Dissociation of pathological and molecular phenotype of variant Creutzfeldt–Jakob disease in transgenic human prion protein 129 heterozygous mice

Emmanuel A. Asante, Jacqueline M. Linehan, Ian Gowland, Susan Joiner, Katie Fox, Sharon Cooper, Olufumilayo Osiguwa, Michelle Gorry, Julie Welch, Richard Houghton, Melanie Desbruslais, Sebastian Brandner, Jonathan D. F. Wadsworth, and John Collinge*

Medical Research Council Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, United Kingdom

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All neuropathologically confirmed cases of variant Creutzfeldt–Jakob disease (vCJD), characterized by abundant florid plaques and type 4 disease-related prion protein (PrP^{Sc}) in the brain, have been homozygous for methionine at polymorphic residue 129 of *PRNP*. The distinctive neuropathological and molecular phenotype of vCJD can be faithfully recapitulated in *Prnp*-null transgenic mice homozygous for human PrP M129 but not V129, where a distinct prion strain is propagated. Here we model susceptibility of 129MV heterozygotes, the most common *PRNP* genotype, in transgenic mice and show that, remarkably, propagation of type 4 PrP^{Sc} was not associated with characteristic vCJD neuropathology. Depending on the source of the inoculum these mice can develop four distinct disease phenotypes after challenge with bovine spongiform encephalopathy (BSE) prions or vCJD (human-passaged BSE) prions. vCJD-challenged mice had higher attack rates of prion infection than BSE-challenged recipients. These data argue that human *PRNP* 129 heterozygotes will be more susceptible to infection with vCJD prions than to cattle BSE prions and may present with a neuropathological phenotype distinct from vCJD.

bovine spongiform encephalopathy | prion disease | prion strains | florid plaques | subclinical infection

According to the "protein-only" hypothesis (1), prions are composed principally or entirely of abnormal isoforms of host-encoded prion protein (PrP) (2, 3). The disease-related isoform, PrP^{Sc}, is derived from its normal cellular precursor, PrP^{C}, by a posttranslational process that involves conformational change. PrP^{Sc} can be distinguished biochemically from PrP^{C} by its partial resistance to proteolysis and detergent insolubility (2, 3). Multiple prion strains are recognized, and these are associated with distinct PrP^{Sc} types that can be distinguished by Western blot analysis with distinct cleavage sites to proteinase K implying different PrP^{Sc} conformations (4–8) and by differences in the glycoform ratios of protease-digested PrP^{Sc} (5, 8).

The appearance of a novel human prion disease, variant Creutzfeldt–Jakob disease (vCJD), in the United Kingdom from 1995 onwards (9), and the experimental confirmation that this is caused by the same prion strain as that causing bovine spongiform encephalopathy (BSE) in cattle (5, 10–12), has led to widespread concern that exposure to the epidemic of BSE poses a distinct and conceivably a severe threat to public health in the United Kingdom and other countries (13). The extremely prolonged and variable incubation periods seen with prion diseases when crossing a species barrier means that it will be some years before the parameters of any human epidemic can be predicted with confidence (13–15). In the meantime, we are faced with the possibility that significant numbers in the population may be incubating this disease and that they might pass it on to others through blood transfusion, blood products, tissue and organ transplantation, and other iatrogenic routes (13, 16–18).

To date, the clinical presentation of human BSE infection has only been recognized as vCJD in individuals homozygous for methionine at polymorphic residue 129 of human PrP. The neuropathological features of vCJD are highly distinct from those seen in classical (sporadic or iatrogenic) Creutzfeldt–Jakob disease (CJD) and are characterized by the presence of abundant florid PrP plaques (9) and the propagation of type 4 disease-related PrP^{Sc} in brain (5). Polymorphism at codon 129 of the human PrP gene (*PRNP*), where either methionine (M) or valine (V) can be encoded, powerfully affects susceptibility to human prion diseases. Homozygotes have increased susceptibility to either sporadic CJD (19) or acquired prion diseases (20–22), and homozygosity or heterozygosity at codon 129 may affect age at onset in inherited prion disease (19, 23–25). We have previously described four human PrP^{Sc} types in brain tissue from patients with CJD: types 1–3 are seen in classical CJD, and type 4 is uniquely seen in vCJD (5, 8, 26). Polymorphism at residue 129 appears to place constraints on the propagation of human PrP^{Sc} types. To date, types 1 and 4 PrP^{Sc} have been found only in humans of the MM genotype, type 3 PrP^{Sc} is seen almost exclusively in individuals with at least one valine allele, and type 2 PrP^{Sc} has been commonly observed in all codon 129 genotypes (5, 8, 26). Transgenic modeling of BSE and vCJD prion infection in mice expressing human, but not mouse, PrP (5, 10, 12, 27) indicates that this polymorphism directly dictates the propagation of distinct PrP^{Sc} conformers, consistent with a conformational selection model of prion transmission barriers (3, 13, 27, 28).

Notably, there appear to be no overlapping preferred PrP^{Sc} conformations for V129 and M129 human PrP that can be generated as a result of exposure to the vCJD/BSE prion strain (27). Depending on the origin of the inoculum and the PrP codon 129 genotype of the host, transmission of cattle BSE prions or vCJD (human-passaged BSE) prions can result in four distinct prion disease phenotypes. Transgenic mice homozygous for human PrP M129 propagate either type 2 or 4 PrP^{Sc} with respective neuropathologies consistent with human sporadic CJD or vCJD (12, 27), whereas transgenic mice homozygous for human PrP V129 either propagate type 5 PrP^{Sc} and a distinct pattern of neuropathology or develop clinical prion disease in the absence of detectable PrP^{Sc} (10, 27). These findings argue that primary BSE prion infection, as well as secondary infection by iatrogenic routes, may not be restricted to a single disease

Conflict of interest statement: J.C. is a director and J.C. and J.D.F.W. are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination, and therapeutics. D-Gen markets one of the routine antibodies (ICSM 35) used in this study.

Abbreviations: CJD, Creutzfeldt–Jakob disease; vCJD, variant CJD; BSE, bovine spongiform encephalopathy; PrP, prion protein.

*To whom correspondence should be addressed. E-mail: j.collinge@prion.ucl.ac.uk.

Table 1. Transmission of BSE and vCJD prions in transgenic 129MV Tg45/152 mice

	Inoculum			129MV Tg45/152 mice				
Aetiology	Code	PRNP codon 129 genotype	Human PrP^{Sc} type*	Clinical signs	Incubation period, days ± SEM	Positive by IB	Positive by IHC	Total affected†
Sporadic CJD	I1197	MM	T1	4/4	203 ± 18	4/4	4/4	4/4
	I1200	MM	T1	4/6	189 ± 9	6/6	4/4	6/6
	I1203	MM	T1	7/10	209 ± 3	9/10	6/7	10/10
	I024	MV	T2	8/9	216 ± 9	6/7	5/6	8/9
	I022	VV	T2	3/7	439 ± 20	7/7	5/7	7/7
Iatrogenic CJD	I026 (DM)	MM	T2	5/8	197 ± 4	7/7	6/6	8/8
	I020 (GH)	MV	T3	5/8	333 ± 10	5/6	4/5	7/8
	I021 (GH)	VV	T3	4/6	336 ± 46	5/5	5/5	6/6
vCJD	I336	MM	T4	0/8	>284	8/8	8/8	8/8
	I344	MM	T4	0/7	>518	7/7	4/4	7/7
BSE	I038	MM‡		4/15§	686 ± 97	2/15	0/15	6/15¶
	I060	MM‡		2/8§	627, 967	0/8	0/8	2/8
	I062	MM‡		1/6	346	1/6	0/3	1/6‖
	I064	MM‡		0/4	>546	0/4	0/4	0/4
	I066	MM‡		3/8§	631 ± 29	0/7	0/5	3/8
129VV Tg152-passaged vCJD	I784	VV	T5	1/7**	385	6/7	3/6	6/7
	I786	VV	T5	0/5	>386	4/5	2/5	4/5

DM, dura mater; GH, growth hormone; IB, immunoblotting; IHC, immunohistochemistry.
*According to classification of Hill *et al.* (8).
†Mice were scored affected by either clinical signs or detection of PrP^{Sc} by immunoblot and/or positive PrP immunohistochemistry.
‡Genotype at corresponding codon of the bovine PrP gene.
§Clinically affected mice showed no detectable PrP^{Sc} or abnormal PrP immunohistochemistry.
¶Two subclinically affected mice showed monoglycosylated dominant PrP^{Sc} by high-sensitivity immunoblotting.
‖Single clinically affected mouse showed monoglycosylated dominant PrP^{Sc} by high-sensitivity immunoblotting.
**Single clinically affected mouse propagated type 2^- PrP^{Sc}.

phenotype in humans with *PRNP* homozygous genotypes. Here we now report analysis of transgenic mice expressing both human PrP M129 and V129 to model susceptibility of the commonest human *PRNP* genotype, 129MV heterozygotes, comprising 51% of the United Kingdom population (21, 29).

Results and Discussion

We generated human PrP 129MV heterozygous transgenic mice by crossing two previously well characterized transgenic lines, 129MM Tg45 mice (12) and 129VV Tg152 mice (5, 10, 27, 30). Human PrP 129MV heterozygous mice, designated 129MV Tg45/152, express human PrP M129 and V129 at similar levels (ratio ≈1:1.5) with a total PrP expression level in the brain comparable to each parental transgenic line (at ≈4- to 6-fold level of pooled normal human brain) (data not shown). These lines were all on a $Prnp^{o/o}$ background and therefore expressed human, but not mouse, PrP.

After challenge with a panel of human and bovine prion isolates characterized previously (5, 10, 12, 27), we found that sporadic or iatrogenic CJD prions, from patients of all three *PRNP* 129 genotypes, transmitted with high clinical attack rates to 129MV Tg45/152 mice (Table 1). Although clinical prion disease was not observed in all prion-infected mice (Table 1), infected recipients that lacked the typical clinical signs of prion disease had abundant PrP^{Sc} in the brain and generally died at postinoculation intervals very close to the group mean incubation periods (Table 1). In contrast, mock-inoculated 129MV Tg45/152 mice survived to advanced age without clinical or neuropathological signs of prion disease or detectable PrP^{Sc} (data not shown).

Remarkably, a 100% infection rate was seen in vCJD prion-inoculated 129MV Tg45/152 mice (Fig. 1 and Table 1), but without clinical signs of prion disease (subclinical infection), and in 14/15 of these mice we readily detected type 4 PrP^{Sc} in brain (Fig. 2*A*). However, although type 4 PrP^{Sc} is faithfully propagated in 129MV Tg45/152 mice, the associated neuropathology was completely distinct from vCJD. Abundant florid plaques are the neuropathological hallmark of vCJD and to date have been found only in association with BSE or vCJD prion infection in hosts with PrP M129 homozygosity (in humans, primates, or transgenic mice) (9, 12, 27, 31). In contrast, type 4 PrP^{Sc} propagation in 129MV Tg45/152 mice was associated with large nonflorid PrP plaques in the corpus callosum (Fig. 3*D*) accompanied by diffuse synaptic PrP deposition and occasional small nonflorid PrP plaques in the brainstem and thalamus (Fig. 3*G*). Spongiform neurodegeneration was observed only at moderate levels in the cerebellum, brainstem, and thalamus (Fig. 3 *A* and *J*). Despite an exhaustive search, only a single PrP florid plaque was found in the brain of one 129MV Tg45/152 mouse propagating type 4 PrP^{Sc} (Fig. 4*A*). Importantly, the absence of florid plaques in the brain of vCJD-inoculated 129MV Tg45/152 mice is not due to a lack of PrP^{C} expression in the cerebral cortex. Challenge of these mice or the two parental lines (129MM Tg45 and 129VV Tg152 mice) with sporadic CJD isolate I024 produced 100% clinical disease in all lines, with significant spongiosis and typical diffuse synaptic PrP deposition throughout the cerebral cortex and other brain areas (data not shown). Overexpression of PrP M129 in transgenic mouse brain also does not seem to be a prerequisite for generation of florid PrP plaques on challenge with BSE or vCJD prions. We also observed abundant florid PrP plaques in vCJD-inoculated 129MM Tg28 mice, a transgenic line expressing endogenous levels of human PrP M129 (Fig. 4 *B* and *C*). Based on these findings we propose that, in the heterozygous state in 129MV Tg45/152 mice, PrP V129 may have a dominant negative effect on florid PrP plaque formation. Because we saw no evidence for recruitment of PrP V129 to type 5 PrP^{Sc} in vCJD-inoculated 129MV Tg45/152 mice, one possibility is that PrP V129 acts to slow the rate of conversion of PrP M129 to type 4 PrP^{Sc} and that this prevents the evolution of florid PrP plaques leading to the uncoupling of vCJD neu-

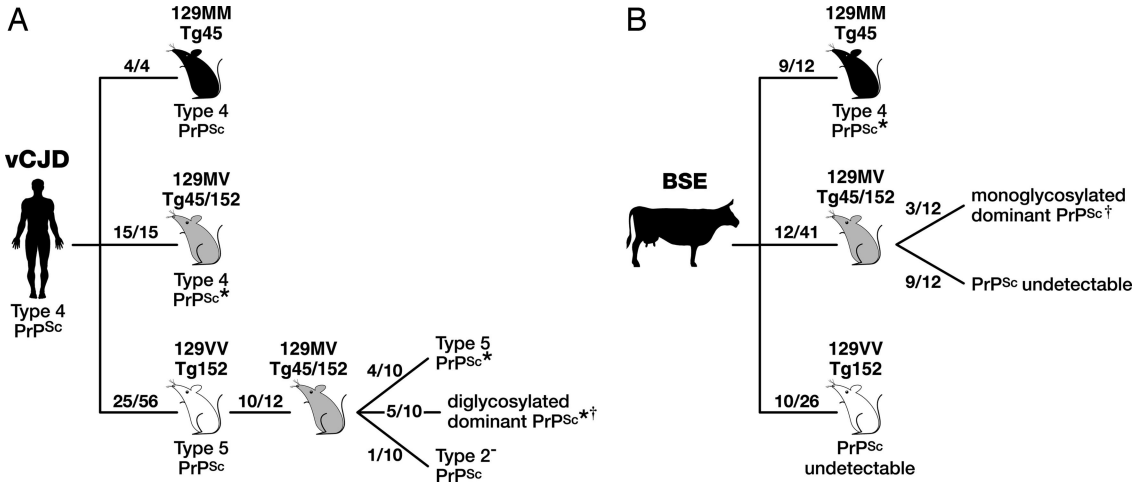

Fig. 1. Summary of transmissions of vCJD (*A*) and BSE (*B*) prions to transgenic mice. The total number of prion-affected mice (both clinically and subclinically infected) is reported for each inoculated group: 129MM Tg45 mice (black), 129VV Tg152 mice (white), and 129MV Tg45/152 mice (gray). Animals were scored by clinical signs, immunoblotting for PrP^{Sc}, and/or PrP immunohistochemistry. Transmissions to 129VV Tg152 and 129MM Tg45 mice have been published (10, 12). In transmissions producing distinct PrP^{Sc} types, the number of samples positive for a particular PrP^{Sc} type is reported as a proportion of the total number of affected mice. *, only subclinical prion infection was seen. †, use of sodium phosphotungstic acid precipitation to detect low levels of PrP^{Sc} precludes precise assignment of PrP^{Sc} type (16).

ropathology from propagation of type 4 PrP^{Sc}. This interpretation is consistent with findings from other transgenic studies showing that the kinetics of PrP^{Sc} propagation from one allelic variant of PrP can be dramatically affected by coexpression of nonhomologous PrP (30, 32, 33).

The absence of type 5 PrP^{Sc} in vCJD-inoculated 129MV Tg45/152 mice is consistent with our previous work indicating that the vCJD prion strain propagates most efficiently from human PrP M129 (with faithful replication of type 4 PrP^{Sc}) (12, 27), whereas a substantial barrier limits propagation of type 5 PrP^{Sc} from human PrP V129 (10, 27). This interpretation was reinforced by direct investigation of the transmission properties of type 5 PrP^{Sc} in 129MV Tg45/152 mice (Table 1 and Fig. 1). Here we observed the presence of a substantial transmission barrier, and, in contrast to type 4 PrP^{Sc}, type 5 PrP^{Sc} did not uniformly breed true in 129MV Tg45/152 mice (Fig. 1 and Table 1). Although 10/12 inoculated mice showed evidence of prion infection, in five subclinically affected mice PrP neuropathology was absent, and high-sensitivity methods (16) were required to demonstrate PrP^{Sc} in brain (Fig. 1 and Table 1). Although the method used precluded accurate molecular typing of this PrP^{Sc} (16) (Fig. 1), PrP^{Sc} typing was possible in the other affected mice that had higher concentrations of PrP^{Sc} in brain. Remarkably, although four subclinically affected mice propagated type 5 PrP^{Sc} (Figs. 1 and 2*B*), the only mouse with clinical prion disease propagated type 2^- PrP^{Sc} (Figs. 1 and 2*B*). Type 2^- PrP^{Sc} is associated with sporadic CJD in human *PRNP* M129 homozygotes (8, 26), but not V129 homozygotes (8). Based on these findings, and because sporadic CJD-like PrP^{Sc} types are seen after secondary passage of type 5 PrP^{Sc} in 129MM Tg35 mice, but not 129VV Tg152 mice (27), we deduce that type 2^- PrP^{Sc} in 129MV Tg45/152 mouse brain is probably generated from PrP M129. The occurrence of a distinct molecular strain type propagated from PrP M129 is consistent with presence of a substantial barrier to propagation of type 5 PrP^{Sc} from PrP V129 in 129MV Tg45/152 mice.

Neuropathologically, propagation of type 5 PrP^{Sc} in 129MV Tg45/152 mice was similar to that seen after secondary passage

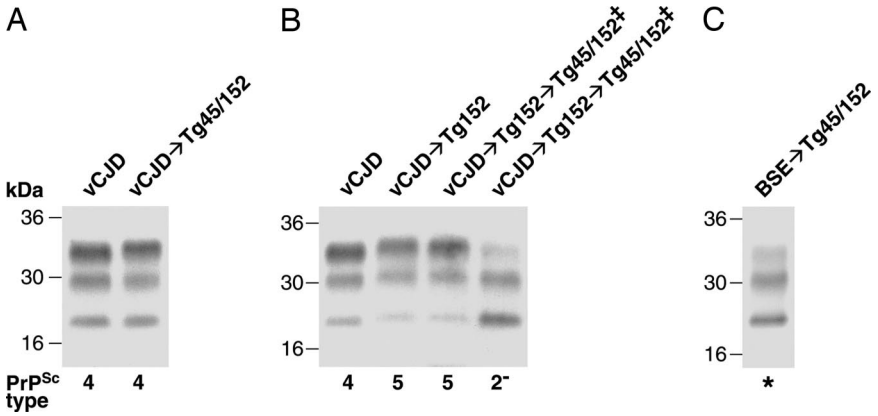

Fig. 2. Molecular strain typing of vCJD and BSE prion transmissions in transgenic mice. (*A* and *B*) Immunoblots of proteinase K-digested brain homogenates from vCJD and vCJD-inoculated transgenic mice. (*C*) Immunoblot of a proteinase K-digested sodium phosphotungstic acid (NaPTA) pellet from transgenic mouse brain homogenate. The provenance of each brain sample is designated above each lane, and the type of PrP^{Sc} detected in each sample is designated below. Immunoblots were analyzed by enhanced chemiluminescence with anti-PrP monoclonal antibody 3F4. ‡, transmissions that result in the propagation of either type 2^- or type 5 PrP^{Sc}. *, use of NaPTA precipitation to detect low levels of PrP^{Sc} precludes precise assignment of PrP^{Sc} type (16).

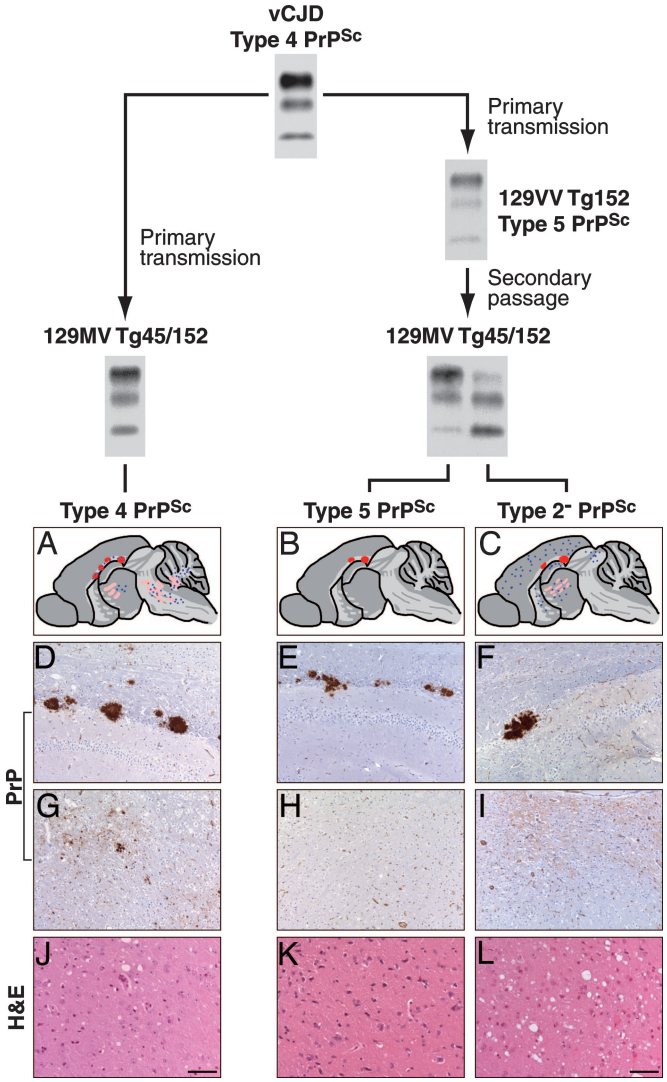

Fig. 3. Neuropathological analysis of transgenic mouse brain. Distinct patterns of neuropathology are seen in 129MV Tg45/152 mice that propagate type 4 PrP^{Sc} (after primary transmission of vCJD prions) or type 5 or 2^- PrP^{Sc} (after secondary transmission of 129VV Tg152-passaged vCJD prions). PrP immunohistochemistry using anti-PrP monoclonal antibody ICSM 35 demonstrates abnormal PrP immunoreactivity comprising nonflorid PrP plaques and diffuse synaptic PrP deposition. Staining with hematoxylin and eosin demonstrates spongiform neurodegeneration. (*A–C*) The regional distribution of spongiosis (blue), diffuse abnormal PrP deposition (pink), and PrP plaques (red). (*D–F*) Corpus callosum. (*G–L*) Thalamus. (Scale bars: 100 μm for *D–L*.)

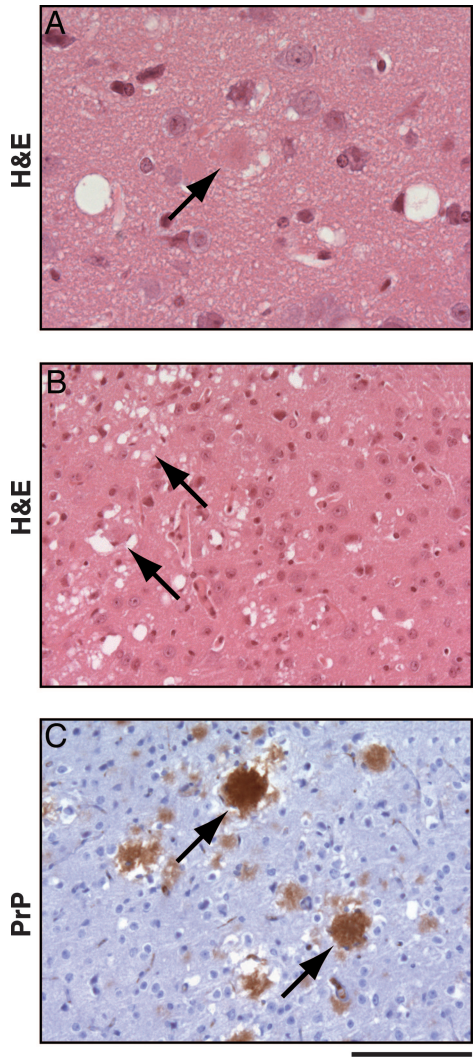

Fig. 4. Neuropathological analysis of transgenic mouse brain. (*A*) Cerebral cortex of a vCJD-inoculated 129MV Tg45/152 mouse propagating type 4 PrP^{Sc} with a single florid plaque (arrow) detected after hematoxylin and eosin staining (H&E). (*B* and *C*) Abundant florid PrP plaques (arrow) in the cerebral cortex of a vCJD-inoculated 129MM Tg28 mouse propagating type 4 PrP^{Sc}. (*B*) Hematoxylin and eosin staining. (*C*) PrP immunohistochemistry using anti-PrP monoclonal antibody ICSM 35. (Scale bar: 200 μm.)

in 129VV Tg152 mice (27) with PrP deposition restricted to nonflorid PrP plaques in the corpus callosum (Fig. 3 *B* and *E*). However, in distinction to 129VV Tg152 mice, no significant spongiosis was detectable throughout the brain (Fig. 3 *B* and *K*). In sharp contrast, type 2^- PrP^{Sc} was associated with widespread spongiosis in the cortex, hippocampus, thalamic nuclei, and tectum (Fig. 3*C*), and extensive synaptic PrP deposition was seen in the thalamus in addition to nonflorid PrP plaques in the corpus callosum (Fig. 3 *C* and *F*). The PrP deposits in the corpus callosum were extracellular, and we did not observe any significant PrP-immunoreactive deposits around blood vessels in any part of the brain examined.

The presence of a substantial transmission barrier and evidence for propagation of distinct prion strain types were also observed after primary transmission of BSE prions to 129MV Tg45/152 mice (Fig. 1 and Table 1). Here only 12/41 BSE-inoculated mice showed evidence of prion infection, and none of the affected mice showed abnormal PrP immunohistochemistry. Nine affected 129MV Tg45/152 mice developed clinical signs of prion disease in the absence of detectable PrP^{Sc} (Fig. 1 and Table 1) similar to primary transmission of BSE prions in 129VV Tg152 mice (10). However, in the absence of abnormal PrP immunohistochemistry or immunoblotting it will be crucial to confirm transmission by secondary passage. Nonetheless, in this regard we previously demonstrated that PrP^{Sc}-negative brain isolates from clinically affected BSE-challenged 129VV Tg152 mice transmit clinical prion disease accompanied by abundant PrP^{Sc} accumulation when inoculated in wild-type FVB mice (27). Thus, clinical prion disease in the absence of detectable PrP^{Sc} is an expected phenotype associated with the interaction of BSE prions and human PrP V129. Notably, the absence of detectable PrP^{Sc} has also been observed in clinically affected BSE-challenged wild-type mice (34).

In the other three affected BSE-inoculated 129MV Tg45/152 mice we detected sporadic CJD-like PrP^{Sc} with a predominance

of monoglycosylated PrP (Fig. 2*C*) similar to findings from primary transmission of BSE prions to 129MM Tg35 mice (12). Collectively, these data suggest that prion propagation from human PrP M129 or V129 is possible in BSE-challenged 129MV Tg45/152 mice. However, we found no evidence for propagation of type 4 PrP^{Sc} or associated vCJD-like neuropathology in these transmissions.

Although caution must be exercised in extrapolating from animal models [even where faithful recapitulation of molecular and pathological phenotypes is possible (12, 27)], our findings suggest that human *PRNP* codon 129 heterozygotes will be susceptible to infection with human-passaged BSE (vCJD) prions but may be less susceptible to primary infection with BSE prions. Depending on the source of the inoculum, multiple disease phenotypes appear possible. Neuropathologically, none of these phenotypes may resemble vCJD, because in the PrP codon 129MV genotype the propagation of type 4 PrP^{Sc} is dissociated from the occurrence of abundant florid PrP plaques. These findings underline the need for continued surveillance and investigation of all forms of human prion disease within the United Kingdom and in other populations with extensive dietary exposure to BSE prions.

Materials and Methods

Generation of Transgenic Mice. Human PrP codon 129MV heterozygous transgenic mice were generated by mating two homozygous lines of transgenic mice, Tg(HuPrP129$M^{+/+}$ *Prnp*$^{o/o}$)-45 mice (12) and Tg(HuPrP129$V^{+/+}$ *Prnp*$^{o/o}$)-152 mice (30). The latter line was originally derived by breeding transgenic HuPrP-V129 Tg152 mice that also expressed mouse PrP (35) with PrP-null mice (36). The heterozygous F_1 offspring were designated Tg(HuPrP129$MV^{+/+}$ *Prnp*$^{o/o}$)-45/152 mice (129MV Tg45/152 mice) and were used in all transmission experiments. RT-PCR, in conjunction with quantitative allele-specific oligonucleotide hybridization, confirmed expression of both V129 and M129 PrP mRNA in 129MV Tg45/152 mice. Immunoblotting confirmed overexpression of human PrP at four to six times that of human brain, and this level of PrP expression is comparable to that seen in the parental lines of transgenic mice (12, 30, 35).

Transmission Studies. All procedures were carried out in a microbiological containment level III facility with strict adherence to safety protocols. Inocula were prepared by using disposable equipment for each inoculum, and inoculations were performed within a class 1 microbiological safety cabinet. BSE tissues were collected under strict aseptic conditions using sterile instrumentation specifically for transmission studies by the United Kingdom Central Veterinary Laboratory (now the Veterinary Laboratories Agency). Four separate BSE inocula, derived from the brainstem of four natural BSE cases (I060, I062, I064, and I066), were studied, together with a pool of brainstems from five natural BSE cases (I038). The BSE pool homogenate (I038) was titrated in RIII wild-type mice at the Veterinary Laboratories Agency and had a titer of $10^{3.3}$ mouse intracerebral LD_{50} units per gram of tissue. These BSE isolates have been used in previous transmission studies (10, 12, 30). Inocula were prepared from the brain of neuropathologically confirmed cases of sporadic CJD and vCJD with consent from relatives and with approval from the Institute of Neurology/National Hospital for Neurology and Neurosurgery Local Research Ethics Committee. Before inoculation the genotype of each transgenic mouse was confirmed by PCR of tail DNA, and all mice were uniquely identified by s.c. transponders. Disposable cages were used, and all cage lids and water bottles were also uniquely identified by transponder and remained with each cage of mice throughout the incubation period. Care of the mice was according to institutional guidelines. Both transgenic and wild-type mice were anesthetized with a mixture of halothane and O_2 and intracerebrally inoculated into the right parietal lobe with 30 μl of a 1% wt/vol brain homogenate prepared in PBS. All mice were thereafter examined daily for clinical signs of prion disease. Mice were killed if exhibiting any signs of distress or once a diagnosis of prion disease was established. The criteria for clinical diagnosis of prion disease in mice were as described in ref. 37.

Neuropathology and Immunohistochemistry. Mice were killed by CO_2 asphyxiation. Brains were fixed in 10% buffered formol saline and then immersed in 98% formic acid for 1 h and paraffin wax-embedded. Serial sections of 4-μm thickness were pretreated by boiling for 10 min in a low-ionic-strength buffer (2.1 mM Tris/1.3 mM EDTA/1.1 mM sodium citrate, pH 7.8) before exposure to 98% formic acid for 5 min. Abnormal PrP accumulation was examined by using anti-PrP monoclonal antibody ICSM 35 (D-Gen, London) on a Ventana automated immunohistochemical staining machine (Ventana Medical Systems, Tucson) by using proprietary secondary detection reagents (Ventana Medical Systems) before development with 3′3 diaminobenzedine tetrachloride as the chromogen. Harris hematoxylin and eosin staining was done by conventional methods. Appropriate controls were used throughout.

Immunoblotting. Ten percent wt/vol brain homogenates were prepared in Dulbecco's PBS (lacking Ca^{2+} or Mg^{2+}) by serial passage through needles of decreasing diameter. Proteinase K digestion (100 μg/ml final protease concentration, 1 h, 37°C), electrophoresis, and immunoblotting were performed as described in ref. 16. Blots were blocked in PBS containing 0.05% vol/vol Tween 20 and 5% wt/vol nonfat milk powder and then probed with anti PrP monoclonal antibody 3F4 (38) in conjunction with an alkaline phosphatase-conjugated secondary antibody and chemiluminescent substrate CDP-Star (Tropix) as described (16). Brain homogenates scored negative for PrP^{Sc} after analysis of 10 μl of 10% brain homogenate were reanalyzed by sodium phosphotungstic acid precipitation of PrP^{Sc} from 250 μl of 10% brain homogenate as described (16). After recovery of PrP^{Sc} by sodium phosphotungstic acid and proteinase K digestion, distinction of differences in glycoform ratios of the three principal amino-terminally truncated PrP fragments is possible; however, this method is not compatible with accurate PrP^{Sc} fragment size analysis used in characterization of prion strain types (16).

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