Immunopathology and Infectious Diseases

Pathogenesis of Chronic Wasting Disease in Cervidized Transgenic Mice

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Chronic wasting disease (CWD) is a fatal, endemic prion disease of wild and captive cervids, including deer, elk, and moose. Typical of prion diseases, CWD is characterized by the conversion of the native, protease-sensitive protein PrP^C to a protease-resistant isoform, denoted as PrP^{RES}. Here we have studied the expression of cervid PrP^C and the pathogenesis of CWD infection in transgenic mice expressing the normal cervid prion protein (Tg[CerPrP] mice). Using tissue-based *in situ* immunohistochemistry protocols, we first identified cervid PrP^C expression in the lymphoid, nervous, hemopoietic, endocrine, and certain epithelial tissues of Tg[CerPrP] mice. Tg[CerPrP] mice were then inoculated with CWD via one of four routes (intracerebral, intravenous, intraperitoneal, or oral); all groups developed spongiform encephalopathy, although the oral route required a larger infecting dose. Incubation periods were 184 ± 13, 218 ± 15, 200 ± 7, and 350 ± 27 days after inoculation, respectively. In longitudinal studies, we tracked the appearance of PrP^{RES} in the brain, spleen, Peyer's patches, lymph nodes, pancreatic islets of Langerhans, bone marrow, and salivary glands of preclinical and terminal mice. In addition, we documented horizontal transmission of CWD from inoculated mice and to un-inoculated cohabitant cage-mates. This work documents the multiroute susceptibility, pathogenesis, and lateral transmission of CWD infection in Tg[CerPrP] mice, affirming this model as a robust system to study this cervid transmissible spongiform encephalopathy. (Am J Pathol 2010; 176:2785–2797; DOI: 10.2353/ajpath.2010.090710)

gressive neurodegenerative diseases that affect humans (Kuru and Creutzfeldt-Jakob disease), sheep and goats (scrapie), cattle (bovine spongiform encephalopathy), felids (feline spongiform encephalopathy), and cervids (chronic wasting disease [CWD]). Prion diseases are so named on the basis of their association with aggregates of conformationally altered and post-translationally modified isoforms (denoted PrP^{RES}) of the normal cellular prion protein (denoted PrP^C). CWD was first recognized in captive mule deer in Colorado in 1967 and subsequently identified as a TSE affecting captive and wild cervids including mule deer, white-tailed deer, elk, and moose. CWD is unique among TSEs in infecting free-ranging wildlife species and in its highly transmissible nature.¹ Although the mechanism(s) of this facile spread remain uncertain, experimental and epidemiological evidence suggests lateral transmission via saliva, urine, and feces.^{2–4}

Although mice of *Mus* species are substantially resistant to CWD infection, FVB mice engineered to express the normal cervid prion protein (PrP^C) transgenically (ie, cervidized mice, Tg[CerPrP] mice) have been shown to be susceptible to CWD infection by intracerebral inoculation.^{5–10} In the present study we used a series of immunohistochemical (IHC) techniques to i) map the distribution of PrP^C in naive Tg[CerPrP] mice, ii) determine the CWD susceptibility of these mice after exposure via a presumed natural route (p.o.) and three parenteral routes (i.v., i.p., and intracerebral [i.c.]), iii) assess the longitudinal accumulation of PrP^{RES} in tissues of inoculated mice, and iv) evaluate the possibility of CWD horizontal transmission to naive, cohabitating mice, thereby simulating lateral transmission in the native cervid host.

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The transmissible spongiform encephalopathies (TSEs), or prion diseases, are uniformly fatal, chronic, and pro-

Materials and Methods

Ethics Statement

The animal experiments were conducted under the guidelines developed by the Colorado State University Animal Care and Use Committee.

Generation and Genotyping of Tg[CerPrP] Mice

The transgenic mice used in this study (1536 Tg[CerPrP] mice) were created and their susceptibility to CWD was established in the Telling laboratory.⁶ Line 1536 was generated as follows: the open reading frame cassette of the CerPrP S2 allele (GenBank accession no. AF009180) was released from plasmid sequences after digestion with Sall and XhoI, and purified open reading frame fragments were ligated to the Sall-cut cosSHa.Tet cosmid expression vector. The cosSHa.Tet cosmid expression vector contains a 49-kb DNA fragment encompassing the Syrian hamster PrP gene and has been used to produce numerous Tg models of prion diseases, including mice in which the species barriers to Syrian hamster, human, and bovine prions were eliminated.^{5,6,11–16} To increase CerPrP expression in transgenic mice, the CerPrP S2 allele plasmid nucleotide sequence was modified by site-directed mutagenesis immediately upstream of the initiating ATG to produce a consensus Kozak translation initiation sequence. Two founders were generated by microinjection of fertilized embryos from *Pmp^{0/0}* knockout mice on an FVB/N background (FVB/*Prnp^{0/0}*), from which a colony of 1536 Tg[CerPrP] mice was developed. Tail-tip DNAs were screened for the presence and appropriate orientation of the CerPrP transgene by conventional and real-time PCR.⁶ The *PrP^{0/0}* mice used in the *PrP^C* mapping studies were kindly donated by Dr. Mark Zabel at Colorado State University (Fort Collins, CO).

Inoculum Preparation

The CWD inoculum (D-10) was prepared as a brain homogenate derived from a terminally ill, naturally infected mule deer (Dr. Michael Miller, Colorado Division of Wildlife, Wildlife Research Center, Fort Collins, CO). This inoculum has proven to be infectious for both deer and the intracerebrally inoculated 1536 Tg[CerPrP] mice in previous studies.^{6,17} The negative control inoculum consisted of a brain homogenate from a CWD-negative white-tailed deer originating outside of the CWD endemic area (courtesy of David Osborn, Warnell School of Forestry, University of Georgia, Athens, GA). Both inocula were homogenized in sterile PBS using a reciprocal homogenizer and diluted to a final concentration of 1% (w/v) using sterile PBS-containing penicillin-streptomycin (100 U/ml).

Inoculation Protocol

Five experimental groups of 1536 Tg[CerPrP] mice ($n = 10$ per group) were used in these studies. Four of the groups consisted of 4- to 6-week-old, mixed-sex, Tg[CerPrP] mice inoculated with CWD prions via one of four

routes: i.c., i.p., i.v., or p.o. The fifth group consisted of age- and sex-matched, naive Tg[CerPrP] mice included to assess the possibility of horizontal transmission (described below). In each cage, two inoculated mice were housed with one sentinel, noninoculated mouse. For i.c. inoculation, mice were sedated through the i.p. injection of a mixture of ketamine (120 mg/kg) and xylazine (16 mg/kg). Mice were inoculated with 30 μ l of a 1% brain homogenate via a 29-gauge needle in the left parietal lobe of the cerebral cortex. For i.p. inoculation, 100 μ l of a 1% brain homogenate was injected via a 29-gauge needle into the right caudal ventral abdomen. For i.v. inoculation, 30 μ l of a 1% brain homogenate was injected through either the left or right tail vein. Oral inoculations were performed by oral instillation of 50 μ l of a 1% brain homogenate administered on each of 2 consecutive days. Mice were not sedated for the i.p., i.v., or p.o. inoculations. A second oral inoculation study used whole brain and is described separately below. Negative control mice ($n = 6$ per route) were inoculated by all routes above with 1% brain homogenate obtained from a CWD-negative white-tailed deer.

Animal Evaluation, Euthanasia, and Necropsy

After inoculation, Tg[CerPrP] mice were sacrificed at either i) a predetermined time point or ii) at the onset of the terminal neurological disease. The criteria used for the diagnosis of central nervous dysfunction in prion-inoculated mice have been published previously¹⁸ and consist of severe ataxia, difficulty in righting from a supine position, tail rigidity, generalized tremors, and/or severe mental obtundation. Tg[CerPrP] mice sacrifice time points were chosen using the previously reported 220-day survival period after i.c. inoculation⁶ and were as follows: 60 days postinoculation (dpi), 120 dpi, 180 dpi, 240 dpi, and terminal disease. In contrast, the sentinel, cohabitant mice were euthanized at either the onset of terminal neurological disease or other clinical disease necessitating humane euthanasia.

At each predetermined sacrifice point, three mice per inoculation group were euthanized (two mice from the CWD-inoculated group and one mouse from the sham-inoculated group) for a total of 12 mice at each time point. One of the two mice from each CWD-inoculated group was perfusion-fixed with paraformaldehyde-lysine-periodate (PLP) fixative and necropsied. The second mouse from this group and the single sham-inoculated mouse were euthanized via i.p. injection of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) and necropsied. Tissues from this group were split between frozen storage (at -80°C) and PLP immersion fixation. At necropsy, samples from all tissues were obtained, including both nervous (brain and spinal cord) and non-nervous (peripheral) tissues. After necropsy, all fixed tissues were immersed in PLP fixative for 24 hours before being moved into 70% ethanol for long-term storage. After less than 5 days in ethanol, tissues were routinely trimmed for histological processing. Whole brains were sectioned coronally to generate a series of 2- to 3-mm thick tissue

slices, representing the following regions of the brain: i) neocortex (at the level of the caudate nucleus), ii) hippocampus and hypothalamus, iii) midbrain (at the level of the colliculi); iv) hindbrain (including the pons and the cerebellum), and v) caudal brainstem (at the level of the obex).

Tissue Collection and Processing

For detection of PrP^C, Tg[CerPrP] and PrP^{0/0} mice were perfusion or immersion fixed with one of two fixatives: 10% neutral buffered formalin or PLP. In all cases, perfusion fixation was performed with a commercially available, gravity-feed system (AutoMate Scientific) and modification of a published protocol, which uses left ventricular fixative injection and right atrial exsanguination.¹⁹ After perfusion, tissues were postfixed in their respective fixative for 12 to 24 hours and transferred to 70% ethanol for long-term storage. For detection of PrP^{RES}, tissues were perfusion-fixed with PLP as described above. To ablate PrP^C immunoreactivity, cassetted tissues were immersed in 88% formic acid for 1 hour and then rinsed in running tap water for 1 hour before histological processing.

Histology and Immunohistochemistry

For all experiments, paraffin-embedded tissue sections (6 μ m) were mounted onto positively charged glass slides and deparaffinized in an oven followed by successive xylene immersions in and rehydrated through graded ethanol. To enhance detection, tissues were subjected to heat-induced epitope retrieval using an automated antigen-retrieval system (Retriever) and a proprietary buffer solution (Target Retrieval Solution, DakoCytomation, Carpinteria, CA).

For the detection of PrP^C, tissues were stained using an automated immunostainer (DakoCytomation), the primary prion protein antibody R505.5 (rabbit polyclonal, a generous gift from Dr. Jan Langeveld, Central Veterinary Institute of Wageningen University, Wageningen, The Netherlands), and a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody. (The R505.5 antibody was elicited in rabbits by immunization with the ovine prion protein peptide 100 to 111 [SQWKNKPSKPKTN]. The reactivity and specificity of this antibody for prion protein have been demonstrated by Pepscan, Western blot, enzyme-linked immunosorbent assay, and radio-immunoprecipitation.²⁰) Antibody deposition was visualized using the chromogen diaminobenzidine (DakoCytomation); slides were counterstained with hematoxylin and incubated with a bluing reagent (0.1% sodium bicarbonate). After immunostaining, slides were dehydrated through graded ethanol, cleared with xylene, and coverslipped.

For detection of PrP^{RES}, an IHC protocol that combined either a one- or two-step immunostaining procedure with tyramide signal amplification (TSA) was used. Previous work in our laboratory has shown that this methodology is more sensitive in the detection of PrP^{RES} than traditional, two-step indirect IHC protocols.²¹ The TSA-PrP^{RES} proto-

col used a proprietary TSA detection kit (PerkinElmer Life and Analytical Sciences, Waltham, MA) and was conducted as follows. After slide rehydration and heat-induced epitope retrieval (as described above), tissue section endogenous peroxidase activity was blocked using 3% H₂O₂ for 60 minutes. Sections were further blocked using a proprietary protein block (TNB, PerkinElmer Life and Analytical Sciences) for 60 minutes and 10% goat serum for 30 minutes. (Slides were incubated with one of two anti-prion antibodies, either the R505.5 antibody or the HRP-conjugated antibody BAR-224. The BAR-224 antibody [Cayman Chemicals, Ann Arbor, MI] is a monoclonal prion protein antibody raised against amino acids 141 to 151 of the ovine prion protein.)

In protocols incorporating the R505.5 antibody, slides were incubated with a HRP-conjugated anti-rabbit Ig secondary antibody. Between all incubation steps, slides were washed three times (5 minutes each) in a TNT wash buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20). After application of the antibodies, slides were sequentially incubated with two proprietary TSA reagents: i) dinitrophenol (DNP) amplification reagent (PerkinElmer Life and Analytical Sciences) for 4 minutes and ii) anti-DNP-HRP (PerkinElmer Life and Analytical Sciences) for 30 minutes. Antibody deposition was visualized using diaminobenzidine, and slides were counterstained with hematoxylin and incubated with a bluing reagent (0.1% sodium bicarbonate). After immunostaining, slides were dehydrated through graded ethanol, cleared with xylene, and coverslipped.

Peptide-Blocking Studies

To confirm the specificity of the anti-PrP^{RES} detection protocol, we conducted a series of complementary peptide-blocking IHC studies in which the anti-prion protein antibody BAR-224 was preincubated with the peptide FGSDYEDRYR, which corresponds with its eliciting antigen, residues 141 to 151 of the ovine prion protein. In such peptide-blocking reactions, the monoclonal antibody was incubated with its eliciting peptide for 60 minutes at room temperature, and this monoclonal antibody-peptide solution was applied to the tissue sections after the application of the TNB protein block and the 10% goat serum block. The remainder of the immunostaining protocol was identical to that described above; however, antibody deposition was visualized using the chromogen 3-amino-9-ethyl-carbazole (DakoCytomation) rather than diaminobenzidine.

Whole-Brain Oral Inoculation Studies

As a follow-up to the p.o. inoculation studies described above, a study was initiated to determine whether larger p.o. doses of CWD prions would affect the susceptibility of Tg[CerPrP] mice to infection. Two groups of naive, 4- to 6-week-old mice ($n = 5$ per group) were orally inoculated with whole brain obtained from either a known CWD-positive or -negative white-tailed deer. Mice were orally inoculated with 0.2 to 0.25 g of whole-brain material

introduced directly into the oral cavity. Mice were not sedated for this procedure as they consumed the inoculum. After inoculation, mice were monitored for the development of clinical neurological disease as described above and euthanized at the onset of either terminal neurological disease or any other severe clinical disease necessitating humane euthanasia. On euthanasia, mice were perfusion-fixed with PLP fixative, and tissues were prepared for immunohistochemical evaluation.

Results

Immunohistochemical Detection of PrP^C in Naive Tg[CerPrP] Mice

To determine the tissue-specific distribution profile of the PrP^C in Tg[CerPrP] mice, we developed IHC protocols to detect PrP^C in paraffin-embedded tissues by evaluating seven primary antibodies, two fixation techniques (perfusion and immersion), and two fixatives (PLP and 10% neutral buffered formalin). The most sensitive PrP^C detection method combined perfusion fixation with PLP and the primary polyclonal antibody R505.5. Cervid PrP^C expression was identified in a surprisingly wide variety of tissues, including those of the nervous, lymphoid, gastrointestinal, hemopoietic, and endocrine systems (Figure 1, Table 1). In general, the immunoreactivity was most prominent in the cytoplasm, usually as finely granular; in the central nervous system (CNS), more coarsely aggregated immunostaining was seen in the neuropil.

CNS PrP^C Expression

PrP^C immunoreactivity was identified in cells morphologically consistent with neurons and glia and within the neuropil (Figure 1A). In contrast, PrP^C expression was not identified within components of the peripheral nervous system, including sections of peripheral nerves identified in skeletal muscle or the plexuses of the enteric nervous system.

Lymphoid PrP^C Expression

PrP^C immunoreactivity was identified within the cortical germinal centers and paracortical and medullary regions, representing macrophages and/or dendritic cells of lymph nodes, spleen, thymus, and Peyer's patches (Figure 1, B and K). In the spleen, immunoreactivity was localized to cells morphologically and microanatomically consistent with lymphocytes and macrophages and/or dendritic cells in the lymphoid nodules and the periaarterial lymphatic sheaths (Figure 1B). In the thymus, immunoreactivity was confined to cortical and medullary lymphoid cells, whereas the thymic epithelial cell population was negative.

Gastrointestinal PrP^C Expression

PrP^C expression was identified within a small number of cells morphologically consistent with small lymphocytes and dendritic cells throughout the alimentary canal (oral cavity, esophagus, stomach, intestine, and colon).

All remaining cell types were negative. Among the extratubular tissues of the gastrointestinal tract, including the salivary glands, tongue, liver, and exocrine pancreas, PrP^C immunoreactivity was identified in the glandular epithelial cells of the salivary glands (Figure 1C), in the cells of the lingual taste buds (Figure 1J), in the resident macrophage population of the liver (Kupffer cells) (Figure 1L), and in the epithelial cells of the pancreatic endocrine islets (Figure 1I).

Urogenital PrP^C Expression

PrP^C immunoreactivity was identified in the renal cortical tubular system, renal glomerulus (Figure 1D), testes, accessory sex glands, and ovary.

Respiratory PrP^C Expression

PrP^C was detected in only a small number of cells within the interalveolar interstitium, probably either interstitial or intravascular macrophages.

Endocrine PrP^C Expression

PrP^C immunoreactivity was detected in the epithelial cells of the pancreatic islets, and the adrenal, thyroid, and parathyroid glands (Figure 1I).

Hematopoietic PrP^C Expression

PrP^C immunoreactivity was detected in megakaryocytes and cells of the myeloid lineage. (To confirm the specificity of the PrP^C detection protocol, matching tissues from PrP^{0/0} mice were identically immunostained and in such animals, no PrP^C immunoreactivity was observed [Figure 1, I–L and U–X].) In addition, PrP^C immunoreactivity was not identified in sections immunostained with a protocol incorporating a negative control antiserum (data not shown) or two immersions in 88% formic acid (Figure 1, E–H, M–P, and Q–T).

Transmission of CWD to Tg[CerPrP] Mice

The inoculation of the Tg[CerPrP] mice with mule deer origin CWD prions resulted in clinical disease in the i.c., i.p., and i.v. inoculated animals with the following survival periods: i.c., 184 ± 13 dpi; i.p., 218 ± 15 dpi; and i.v., 200 ± 7 dpi, respectively (Table 2). The clinical disease was characterized by ataxia, hyperactivity, tail rigidity, and, terminal mental obtundation. In contrast, Tg[CerPrP] mice inoculated orally with the same 1% CWD brain homogenate remained healthy for >700 dpi, as did all sham-inoculated controls (Table 2).

Neuropathology in Inoculated Tg[CerPrP] Mice

Independent of inoculation route, CWD-infected mice developed similar histopathological lesions. These changes, in the hippocampus, hypothalamus, cerebellum, and brainstem, consisted of raggedy rarefaction of the neuropil and

Figure 1. Cervid PrP^C expression in tissues of naive Tg[CerPrP] mice. All tissues were from PLP-perfused, noninoculated mice stained using the IHC protocol detailed in the *Materials and Methods*. Each of the two sets of three rows represents identical tissues that were stained with the R505.5 antibody. The **middle row** of each set represents tissues that have been twice treated with 88% formic acid, whereas the **bottom row** represents tissues from PrP^{0/0} mice. **Row 1:** PrP^C immunoreactivity (brown) in (A) neurons and neuropil, (B) splenic nucleated cells, (C) salivary gland epithelial cells, and (D) renal tubular epithelial cells. **Row 3:** Mild to marked PrP^C immunoreactivity (brown) in (I) pancreatic islet cells, (J) lingual taste buds, (K) lymph node nucleated cells, and (L) hepatic Kupffer cells. Note the lack of PrP^C immunoreactivity observed in the tissues treated with 88% formic acid (**row 2, E–H, and row 5, Q–T**) or in the tissues from the PrP^{0/0} mice (**rows 3 and 6**). Scale bars: 100 μ m (A, E, and I); 40 μ m (B, F, and J); 40 μ m (C, G, and K); 30 μ m (D, H, and L); 120 μ m (M, Q, and U); 45 μ m (N, R, and V); 25 μ m (O, S, and W); 20 μ m (P, T, and X).

Table 1. PrP^C Expression in the Tissues of 1536 Tg[CerPrP] Cervidized Transgenic Mice as Determined by Immunohistochemical Analysis

Central nervous system		Urogenital system	
Neuronal cell bodies	Positive	Renal tubules	Positive
Oligodendrocytes	Negative	Glomerulus	Positive
Astrocytes	Negative	Urinary epithelium	Negative
Neuropil	Positive	Prostate	Positive
Ependymal cells	Positive	Vesicular gland	Positive
Peripheral nervous system		Coagulating gland	Negative
Ganglion neuronal cell bodies	Negative	Testes	Positive
Axons	Negative	Uterine endometrium	Negative
Cardiovascular system		Vaginal epithelium	Negative
Cardiac myocytes	Negative	Ovary	Positive
Endothelial cells	Positive	Respiratory system	
Lymphoid system		Respiratory epithelium	Negative
Lymph node cortical follicles	Positive	Type I pneumocytes	Negative
Lymph node paracortex	Positive	Type II pneumocytes	Negative
Lymph node medulla	Positive	Alveolar macrophages	Positive
Splenic lymphatic sheaths	Positive	Endocrine system	
Thymus	Positive	Pancreatic islet cells	Positive
Gastrointestinal system		Adrenal medullary cells	Positive
Salivary glands	Negative	Adrenal cortical cells	Positive
Tongue epithelium	Negative	Thyroid epithelium	ND
Taste buds	Positive	Parathyroid epithelium	ND
Esophagus	Negative	Other tissues	
Gastric epithelium	Negative	Myocytes	Negative
Submucosal glands	Negative	Bone marrow	Positive
Peyer's patches	Positive	Adipose tissue	Negative
Enterocytes	Negative	Keratinocytes	Negative
Pancreatic exocrine cells	Negative	Hair follicles/adnexae	Negative
Liver (hepatocytes)	Negative	Bone marrow	Positive
Liver (nonhepatocytes)	Positive	Osteocytes, osteoclasts, and chondrocytes	Negative

All tissues were obtained from PLP perfusion-fixed, adult 1536 Tg[CerPrP] mice and stained using the R505.5 primary antibody and the IHC protocol detailed in the text. In the tissues denoted as positive, PrP^C immunoreactivity varied in intensity, but the reactivity was uniformly cytoplasmic and granular in nature.

ND, not done.

perikaryon (spongiform degeneration) with the adjacent deposition of pale eosinophilic plaques. The most extensive lesions were identified in the cellular layers of the hippocampus and the cerebellum. In the cerebellum, there was extensive neuronal loss within the Purkinje cell and granular layers with rarefaction of the neuropil and plaque accumulation (Figure 2A). Likewise in the hippocampus, there was loss of neurons within cellular layers as well as neuropil rarefaction and eosinophilic plaque accumulation (Figure 2C). No lesions were observed in sham-inoculated Tg[CerPrP] mice (Figures 2, B and D).

PrP^{RES} Distribution in Tg[CerPrP] Mice

In mice successfully infected by each of the three routes, PrP^{RES} depositions differed chiefly in the sequence of

first detection in target organs and cell populations and, in some instances as noted, in the spectrum of PrP^{RES}-positive cell phenotype (Figure 3). The earliest detection of PrP^{RES} in the CNS was at 60 dpi, in the gray and white matter of the frontal cortex, the hippocampus, and the hypothalamus, most commonly in regions of neuropil spongiosis (Figure 4A). The earliest detection of PrP^{RES} in a peripheral tissue was at 60 dpi in the spleen, liver, and mesenteric lymph nodes. In the spleen, the PrP^{RES} appeared as granular deposits suggestive of cytoplasmic processes of follicular dendritic cells in lymphoid follicles (Figure 4B). In the liver, the PrP^{RES} was detected in angular-shaped, sinusoidal-lining cells suggestive of Kupffer cells (Figure 4C). In all lymphoid tissues, PrP^{RES} was detected in a small number of cells within the lym-

Table 2. Transmission of CWD Prions to Transgenic Mice After Multiroute Inoculation

	Route of inoculation					
	i.c.	i.p.	i.v.	p.o. 1%	Contact	p.o. WB
Days to disease	184 ± 13	218 ± 15	200 ± 7	NA	See text	350 ± 27
No. mice with TSE/total no. mice	3/3	3/3	3/3	0/10	3/20*	3/5

Time to clinical disease and number of mice evaluated at the time of terminal disease are represented for the i.c., i.p., i.v., and p.o. inoculated 1536 Tg[CerPrP] mice. The results of two p.o. inoculation studies are summarized: one performed with a 1% inoculum (p.o. 1%) and the second with a whole-brain (p.o. WB) inoculum (see text for details). The contact transmission mice represent naïve transgenic mice, which were cohabitant with inoculated counterparts (see text for details). For all animals, CWD infectivity was confirmed by IHC. None of the sham-inoculated mice (*n* = 6 per route) developed clinical neurological disease nor demonstrated PrP^{RES} accumulations in the brain. *In the contact transmission group, only 15 of these 20 mice were observed beyond 600 dpi of cage-mates.

NA, not applicable.

Figure 2. Neuropathology in CWD-inoculated Tg[CerPrP] mice. All tissues are from CWD-inoculated, PLP-perfused mice stained with H&E. Panels at left (**A** and **C**) are tissues from a terminal i.v. inoculated Tg[CerPrP] mouse sacrificed at 201 dpi. Panels at right (**B** and **D**) are tissues obtained from a sham i.v. inoculated Tg[CerPrP] mouse sacrificed at 201 dpi. **A:** Loss of neuronal cell bodies within the granular and Purkinje cell layers of the cerebellum, raggedy rarefaction of the neuropil, and the accumulation of eosinophilic plaques (outlined by arrows). **C:** Similar loss of neuronal cell bodies within the hippocampus, as well as rarefaction of the neuropil and eosinophilic plaques (outlined by arrows). In contrast, lack of neuropathological changes in sham-inoculated negative controls (**B** and **D**). Scale bars: 50 μ m (**A**); 40 μ m (**B**); 75 μ m (**C** and **D**).

phoid follicles, which is morphologically suggestive of either macrophages and/or dendritic cells (Figure 4D).

At subsequent time points PrP^{RES} deposits were detected in the bone marrow, pancreatic islets, Peyer's patches, tongue, and salivary, adrenal, and pituitary

glands. In the bone marrow, PrP^{RES} deposits were finely granular to coarsely clumped and localized to a small number of nucleated cells (Figure 4E), which were interpreted to be of myeloid origin (nonerythroid/nonlymphoid). In the pancreas, PrP^{RES} was limited to the cytoplasm of the islets of Langerhans (Figure 4F). In the tongue, PrP^{RES} was detected in two microanatomic locations: within the cells of the taste bud and cells of lingual glands (Figure 4, G and H). Within the lingual glands of the tongue, PrP^{RES} accumulations were limited to the serous glands (Figure 4H) with mucous glands lacking detectable PrP^{RES} (data not shown).

In the salivary glands, PrP^{RES} was detected in the serous epithelial cells of the submandibular salivary gland (Figure 4I). Within the adrenal gland, PrP^{RES} was observed in the glandular cells of the adrenal medulla. Within one of two terminal i.v. inoculated mice, PrP^{RES} deposits were seen in the pituitary gland, within cells of the neurohypophysis. PrP^{RES} was not detected at any time point in any tissues from the following systems: musculoskeletal, reproductive, peripheral nervous, respiratory, or cardiovascular. Moreover, PrP^{RES} was not identified in any of the tissues obtained from the sham-inoculated Tg[CerPrP] mice.

Successful Transmission of CWD to Orally Inoculated Tg[CerPrP] Mice

In the initial p.o. inoculation study (above), none of the inoculated Tg[CerPrP] mice developed neurological disease (>700 dpi) or accumulated PrP^{RES} in either brain or spleen (data not shown). To determine the role of inoculum dose in oral susceptibility, we inoculated a second group ($n = 5$) of Tg[CerPrP] mice with undiluted CWD-positive or CWD-negative brain. Three of the five CWD-inoculated mice developed progressive and terminal neurological disease characterized by ataxia and mental obtundation, at 323, 350, and 378 dpi, and accumulated PrP^{RES} in nervous and non-nervous tissues. The largest and most numerous immunoreactive aggregates were detected in the caudal aspects of the brain (obex, brainstem, and cerebellum) with decreasing amounts in the mid- and forebrain regions, including the hippocampus and hypothalamus. (Figure 5, A and B) with rare PrP^{RES} detected in the neocortex. In the non-CNS tissues from these three mice, PrP^{RES} was detected in the spleen (three of three), Peyer's patches (three of three), mesenteric lymph nodes (three of three), and pancreatic islets (two of three) (Figure 5C). PrP^{RES} was not detected in the remainder of the peripheral tissues evaluated, including all remaining tissues from the gastrointestinal, urogenital, endocrine, and musculoskeletal systems. None of the sham-inoculated animals developed clinical disease nor did tissues from these mice contain PrP^{RES} immunoreactivity. Thus, a 100% fold increment in CWD brain homogenate dosage produced infection and disease by the oral route.

Figure 3. Longitudinal CWD PrP^{RES} detection in Tg[CerPrP] mice after i.c., i.p., or i.v. inoculation. For each of the three routes of inoculation (green, i.c.; blue, i.p.; red, i.v.), tissues are listed by earliest sacrifice time point (60, 120, 180, and 240 dpi) at which PrP^{RES} was detected by IHC. For each time point and each route, only those tissues in which the identification of PrP^{RES} represented a new appearance are listed. The transition from solid to dashed line represents the approximate onset of terminal clinical disease. Tissues listed at the last time point represent IHC findings in terminal animals. LN, lymph nodes.

Figure 4. CWD PrP^{RES} in tissues of i.c., i.p., and i.v. inoculated Tg[CerPrP] mice. All tissues were obtained from PLP-perfused, CWD-inoculated mice and were immunostained using the IHC protocol detailed in *Materials and Methods*. **A:** PrP^{RES} (brown) in the hippocampus (in the hilus fasciae dentatae) at 60 dpi. **B:** PrP^{RES} in the spleen, most notably within large cells of the marginal zone probably representing macrophages and/or dendritic cells at 60 dpi. **C:** Presence of PrP^{RES} in the sinusoidal lining cells of the liver (probable Kupffer cells) at 120 dpi. **D:** PrP^{RES} within the mesenteric lymph node, most notably in large cells probably representing macrophages and/or dendritic cells at 180 dpi. **E:** PrP^{RES} in the bone marrow at 193 dpi. **F:** PrP^{RES} in islet cells of the pancreas at 193 dpi. **G:** PrP^{RES} in the cells of a lingual taste bud. **H:** PrP^{RES} in cells of the mucus lingual glands. **I:** PrP^{RES} in serous cells of the submandibular salivary gland at 233 dpi. Scale bars: 200 μ m (**A**); 50 μ m (**B**); 10 μ m (**C**); 60 μ m (**D**); 25 μ m (**E** and **F**); 25 μ m (**G**); 60 μ m (**H**); 25 μ m (**I**).

Horizontal Transmission of CWD to Contact-Exposed Sentinel Tg[CerPrP] Mice

To evaluate for the possibility of horizontal CWD transmission in Tg[CerPrP] mice, one naive Tg[CerPrP] mouse was housed as a cohabitant with two CWD-inoculated mice. At each of the predetermined sacrifice time points (60, 120, 180, and 240 dpi), two CWD-inoculated mice

and one sham-inoculated mouse were euthanized and necropsied. Thus, at the completion of each of these sacrifices, one of the five cohabitant mice assigned per inoculation group was left alone in the cage, resulting in sentinel mice that were exposed to the CWD-inoculated mice for either 60, 120, 180, or 240 days or until terminal disease developed in inoculated cage-mates (171 to 233 days).

Figure 5. CWD PrP^{RES} in tissues of p.o. inoculated and horizontally infected Tg[CerPrP] mice. All tissues were obtained from either a terminally ill, PLP-perfused, p.o. inoculated mouse sacrificed at 350 dpi (**A–C**) or from a terminally ill, PLP-perfused, noninoculated mouse that cohabitated with an i.c. inoculated mice that was sacrificed because of terminal neurological disease (**D–F**). All tissues were immunostained using the IHC protocol detailed in *Materials and Methods*. Each panel demonstrates PrP^{RES} immunoreactivity (brown) in selected tissues. **A:** PrP^{RES} in the cervical spinal cord, including heavy deposition surrounding the central canal. **B:** PrP^{RES} in the granular layer of the cerebellum. **C:** PrP^{RES} in the cells of the Peyer's patch which, based on size and cytomorphologic characteristics, are probably macrophages and/or dendritic cells. **D:** PrP^{RES} in the brainstem. **E:** PrP^{RES} in the hippocampus (hilus fasciae dentatae). **F:** PrP^{RES} in the pancreatic islets. Scale bars: 25 μ m (**A**); 75 μ m (**B**); 200 μ m (**C**); 25 μ m (**D**); 60 μ m (**E**); 50 μ m (**F**).

Figure 6. Blocking of PrP^{RES} immunoreactivity by target peptide before incubation. All tissues were obtained from a terminally ill, PLP-perfused, i.p. inoculated Tg[CerPrP] mouse sacrificed at 233 dpi. Tissues were immunostained using an IHC protocol incorporating the BAR-224 monoclonal antibody, amplification using TSA reagents and a peptide blocking step. **Top row** (no blocking): PrP^{RES} immunoreactivity (red) in (A) brainstem neuropil, (B) splenic nucleated cells, (C) pancreatic islet epithelial cells, and (D) hepatic Kupffer cells. **Bottom row** (blocking): Pre-application incubation of the primary antibody with its eliciting peptide results in complete to near-complete abrogation of PrP^{RES} immunoreactivity in matched tissue sections (E–H). Scale bars: 100 μ m (A and E); 100 μ m (B and F); 80 μ m (C and G); 27.5 μ m (D and H).

After the sacrifice of their inoculated cage-mates, the cohabitant mice were allowed to live out the remainder of the study (600 dpi) or until the onset of either i) clinical neurological disease or ii) clinical signs of any other disease which necessitated humane euthanasia. Of these 20 sentinel mice, 3 demonstrated clinical neurological dysfunction and had immunohistochemically detectable PrP^{RES} within the brain, although only 15 were observed over a long enough observation period (600 dpi) to allow for detection of infection and disease. The first affected mouse was housed with two i.c. inoculated mice for 193 days and was sacrificed at 543 days postexposure. The second affected mouse was housed with two i.p. inoculated mice for 233 days and was sacrificed at 562 days postexposure. The third affected mouse was housed with two i.v. inoculated mice for 201 days and was sacrificed at 551 days postexposure. These three mice lived for 350, 329, and 350 days, respectively, after the sacrifice of their inoculated cage-mates. Thus, of the 15 mice exposed to infected cage-mates that survived to the end of the study (600 dpi), 3 developed a TSE.

Lesions and PrP^{RES} in Horizontally Infected Tg[CerPrP] Mice

Neuropathological findings in the affected Tg[CerPrP] mice were identical to those seen in the i.c., i.p., and i.v. inoculated mice. PrP^{RES} distribution in the brains of horizontally infected mice was similar to that in the high-dose p.o. inoculated mice, with heavy PrP^{RES} deposits in the obex, brainstem, and cerebellum (Figure 5, D–E) and

smaller, less frequent PrP^{RES} deposits in the hippocampus, hypothalamus, and neocortex. PrP^{RES} was also detected in the spleen, Peyer's patches, and pancreatic islets (Figure 5F). PrP^{RES} was not detected in the remainder of the peripheral tissues evaluated, including all remaining tissues from the gastrointestinal, urogenital, endocrine, and musculoskeletal systems.

Abrogation of PrP^{RES} Immunoreactivity Using Peptide Blocking

To confirm the specificity of the PrP^{RES} IHC detection protocols, tissues previously identified as PrP^{RES}-positive were subjected to an IHC protocol, which incorporated a preincubation of the primary, anti-prion antibody with its eliciting peptide. In these experiments, there was near-complete to complete abrogation of PrP^{RES} immunoreactivity when such a peptide, preincubation step was incorporated (Figure 6, A–H).

Discussion

Although the effectiveness of peripheral inoculation in prion diseases seems to rely, in part, on the expression of PrP^C in peripheral (non-CNS) tissues, there are few published data regarding the systemic expression patterns of PrP^C in the tissues of transgenic mice expressing PrP^C of alternate species.^{22–25} In the initial characterization of the 1536 Tg[CerPrP] strain used in these studies, cervid PrP was detected only in the brain by dot and Western blot-

ting using monoclonal antibody 6H4.⁶ To expand on the initial characterization of 1536 Tg[CerPrP] mice we used PLP fixation and the polyclonal anti-prion antibody R505.5 to identify PrP^C expression in cell populations in the nervous, lymphoid, endocrine, reproductive, gastrointestinal, and hematopoietic systems (Table 1): a pattern of expression similar to that reported for expression of native species PrP^C in rodents and cervids.^{26–30} Morphologically, the pattern of PrP^C immunoreactivity varied widely between individual cell and tissue types from finely granular to punctate to aggregate in nature. Such a pattern of PrP^C immunoreactivity is similar to that described in other PrP^C mapping studies in the adult mouse^{28,31} and may be reflective of the varied, and often high, level of protein expressed in the immunopositive cells. In certain cell types (ie, splenic lymphocytes and brain neurons) the high levels of protein might saturate the detection system, resulting in aggregation of the chromogen. Based on the lack of corresponding immunoreactivity in the PrP^{0/0} mice, we are confident that the protocol used to detect PrP^C produced accurate and specific results. Finally, these findings provide the first evidence suggesting that the cosTet vector system is capable of manifesting peripheral expression of a prion protein of a heterologous species and complement recent work confirming this vector system as being capable of manifesting widespread prion protein expression in the mouse.^{5–11,13,14,32–35}

In these studies, we used the combination of clinical disease and the immunohistochemical detection of PrP^{RES} to document successful CWD infection in the inoculated Tg[CerPrP] mice. The use of such an approach is justified by previously published prion pathogenesis and PrP^{RES} mapping studies, including those evaluating longitudinal patterns of CWD prion accumulation in native cervid species.^{17,21,36} Although we did not include concurrent immunoblot analysis of all tissue samples, we believe the sensitivity of the TSA-based immunohistochemical detection techniques used provided sensitivity that was more than equivalent to tissue homogenate Western blotting. Moreover, we believe that the results from the peptide blocking and PrP^{0/0} studies confirm the specificity of the PrP^{RES} longitudinal mapping results.

We demonstrate the susceptibility of Tg[CerPrP] mice to CWD after the i.c., i.v., i.p., and p.o. routes, expanding on previously published work.⁶ Although this is the first report documenting the susceptibility of 1536 Tg[CerPrP] mice to infection with CWD after i.p., i.v., and p.o. routes of inoculation, this is not the first report documenting oral susceptibility of cervidized mice to CWD. Trifilo et al⁹ demonstrated that oral inoculation of two other lines of cerPrP transgenic mice produced clinical TSE in both lines at 370 ± 26 and 381 ± 55 dpi, respectively,⁹ which is similar to our findings of 350 ± 27 dpi. The failure of the initial group of orally inoculated mice to demonstrate susceptibility to CWD is probably the result of an insufficient inoculum dose, a point that is emphasized by both the successful inoculation of three of five mice fed a whole-brain inoculum and the successful p.o. inoculation

study cited above, in which a more concentrated (5%) brain homogenate proved successful.⁹

Through serial sacrifice, we were able to demonstrate longitudinal patterns of PrP^{RES} tissue tropism suggestive of a three-phase pathogenesis: i) an early lymphoreticular system-associated phase characterized by the accumulation of PrP^{RES} in lymphoid compartments (spleen and/or lymph node), ii) a neuroinvasive phase, whereby PrP^{RES} became detectable in the CNS, and iii) the terminal accumulation of PrP^{RES} within peripheral tissues. The first two phases are most evident in the temporal patterns of PrP^{RES} accumulation in the i.v. and i.p. inoculated mice in which the early lymphoreticular system accumulation phase was demonstrated. In these tissues, PrP^{RES} was observed within likely follicular dendritic cells, cells that have been shown to be important enablers of prion disease propagation and neuroinvasion.^{21,37–43}

Detection of splenic PrP^{RES} at a time point (60 dpi) preceding PrP^{RES} detection in the brain (120 dpi) suggests that splenic-mediated neuroinvasion plays a role in Tg[CerPrP] mice, as has been theorized for scrapie prion transit via nerve fibers associated with follicular dendritic cell-rich splenic germinal centers as conduits to the thoracic spinal cord.^{44,45} Although splenic-mediated neuroinvasion remains the most likely possibility, we cannot exclude the possibility of hematogenous neuroinvasion, a phenomenon that, although little investigated, finds support in the literature and in the detection of infectious prions in blood.^{46–52}

The third phase of CWD pathogenesis in Tg[CerPrP] mice was marked by the accumulation of PrP^{RES} in peripheral tissues (denoted terminal PrP^{RES} dissemination). In this phase, PrP^{RES} deposits were detected in the bone marrow, pancreatic islets, Peyer's patches, mesenteric lymph nodes, adrenal gland, pituitary gland, tongue, and salivary gland. Such a pattern is typical of the progressive vagal and sympathetic nervous system-mediated, centrifugal dissemination of prions from the brain in the later to terminal stages of prion disease as described in previous mouse, sheep, and hamster models.^{24,53–56}

In the successfully infected Tg[CerPrP] mice, there were exceptions to the proposed two- to three-step pathogenesis, ie, instances in which PrP^{RES} was identified in peripheral tissues either before or simultaneous with its identification in the CNS. Such cases included the identification of PrP^{RES} in the spleen or liver of i.v., i.p., and i.c. inoculated mice sacrificed at 60 dpi or in the pancreatic islets of i.v. inoculated animals sacrificed at 120 dpi. These findings support the presence of a transient, postinoculation period of hematogenous prion trafficking, which results in the delivery of inoculum-origin prions to peripheral tissues. Such a phenomenon, described as a spillover "prionemia," has been reported after the i.p. or i.c. inoculation of scrapie or Creutzfeldt-Jakob disease prions, such that infectious prions accumulate in peripheral tissues (bone marrow, adrenal gland, salivary gland, and spleen), either before or coincident with the identification of prions in the brain and that infectious prions can be detected in the blood immediately after i.p. and i.c. inoculation.^{43,57–59}

The more novel detection of PrP^{RES} in the salivary glands, bone marrow, neuroendocrine system, and neurosensory cells of the tongue confirms the utility of Tg[CerPrP] mice as effective surrogates for CWD infection. Moreover, serial sacrifice and longitudinal PrP^{RES} accumulation data offer insights into the manner by which prions traffic to these tissues. In the salivary glands, we identified PrP^{RES} only after it was identified in the CNS, which suggests that centrifugal spread along neural pathways is responsible for prion delivery. The salivary glands secrete in response to stimuli provided by the autonomic nervous system, including fibers of both the parasympathetic and sympathetic systems via the medulla oblongata and lateral hypothalamus.^{60,61} Although we did not track the accumulation of prions along specific peripheral and cranial nerve tracts, in our terminal i.v. and i.p. inoculated mice we did identify significant deposits of PrP^{RES} within the pons and the ventromedial hypothalamus, which have been described as the salivary centers of the brain.⁶¹ Finally, the pattern of PrP^{RES} deposition in the salivary glands, with PrP^{RES} accumulations present on the surface of acinar epithelial cells, is similar to that reported in an IHC study published in scrapie-affected sheep⁶² and suggests that salivary secretion of prions may result from normal, physiological activity of glands during the process of saliva secretion.⁶³

The demonstration of PrP^{RES} in the bone marrow of Tg[CerPrP] mice, although novel in the CWD system, is consistent with our identification of PrP^C in this compartment and with published work in murine models of scrapie.^{43,64} Although the route by which PrP^{RES} accumulated in the bone marrow is uncertain, the weight of the evidence suggests a pathway of hematogenous trafficking, as demonstrated by studies showing the feasibility of blood-borne transmission in several prion diseases (bovine spongiform encephalopathy, scrapie, CWD, and new variant Creutzfeldt-Jakob disease), as well as work detecting prions in the blood of preclinical, scrapie-infected hamsters, and CWD-infected deer.^{46–50} Identification of PrP^{RES} in the endocrine system of cervidized mice has not been reported previously but resembles findings in orally inoculated and naturally infected mule deer, in which PrP^{RES} was demonstrated in the adrenal medulla, islets of Langerhans, and pituitary of animals with late-stage CWD.^{36,65} PrP^{RES} has also been seen in the pancreas of goats and sheep with natural scrapie and in hamsters and mice with experimental scrapie.^{66–68} Because PrP^{RES} was detectable in endocrine tissues only after its detection in the brain, centrifugal dissemination via the vagus nerve, splanchnic nerves, and fibers from the hypothalamus to the pancreatic islets, adrenal gland, and pars nervosa of the pituitary gland, respectively, is likely.^{69,70}

The identification of PrP^{RES} in the lingual taste buds and glands is intriguing. PrP^{RES} has been reported previously in the tongues of hamsters i.c. inoculated with transmissible mink encephalopathy, in hamsters p.o. inoculated with scrapie, in transgenic mice p.o. inoculated with CWD, and in sheep with natural scrapie.^{9,71–73} Using IHC and confocal microscopy, Bessen and colleagues⁵⁴ demonstrated that PrP^{RES} accumulates within the nerve

fibers, taste buds, and stratified squamous epithelium in the fungiform papillae as well as within nerve fascicles and skeletal muscle cells. Trifilo et al⁹ demonstrated PrP^{RES} in occasional taste buds of p.o. inoculated cervidized transgenic mice. We found PrP^{RES} in the tongue only late in disease (180 to 240 dpi), after its appearance in the brain, again suggesting centrifugal spread, probably via cranial nerve fibers (facial [VII] and trigeminal [V]), a pathway proven in hamsters inoculated with transmissible mink encephalopathy.⁷¹ In addition to taste buds, we identified PrP^{RES} in the lingual glands, consistent with the findings of Trifilo et al,⁹ suggesting centrifugal dissemination via the hypoglossal nerve.^{9,74}

In association with the direct CWD inoculation studies, we demonstrated, for the first time, the horizontal transmission of CWD prions in a rodent model system. Whereas lateral transmission is a prominent feature of CWD and scrapie, little published information exists in rodent models.^{1,75,76} Although questions remain regarding the nature of this transmission, based on the identical pattern of PrP^{RES} accumulation seen in naturally and experimentally (p.o.) infected cervids orally infected mice in this study, we reason that the contact-exposed mice were infected by the oral route.^{17,21,65,77,78} The precedent for the horizontal transmission of pathogens between cohabitant cage-mates is provided by work detailing the horizontal spread of hantavirus and *Helicobacter pylori* between murine cage-mates.^{79,80} The vehicle responsible for the spread of prions between cage-mates is uncertain, but other studies in cervids and rodent model systems support the plausibility of biological fluids and excreta as transmission vehicles.^{48,81,82} Given the presence of PrP^{RES} in the taste buds of inoculated Tg[CerPrP] mice, horizontal spread could be linked to the shedding of prion-infected cells into oronasal secretions.⁷¹ In addition to direct animal-to-animal contact or animal-to-excreta contact, coprophagy is also plausible, as evidenced by the transmission of CWD and scrapie by exposure of naive animals to a contaminated environment.^{82,83}

In summary, cervidized transgenic mice substantially recapitulate the clinical, neuropathological, and PrP^{RES} tropism, and transmission patterns reported in the native cervid species^{21,36,65,77,78,84} and studies in Tg[CerPrP] mice can provide additional insights into the trafficking, shedding, and lateral transmission of CWD prions.

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References

1. Miller MW, Williams ES: Prion disease: horizontal prion transmission in mule deer. *Nature* 2003, 425:35–36

2. Miller MW, Wild MA, Williams ES: Epidemiology of chronic wasting disease in captive Rocky Mountain elk. *J Wildl Dis* 1998, 34:532–538
3. Miller MW, Wild MA: Epidemiology of chronic wasting disease in captive white-tailed and mule deer. *J Wildl Dis* 2004, 40:320–327
4. Williams ES, Young S: Spongiform encephalopathies in Cervidae. *Rev Sci Tech* 1992, 11:551–567
5. Angers RC, Browning SR, Seward TS, Sigurdson CJ, Miller MW, Hoover EA, Telling GC: Prions in skeletal muscles of deer with chronic wasting disease. *Science* 2006, 311:1117
6. Browning SR, Mason GL, Seward T, Green M, Eliason GA, Mathiason C, Miller MW, Williams ES, Hoover E, Telling GC: Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *J Virol* 2004, 78:13345–13350
7. Kong Q, Huang S, Zou W, Vanegas D, Wang M, Wu D, Yuan J, Zheng M, Bai H, Deng H, Chen K, Jenny AL, O'Rourke K, Belay ED, Schonberger LB, Petersen RB, Sy MS, Chen SG, Gambetti P: Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. *J Neurosci* 2005, 25:7944–7949
8. Tamgüney G, Giles K, Bouzamondo-Bernstein E, Bosque PJ, Miller MW, Safar J, DeArmond SJ, Prusiner SB: Transmission of elk and deer prions to transgenic mice. *J Virol* 2006, 80:9104–9114
9. Trifilo MJ, Ying G, Teng C, Oldstone MB: Chronic wasting disease of deer and elk in transgenic mice: oral transmission and pathobiology. *Virology* 2007, 365:136–143
10. LaFauci G, Carp RI, Meeker HC, Ye X, Kim JI, Natelli M, Cedeno M, Petersen RB, Kascsak R, Rubenstein R: Passage of chronic wasting disease prion into transgenic mice expressing Rocky Mountain elk (*Cervus elaphus nelsoni*) PrPC. *J Gen Virol* 2006, 87:3773–3780
11. Scott MR, Safar J, Telling G, Nguyen O, Groth D, Torchia M, Koehler R, Tremblay P, Walther D, Cohen FE, DeArmond SJ, Prusiner SB: Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. *Proc Natl Acad Sci USA* 1997, 94:14279–14284
12. Telling GC: Prion protein genes and prion diseases: studies in transgenic mice. *Neuropathol Appl Neurobiol* 2000, 26:209–220
13. Telling GC, Scott M, Hsiao KK, Foster D, Yang SL, Torchia M, Sidle KC, Collinge J, DeArmond SJ, Prusiner SB: Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc Natl Acad Sci USA* 1994, 91:9936–9940
14. Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, Cohen FE, DeArmond SJ, Prusiner SB: Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* 1995, 83:79–90
15. Scott MR, Kohler R, Foster D, Prusiner SB: Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Sci* 1992, 1:986–997
16. Baron T: Mouse models of prion disease transmission. *Trends Mol Med* 2002, 8:495–500
17. Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA: Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (*Odocoileus hemionus*). *J Gen Virol* 1999, 80(Pt 10):2757–2764
18. Carlson GA, Kingsbury DT, Goodman PA, Coleman S, Marshall ST, DeArmond S, Westaway D, Prusiner SB: Linkage of prion protein and scrapie incubation time genes. *Cell* 1986, 46:503–511
19. Petrusz P, Bullock G. Edited by H. Hedrich: *The laboratory mouse*. Elsevier Academic Press 2004
20. Garssen GJ, Van Keulen LJ, Farquhar CF, Smits MA, Jacobs JG, Bossers A, Melen RH, Langeveld JP: Applicability of three anti-PrP peptide sera including staining of tonsils and brainstem of sheep with scrapie. *Microsc Res Tech* 2000, 50:32–39
21. Sigurdson CJ, Barillas-Mury C, Miller MW, Oesch B, van Keulen LJ, Langeveld JP, Hoover EA: PrP(CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. *J Gen Virol* 2002, 83:2617–2628
22. Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A: Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature* 1996, 379:339–343
23. Glatzel M, Aguzzi A: PrP(C) expression in the peripheral nervous system is a determinant of prion neuroinvasion. *J Gen Virol* 2000, 81:2813–2821
24. Blättler T, Brandner S, Raeber AJ, Klein MA, Voigtlander T, Weissmann C, Aguzzi A: PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature* 1997, 389:69–73
25. Le Pichon CE, Firestein S: Expression and localization of the prion protein PrP(C) in the olfactory system of the mouse. *J Comp Neurol* 2008, 508:487–499
26. Burthorn J, Urban B, Pain A, Roberts DJ: The normal cellular prion protein is strongly expressed by myeloid dendritic cells. *Blood* 2001, 98:3733–3738
27. Liu T, Li R, Wong BS, Liu D, Pan T, Petersen RB, Gambetti P, Sy MS: Normal cellular prion protein is preferentially expressed on subpopulations of murine hemopoietic cells. *J Immunol* 2001, 166:3733–3742
28. Ford MJ, Burton LJ, Morris RJ, Hall SM: Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience* 2002, 113:177–192
29. Mathiason C, Foos T, Eliason GA, Sigurdson CJ, Miller M, Lewis-Weis L, Fischer J, Hoover E: Cervid PrPc Quantitation and Tissue Mapping. Presented at the College of Veterinary Medicine and Biomedical Sciences Research Day (Microbiology, Immunology, and Pathology). 2004 January 29. Fort Collins, CO.
30. Zhang CC, Steele AD, Lindquist S, Lodish HF: Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci USA* 2006, 103:2184–2189
31. Brown KL, Ritchie DL, McBride PA, Bruce ME: Detection of PrP in extraneural tissues. *Microsc Res Tech* 2000, 50:40–45
32. Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL, Welch J, Hill AF, Lloyd SE, Wadsworth JD, Collinge J: BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J* 2002, 21:6358–6366
33. Prusiner SB, Scott M, Foster D, Pan KM, Groth D, Mirenda C, Torchia M, Yang SL, Serban D, Carlson GA, Hoppe PC, Westaway D, DeArmond SJ: Transgenic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* 1990, 63:673–686
34. Scott M, Foster D, Mirenda C, Serban D, Coufal F, Walchli M, Torchia M, Groth D, Carlson G, DeArmond SJ, Westaway D, Prusiner SB: Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* 1989, 59:847–857
35. Tremblay P, Bouzamondo-Bernstein E, Heinrich C, Prusiner SB, DeArmond SJ: Developmental expression of PrP in the post-implantation embryo. *Brain Res* 2007, 1139:60–67
36. Sigurdson CJ, Spraker TR, Miller MW, Oesch B, Hoover EA: PrP(CWD) in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease. *J Gen Virol* 2001, 82:2327–2334
37. Muramoto T, Kitamoto T, Tateishi J, Goto I: Accumulation of abnormal prion protein in mice infected with Creutzfeldt-Jakob disease via intraperitoneal route: a sequential study. *Am J Pathol* 1993, 143:1470–1479
38. Mabbott NA, Mackay F, Minns F, Bruce ME: Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. *Nat Med* 2000, 6:719–720
39. Mabbott NA, MacPherson GG: Prions and their lethal journey to the brain. *Nat Rev Microbiol* 2006, 4:201–211
40. Mabbott NA, Young J, McConnell I, Bruce ME: Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. *J Virol* 2003, 77:6845–6854
41. Mohan J, Bruce ME, Mabbott NA: Follicular dendritic cell dedifferentiation reduces scrapie susceptibility following inoculation via the skin. *Immunology* 2005, 114:225–234
42. Huang FP, Farquhar CF, Mabbott NA, Bruce ME, MacPherson GG: Migrating intestinal dendritic cells transport PrP^{Sc} from the gut. *J Gen Virol* 2002, 83:267–271
43. Kimberlin RH, Walker CA: The role of the spleen in the neuroinvasion of scrapie in mice. *Virus Res* 1989, 12:201–211
44. Prinz M, Heikenwalder M, Junt T, Schwarz P, Glatzel M, Heppner FL, Fu YX, Lipp M, Aguzzi A: Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion. *Nature* 2003, 425:957–962
45. Glatzel M, Heppner FL, Albers KM, Aguzzi A: Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. *Neuron* 2001, 31:25–34
46. Hunter N, Foster J, Chong A, McCutcheon S, Parnham D, Eaton S, MacKenzie C, Houston F: Transmission of prion diseases by blood transfusion. *J Gen Virol* 2002, 83:2897–2905

47. Llewellyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, Will RG: Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* 2004, 363:417–421
48. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ, Mason GL, Hays SA, Hayes-Klug J, Seelig DM, Wild MA, Wolfe LL, Spraker TR, Miller MW, Sigurdson CJ, Telling GC, Hoover EA: Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science* 2006, 314:133–136
49. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW: Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004, 364:527–529
50. Saá P, Castilla J, Soto C: Presymptomatic detection of prions in blood. *Science* 2006, 313:92–94
51. Viegas P, Chaverot N, Enslin H, Perriere N, Couraud PO, Cazaubon S: Junctional expression of the prion protein PrP^C by brain endothelial cells: a role in trans-endothelial migration of human monocytes. *J Cell Sci* 2006, 119:4634–4643
52. Banks WA, Niehoff ML, Adessi C, Soto C: Passage of murine scrapie prion protein across the mouse vascular blood-brain barrier. *Biochem Biophys Res Commun* 2004, 318:125–130
53. Beekes M, McBride PA: The spread of prions through the body in naturally acquired transmissible spongiform encephalopathies. *FEBS J* 2007, 274:588–605
54. Mulcahy ER, Bartz JC, Kincaid AE, Bessen RA: Prion infection of skeletal muscle cells and papillae in the tongue. *J Virol* 2004, 78:6792–6798
55. Borchelt DR, Koliatsos VE, Guarneri M, Pardo CA, Sisodia SS, Price DL: Rapid anterograde axonal transport of the cellular prion glycoprotein in the peripheral and central nervous systems. *J Biol Chem* 1994, 269:14711–14714
56. Moya KL, Hassig R, Creminon C, Laffont I, Di Giamberardino L: Enhanced detection and retrograde axonal transport of PrP^C in peripheral nerve. *J Neurochem* 2004, 88:155–160
57. Bruce ME: Agent replication dynamics in a long incubation period model of mouse scrapie. *J Gen Virol* 1985, 66(Pt 12):2517–2522
58. Field EJ, Caspary EA, and Joyce G: Scrapie agent in blood. *Vet Rec* 1968, 83:109–110
59. Millson GC, Kimberlin RH, Manning EJ, Collis SC: Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. *Vet Microbiol* 1979, 5:89–99
60. Zoukhri D, Kublin CL: Impaired neurotransmitter release from lacrimal and salivary gland nerves of a murine model of Sjögren's syndrome. *Invest Ophthalmol Vis Sci* 2001, 42:925–932
61. Proctor GB, Carpenter GH: Regulation of salivary gland function by autonomic nerves. *Auton Neurosci* 2007, 133:3–18
62. Vascellari M, Nonno R, Mutinelli F, Bigolaro M, Di Bari MA, Melchioti E, Marcon S, D'Agostino C, Vaccari G, Conte M, De Grossi L, Rosone F, Giordani F, Agrimi U: PrP^{Sc} in salivary glands of scrapie-affected sheep. *J Virol* 2007, 81:4872–4876
63. McManaman JL, Reyland ME, Thrower EC: Secretion and fluid transport mechanisms in the mammary gland: comparisons with the exocrine pancreas and the salivary gland. *J Mammary Gland Biol Neoplasia* 2006, 11:249–268
64. Eklund CM, Kennedy RC, Hadlow WJ: Pathogenesis of scrapie virus infection in the mouse. *J Infect Dis* 1967, 117:15–22
65. Fox KA, Jewell JE, Williams ES, Miller MW: Patterns of PrP^{CWD} accumulation during the course of chronic wasting disease infection in orally inoculated mule deer (*Odocoileus hemionus*). *J Gen Virol* 2006, 87:3451–3461
66. Ye X, Carp RI, Yu Y, Kozielski R, Kozlowski P: Hyperplasia and hypertrophy of B cells in the islets of Langerhans in hamsters infected with the 139H strain of scrapie. *J Comp Pathol* 1994, 110:169–183
67. Pattison IH, Millson GC: Further observations on the experimental production of scrapie in goats and sheep. *J Comp Pathol* 1960, 70:182–193
68. McBride PA, Eikelenboom P, Kraal G, Fraser H, Bruce ME: PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J Pathol* 1992, 168:413–418
69. Loewy AD, Franklin MF, Haxhiu MA: CNS monoamine cell groups projecting to pancreatic vagal motor neurons: a transneuronal labeling study using pseudorabies virus. *Brain Res* 1994, 638:248–260
70. Kesse WK, Parker TL, Coupland RE: The innervation of the adrenal gland. I. The source of pre- and postganglionic nerve fibres to the rat adrenal gland. *J Anat* 1988, 157:33–41
71. DeJoia C, Moreaux B, O'Connell K, Bessen RA: Prion infection of oral and nasal mucosa. *J Virol* 2006, 80:4546–4556
72. Thomzig A, Kratzel C, Lenz G, Kruger D, Beekes M: Widespread PrP^{Sc} accumulation in muscles of hamsters orally infected with scrapie. *EMBO Rep* 2003, 4:530–533
73. Andréoletti O, Simon S, Lacroux C, Morel N, Tabouret G, Chabert A, Lugan S, Corbiere F, Ferre P, Foucras G, Laude H, Eychenne F, Grassi J, Schelcher F: PrP^{Sc} accumulation in myocytes from sheep incubating natural scrapie. *Nat Med* 2004, 10:591–593
74. Saraswathi P: Neurohistological observations on the tongue of the monkey (*Macaca radiata*). *J Anat Soc India* 2003, 52:7–12
75. Williams ES: Chronic wasting disease. *Vet Pathol* 2005, 42:530–549
76. Morris JA, Gajdusek DC, Gibbs CJ Jr: Spread of scrapie from inoculated to uninoculated mice. *Proc Soc Exp Biol Med* 1965, 120:108–110
77. Spraker TR, Zink RR, Cummings BA, Sigurdson CJ, Miller MW, O'Rourke KI: Distribution of protease-resistant prion protein and spongiform encephalopathy in free-ranging mule deer (*Odocoileus hemionus*) with chronic wasting disease. *Vet Pathol* 2002, 39:546–556
78. Spraker TR, Zink RR, Cummings BA, Wild MA, Miller MW, O'Rourke KI: Comparison of histological lesions and immunohistochemical staining of proteinase-resistant prion protein in a naturally occurring spongiform encephalopathy of free-ranging mule deer (*Odocoileus hemionus*) with those of chronic wasting disease of captive mule deer. *Vet Pathol* 2002, 39:110–119
79. Karita M, Matsumoto S, Kamei T, Shinohara K, Sugiyama T: Direct transmission of *H. pylori* from challenged to nonchallenged mice in a single cage. *Dig Dis Sci* 2005, 50:1092–1096
80. Botten J, Mirowsky K, Ye C, Gottlieb K, Saavedra M, Ponce L, Hjelte B: Shedding and intracage transmission of Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*) model. *J Virol* 2002, 76:7587–7594
81. Haley NJ: Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. *PLoS One* 2009, 4:e4848
82. Safar JG, Lessard P, Tamguney G, Freyman Y, Deering C, Letessier F, Dearmond SJ, Prusiner SB: Transmission and detection of prions in feces. *J Infect Dis* 2008, 198:81–89
83. Miller MW, Williams ES, Hobbs NT, Wolfe LL: Environmental sources of prion transmission in mule deer. *Emerg Infect Dis* 2004, 10:1003–1006
84. Hamir AN, Gidlewski T, Spraker TR, Miller JM, Creekmore L, Crocheck M, Cline T, O'Rourke KI: Preliminary observations of genetic susceptibility of elk (*Cervus elaphus nelsoni*) to chronic wasting disease by experimental oral inoculation. *J Vet Diagn Invest* 2006, 18:110–114