrP<sup>Sc</sup> but at much lower levels than Tg4053 neurospheres (Fig. 2). Our results suggest that infection is more rapidly established in Tg4053 neurospheres than in FVB cultures.

Klohn et al. (30) established a cell blot assay using a subclone of N2a cells; at high dilutions of RML prions, few cells were initially infected, but on subsequent passage, the prions spread to additional cells. A similar spread appears to occur in our FVB and Tg4053 neurosphere cultures. Our results suggest that high levels of PrP<sup>C</sup> in neurospheres increase the rate of PrP<sup>Sc</sup> formation, in accord with earlier studies in Tg mice (6). Whether Tg4053 neurospheres can be infected with lower doses of prions than FVB neurospheres remains to be established.

**Table 1. Cell lysates from Tg4053 neurosphere cultures, but not from Prnp<sup>0/0</sup> cultures, infected with RML prions transmit disease to mice**

<table>
<thead>
<tr>
<th>Mice inoculated</th>
<th>Inculum (total protein)</th>
<th>Incubation time (no. sick/no. inoculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg4053</td>
<td>Infected Tg4053 cell lysate (4 µg)</td>
<td>75 ± 3.8 days (5/5)</td>
</tr>
<tr>
<td>Tg4053</td>
<td>Infected Prnp&lt;sup&gt;0/0&lt;/sup&gt; cell lysate (40 µg)</td>
<td>— (0/5)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tg4053</td>
<td>RML brain homogenate (40 µg)</td>
<td>50 ± 2 days (16/16)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVB/NCr</td>
<td>Infected Tg4053 cell lysate (4 µg)</td>
<td>171 ± 2 days (5/5)</td>
</tr>
<tr>
<td>FVB/NCr</td>
<td>Infected Prnp&lt;sup&gt;0/0&lt;/sup&gt; cell lysate (40 µg)</td>
<td>— (0/5)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVB/NCr</td>
<td>RML brain homogenate (40 µg)</td>
<td>127 ± 2 days (18/18)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Mice inoculated with lysate from Prnp<sup>0/0</sup> neurospheres exhibited no signs of illness and were killed for histopathological analysis when the corresponding group inoculated with Tg4053 lysates became ill.

† Previously published data (5).

‡ Previously published data (4).

**Fig. 5.** PrP<sup>Sc</sup> levels increase with passage in Tg4053 cultures, indicating that neurospheres can serve as a prion bioassay. Two independent Tg4053 isolates were incubated in triplicate with four 10-fold dilutions of 10% RML-infected brain homogenate starting at 1:50. At each passage shown, cell lysates were prepared and either left undigested (−) or digested (+) with PK. Undigested lysate containing 20 µg of protein and PK-digested lysates originally containing 20, 40, or 80 µg of protein were blotted and immunostained for PrP after denaturation with GdnSCN. At high dilutions of RML, the PrP<sup>Sc</sup> signal increased with passage.
In contrast to our results in which infected neurosphere cultures remained healthy and stably infected, the primary cultures infected with sheep scrapie prions consistently showed apoptosis (17). These primary neuronal cultures were maintained only for 28 days and could not be subpassaged, making it difficult to distinguish de novo-generated infectivity from the original inoculum. Neurospheres may be the first primary culture system to propagate prions stably similar to the few immortalized cell lines that are capable of replicating mouse prions.

Our results also demonstrate that prions can replicate in cells with CNS stem-cell properties; whether replication occurs in endogenous stem cells of infected animals is not known. Although we have not tested stem-cell activity in our cultures, similar neurosphere cultures have been shown to be capable of differentiation into the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes (22, 25, 27). Most cells in our neurosphere cultures express vimentin, which has been found in human fetal brain cell cultures (31) and is a commonly used marker for CNS stem cells, and nestin, a major cytoskeletal protein in neuronal progenitor cells (26). Most cells in neuroepithelium are nestin-positive before neurogenesis (32). Thus, our neurosphere cultures are likely to possess cells with CNS stem-cell properties. The presence of adherent cells that are highly positive for the glial marker glial fibrillary acidic protein or for the neuronal marker microtubule-associated protein-2 supports this view.

Immunofluorescent staining after GdnSCN denaturation allowed us to determine that almost all cells in infected Tg4053 cultures produce PrPSc. The subcellular distribution of PrPSc has been difficult to determine because of the poor immunoreactivity of native PrPSc and the deleterious effect of GdnSCN on cell morphology (28). Previous work has shown that PrPSc accumulates as aggregates in the cytoplasm of ScN2a cells (15). Similar results were also obtained in prion-infected peripheral neuroglial cells from sheep (18). A GFP–PrP fusion protein, which binds to PrPSc but does not itself convert to PrPSc, shows a similar distribution without the need for denaturants (33). Denaturation by GdnSCN followed by immunostaining showed a significant increase in fluorescence intensity and revealed punctate deposits in infected Tg4053 neurosphere cultures. Over 95% of the cells in infected Tg4053 cultures showed this pattern of staining, which was not seen in uninfected cultures. This finding is in contrast to persistently infected N2a cell cultures, in which a low fraction of the cells contains PrPSc. Even highly prion-susceptible subclones of N2a are unstable, with decreasing numbers of cells infected on repeated passaging (12, 30, 34).

In summary, PrPSc-positive Tg4053 neurospheres were readily detected 56 days after inoculation with RML-infected brain homogenate at a dilution of 1:50,000. This result suggests that neurospheres can be used as a sensitive bioassay for mouse prions. Neurospheres produced from Tg mice may offer a bioassay not only for mouse prions but also for other prions, including those from cattle and humans. How rapid a neurosphere bioassay can be made is unclear. In Fig. 5, no additional sensitivity was obtained over that found at the fourth passage by a fifth passage. Neurospheres may not only provide a novel system for the bioassay of prion infectivity but may also offer new approaches to studying the replication of prions as well as the spread of prions from one cell to another.

Materials and Methods

Mice. FVB, Prnp0/0, and Tg4053 mice were used. Prnp0/0 mice, which lack PrP, and Tg4053 mice, which express ~8-fold higher levels of PrPc than non-Tg mice, have been described previously (5, 8). Mice were bred and housed at the McLaughlin Research Institute.

Prion Isolates. The RML isolate of the Chandler prion strain was a 10% (wt/vol) brain homogenate from clinically ill CD-1 mice as described previously (35, 36). Lyases from neurosphere cultures to test for prion infectivity were produced by washing the cells in sterile PBS and subjecting them to three cycles of freeze–thaw to kill the cells. They were then passaged successively through smaller-gauge needles (18–20–22–25–27 gauge) and stored at −80°C. Protein concentration in the lysates was determined by the bicinechonic acid assay as recommended by the manufacturer (Pierce).

Antibodies. Recombinant anti-PrP Fabs D18 and D13 have been described previously (37). These humanized anti-PrP Fabs were used at 0.5 μg/ml for immunoblots and 5 μg/ml for immunochemistry; binding was detected by using goat anti-human F(ab)2 polyclonal antibody conjugated with either peroxidase or fluorescein (Pierce). Mouse monoclonal antibodies to nestin, vimentin, and the housekeeping gene GAPDH were diluted as recommended by the manufacturer (Chemicon); peroxidase- or Alexa Fluor 546-conjugated goat anti-mouse IgG (H and L chains; Molecular Probes) was used as the secondary antibody.

Neurosphere Isolation. Isolation of neurosphere lines used methods similar to those described previously (24, 27). Embryos from mice were harvested at embryonic day 13 (E13) to E15, where E0 is the day the postcoital vaginal plug forms. The brains were removed and transferred to a 35-mm plate containing serum-free DMEM supplemented with 2 mM glutamine, 100 units of penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were mechanically dissociated by trituration with a 200-μl Gilson pipette, and the resulting cell suspension was filtered through a 45-μm cell strainer (Falcon). The cells were centrifuged at 100 × g, and the pellet was resuspended in neurobasal medium with N2 supplement (GIBCO/Invitrogen), 2 mM L-glutamine, penicillin, streptomycin, 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (both human recombinant from GIBCO/Invitrogen), and 10 ng/ml mouse recombinant leukemia inhibitor factor (Chemicon). Cells harvested from individual fetal brains were suspended in 15 ml of medium in non-tissue culture-treated T75 flasks (Nunc) and cultured for 2 days in a humidified incubator at 37°C with 5% CO2 in air. Nonadherent cell clusters were collected and recultured in uncoated T75 flasks. After 4–7 days of culture, distinct spheres of cells (neurospheres) were observed. Neurosphere cultures were fed every 3–4 days by replacing 7–10 ml of old medium with fresh medium and were passaged by mechanical dissociation and washing when the medium started to change color from red to orange/yellow followed by reculturing at a 1:4 dilution (every 10–40 days). At every passage, some cells or cell clumps initially attach to the substrate but grow as mounds of cells that later detach to become neurospheres (see Fig. 9, which is published as supporting information on the PNAS web site). During culture, extensive attachment was prevented by gently knocking the flasks every other day. The property of dissociated neurospheres to attach was exploited for immunostaining by plating cells at low density on tissue culture-grade coverslips, chamber slides, or poly-L-lysine-coated coverslips.

Immunocytochemistry. To determine whether neurosphere cells express PrPC and other markers, neurospheres were dissociated by trituration and grown for 4–5 days on tissue culture-treated glass chamber slides. Cells were fixed with 4% paraformaldehyde for 30 min followed by three 10-min washes with PBS. The cells then were permeabilized by incubation in 0.3% Triton X-100 in PBS for 5 min at room temperature followed by three 5-min washes with PBS and blocking with 10% normal goat serum. Primary antibodies were added and incubated overnight at 4°C. The cells were washed three times and incubated with the
appropriate secondary antibodies for 1 h at room temperature, washed three times, rinsed with water, and coverslipped with antifade mounting medium (Molecular Probes). Fluorescence was detected and digital images were taken with a Nikon TE2000 photomicroscope. The epitopes detected by D18 and D13 Fab s are buried in PrPSc, so denaturation is required for PrPSc detection (38, 39). For in situ detection of PrPSc in neurosphere cultures, the neurospheres were tritutrated, filtered, fixed, permeabilized, and denatured with 3 M GdnSCN. Cell suspensions were adhered to an albumin-coated microscope slide; PrP was detected as described earlier. Nuclei were counterstained with DAPI. For quantification of PrP immunofluorescence, stacks of images along the z axis were acquired with a Quantix-57, 12-bit cooled CCD camera (Photometrics, Tucson, AZ) and METAMORPH software ( Molecular Devices). For quantitative comparisons, samples were analyzed during the same session by using identical acquisition settings. Image stacks were deconvoluted with AUTODEBLUR (AutoQuant Imaging, Troy, NY), and regions identical acquisition settings. Image stacks were deconvoluted and regions were transferred to the original image stack, and PrP fluorescence intensity was measured in the best focus plane by using METAMORPH. For qualitative illustrations, the in-focus planes of the image stack were background subtracted and corrected for shading before generating a maximum projection.

**Immunoblots. Cell blots.** The cell blot technique has been described (12). Briefly, cells adherent to plastic coverslips were transferred to a nitrocellulose membrane saturated with lysis buffer (150 mM NaCl/10 mM Tris, pH 7.5/0.5% sodium deoxycholate/0.5% Triton X-100). The membrane was dried, either left undigested or incubated with PK, denatured with 3 M GdnSCN, and immunostained.

**Western blots.** Neurosphere lysates were clarified by centrifugation at 30000 x g, and the protein concentration was determined by the bicinchoninic acid assay. For PrPSc detection, 500 µg of total protein was treated with 5 µg of PK at 37°C for 1 h, followed by addition of Pefabloc (Fluka) to a final concentration of 4 mM. Insoluble proteins were collected by centrifugation at -18,000 x g for 30 min at room temperature, resuspended in sample buffer, and subjected to SDS-PAGE and transferred to a nitrocellulose membrane. PrP was detected by using D13 or D18 Fab and chemiluminescence ( SuperSignal West Pico Kit; Pierce). Samples for PrPSc detection were not treated with PK. Blots were stripped and reprobed with anti-GAPDH to normalize protein loading and transfer.

**Dot blots.** Cell lysates either left untreated or treated with PK were loaded into a 96-well, dot blot apparatus and transferred onto a nitrocellulose membrane by applying vacuum. The membrane was air dried, probed with anti-PrP, and developed as described for Western blots.

**Prion-Incubation Time.** Mice were inoculated intracerebrally by using a 26-gauge needle with 20 µl of brain homogenate or culture lysate under isoflurane anesthesia. Inoculated mice were examined for neurological dysfunction once every week for the first month after inoculation and three times per week thereafter as described (36). Brains were harvested from terminally ill mice and their controls; one-half was immediately frozen for biochemical analyses, and the other half was fixed in 10% buffered formalin solution for histopathology.

We thank Dr. William Provance of the McLaughlin Research Institute Imaging Core Facility for valuable assistance with fluorescence microscopy and quantification. This work was funded by grants from the National Prion Research Program, U.S. Department of Defense ( Grants DAMD17-03-1-0321 and DAMD17-03-1-0425) and from the National Institutes of Health ( Grants NS41997, AG02132, AG10770, and AG021601) U.S. Public Health Service.

Supplementary information

Prion infection of mouse neurospheres

Ranjit K. Giri, Rebecca Young, Rose Pitstick, Stephen J. DeArmond, Stanley B. Prusiner, and George A. Carlson
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 6. Neurosphere cultures showing aggregates of cells growing attached to the substrate (A) or freely floating (B). The bars in each micrograph represent 50 µm.

Supplementary Fig. 7. Expression of PrP\textsuperscript{C}, nestin, vimentin, microtuble associated protein-2 (MAP-2), and glial fibrillary acidic protein (GFAP) in FVB neurosphere cultures. Dissociated neurospheres were cultured on tissue culture–treated slides for 4–5 days, fixed, permeabilized, and immunostained for fluorescence microscopy. Humanized anti-PrP Fab D18 was revealed with fluorescein labeled anti-human F(ab)\textsubscript{2}. Monoclonal antibodies against nestin, vimentin, and GFAP were revealed with alexafluor-546–labeled goat anti-mouse IgG (H & L); monoclonal anti-MAP-2 was detected with fluorescein-labeled goat anti-mouse. (A) Comparison of nestin and PrP expression in adherent neurosphere cultures from FVB (top row) and Prnp\textsuperscript{0/0} (bottom row) mice. Note the absence of PrP staining in Prnp\textsuperscript{0/0} cultures, demonstrating the specificity of the anti-PrP Fab. (B) Co-expression of vimentin and PrP in FVB neurosphere cultures. (C) Expression of markers for the glial (GFAP) and neuronal lineages in adherent neurosphere-derived cells.

Supplementary Fig. 8. Most cells in infected neurosphere cultures produce PrP\textsuperscript{Sc}. The ability of GdnSCN to reveal epitopes hidden in PrP\textsuperscript{Sc} was used as a marker for infected cells. Uninfected and infected Tg4053 neurospheres at passage 8 postinfection were harvested and triturated to obtain single cells/small cell clumps and
recultured on poly-L-Lysine–coated glass coverslips. After 3 days, the adherent cells on were fixed, permeabilized, denatured or not with GdnSCN, and stained for PrP using D13 Fab (green). Nuclei were stained with DAPI (blue). Exposure of all panels is equivalent. Stacks of images along the z-axis were taken using a Nikon TE2000-E inverted fluorescence microscope run using Metamorph software (Molecular Device Corp.). Three central and best-focused images from each stack were harvested, maximum projection was performed, and scale of the images was adjusted equally. Three representative panels for each condition are shown. More than 95% of individual cells in infected neurosphere show the bright, denaturation-dependent, granular staining indicative of PrP\textsuperscript{Sc} that is not seen in noninfected cultures.

**Supplementary Fig. 9.** Tg4053 neurosphere cultures produce infectious prions. (A) Inoculation of infected neurosphere lysates produces typical prion disease pathology in mice. FVB mice were injected intracerebrally either with lysate containing 4 µg of protein from Tg4053 neurospheres 6 passages (109 days) after incubation with RML scrapie isolate (right column) or with lysate containing 40 µg of total protein from Prnp\textsuperscript{0/0} neurospheres exposed to RML isolate 109 days earlier (left column). Hematoxylin and eosin staining of the hippocampal CA1 region shows a pattern of vacuolation typical of prion disease after inoculation with infected Tg4053 neurosphere lysates (2) and no remarkable neuropathologic changes from Prnp\textsuperscript{0/0} neurosphere lysates (1). GFAP immunohistochemistry shows intense reactive astrocytic gliosis in the hippocampus with Tg4053 neurospheres (4) and only mild age-related astrogliosis in mice inoculated with Prnp\textsuperscript{0/0} neurospheres (3). PrP immunohistochemistry shows the characteristic finely and coarsely granular PrP\textsuperscript{Sc} deposits in the gray and white matter after inoculation with
infected Tg4053 neurospheres (6), which are not seen with inoculation of Prnp\(^{0/0}\) neurospheres (5). cc, corpus callosum; e, ependymal lining of the lateral ventricle; Py, pyramidal cell layer of the hippocampal CA1 region. The bar in panel 4 represents 100 µm and also applies to panels 1–3. The bar in panel 6 represents 60 µm and also applies to panel 5. Tg4053 mice also were inoculated with neurosphere lysates from Tg4053 or Prnp\(^{0/0}\) mice. (B) Mice inoculated with infected neurosphere lysates produce PrP\(^{Sc}\). Brain homogenates containing 250 µg of protein were subjected to PK digestion, electrophoresed, and Western blotted along with an undigested sample containing 40 µg of protein. Western blots of brain homogenates from individual Tg4053 mice (top panel) and FVB mice (bottom panel) inoculated with lysate from Prnp\(^{0/0}\) neurospheres (Prnp\(^{0/0}\)) or lysate from Tg4053 neurospheres that had been incubated with prions. PK-digested (+) or undigested (-) samples are indicated. (MW markers? Nothing unusual here so probably not necessary.)
Uninfected Tg4053 NS
Without GdnSCN

With GdnSCN

Infected Tg4053 NS
Without GdnSCN

With GdnSCN