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## Production of cattle lacking prion protein

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## Abstract

Prion diseases are caused by propagation of misfolded forms of the normal cellular prion protein PrP<sup>C</sup>, such as PrP<sup>BSE</sup> in bovine spongiform encephalopathy (BSE) in cattle and PrP<sup>CJD</sup> in Creutzfeldt-Jakob disease (CJD) in humans<sup>1</sup>. Disruption of PrP<sup>C</sup> expression in mice, a species that does not naturally contract prion diseases, results in no apparent developmental abnormalities<sup>2–5</sup>. However, the impact of ablating PrP<sup>C</sup> function in natural host species of prion diseases is unknown. Here we report the generation and characterization of PrP<sup>C</sup>-deficient cattle produced by a sequential gene-targeting system<sup>6</sup>. At over 20 months of age, the cattle are clinically, physiologically, histopathologically, immunologically and reproductively normal. Brain tissue homogenates are resistant to prion propagation *in vitro* as assessed by protein misfolding cyclic amplification<sup>7</sup>. PrP<sup>C</sup>-deficient cattle may be a useful model for prion research and could provide industrial bovine products free of prion proteins.

To generate PrP<sup>C</sup>-deficient (*PRNP*<sup>−/−</sup>) cattle, we transfected a male Holstein primary fetal fibroblast line 6594 with first and second knockout (KO) vectors (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors)<sup>6</sup> to sequentially disrupt the two alleles of *PRNP*. *PRNP*<sup>−/−</sup> fetal cell lines were established at 40–60 d of gestation and three of the *PRNP*<sup>−/−</sup> fetal cell lines (5211, 5232 and 4296) were recloned to produce calves (Table 1 and Fig. 1a). To verify that the calves possess the *PRNP*<sup>−/−</sup> genotype, we collected ear biopsies and established fibroblast cell lines for genotyping. Genotyping was done by genomic PCR specific to each gene targeting event<sup>6</sup> (primer pairs: neoF7 × neoR7 and puroF14 × puroR14, Fig. 1b), followed by sequence

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## COMPETING INTERESTS STATEMENT

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analysis. Negative PCR analysis<sup>6</sup> was also carried out to confirm the absence of wild-type *PRNP* alleles (primer pairs: BPrPex3F × BPrPex3R, Fig. 1b). All calves born were *PRNP*<sup>-/-</sup>.

To demonstrate functional inactivation of the *PRNP* gene in these calves, we extracted mRNA and protein from the *PRNP*<sup>-/-</sup> fibroblasts. Wild-type calves served as controls. For mRNA expression analysis, we performed RT-PCR<sup>6</sup> (primer pairs: PrPmF3 × PrPmR3, Fig. 1c) and confirmed the disruption of *PRNP*-specific mRNA expression in *PRNP*<sup>-/-</sup> calves. For protein expression analysis, we performed PrP-specific western blot analyses on fibroblasts (Fig. 1d), peripheral blood lymphocytes (Fig. 1e) and brain stem (Fig. 1f) from wild-type and *PRNP*<sup>-/-</sup> calves using the mouse anti-bovine PrP monoclonal antibody F89. We detected PrP-specific bands in the wild-type calves, whereas no reaction was observed in *PRNP*<sup>-/-</sup> calves and negative control mouse fibroblasts. These data clearly demonstrate that the *PRNP* gene is functionally inactivated in the *PRNP*<sup>-/-</sup> calves.

*PRNP*<sup>-/-</sup> cattle were monitored for growth and general health status from birth to 20 months of age. Mean birth weight was 46 kg and average daily gain was 0.91 kg/d to 10 months. Both values were in the normal range for Holstein bulls. Serum chemistry was evaluated at 6 months of age and compared with published reference ranges. All the values for *PRNP*<sup>-/-</sup> calves (*n* = 12) were well within the reference range (Supplementary Table 1) and obvious abnormalities were not observed. General physical examinations included the following parameters: body temperature, heart rate, heart sound, jugular vein distension, respiratory rate, respiratory sound, presence of cough, nasal discharge, eye abnormalities, appetite, general behavior (alert and active, sluggish, hyperactive), gait, posture, joints, hooves, feces (diarrhea, constipation) and genitalia and umbilical cord (dry, enlarged, inflamed, infected). All parameters were normal for all *PRNP*<sup>-/-</sup> cattle (*n* = 12).

At 10 months of age, eight pairs of *PRNP*<sup>-/-</sup> and age-, sex- and breed-matched wild-type control cattle were given an extensive clinical examination (consisting of 122 parameters). These examinations were done according to the diagnostic evaluation of ruminants suspected of transmissible spongiform encephalopathy (TSE) as described in the European TSE guideline “Surveillance and diagnostic of TSEs in ruminants”<sup>8,9</sup>. The clinical evaluation included a general examination of all organic systems and a detailed examination of the nervous system. Examination of the nervous system was focused on the following aspects: (i) evaluation of mental status, studied by observation of animal behavior and reactions to stimulation (approaching, menace, sounds and light); (ii) evaluation of sensory function in limbs and trunk, including study of superficial sensitivity, medular reflexes and conscious proprioception; (iii) evaluation of motor function in limbs and trunk by studying muscular tone, motor irritability (presence of muscle fasciculation and tremor) and gait abnormalities and, finally, (iv) evaluation of cranial nerves by observation of disorders in the corresponding innervated regions.

All animals (*PRNP*<sup>-/-</sup> and control cattle) appeared healthy in the general clinical examination. The nervous system evaluation revealed little change other than a mild increased reaction to external stimulation (menace and sounds) in 3/8 *PRNP*<sup>-/-</sup> cattle compared to 1/8 control cattle.

Blood samples were taken for hematological analysis from five pairs of *PRNP*<sup>-/-</sup> and control cattle matched for age-, sex- and breed, at 10 and 12 months of age. The means for various hematological parameters from the two samples were compared between *PRNP*<sup>-/-</sup>, control cattle and published reference ranges (Supplementary Table 2). *PRNP*<sup>-/-</sup> cattle had slightly lower values for mean corpuscular volume and mean corpuscular hemoglobin compared to wild-type cattle; both groups were low compared to reference values. However, other measures of erythrocyte characteristics were normal for both groups. *PRNP*<sup>-/-</sup> cattle had higher values for white blood cell and neutrophil counts compared to controls, but values for both groups

were well within the reference range. Overall, hematological analysis did not reveal obvious unusual characteristics in *PRNP*<sup>-/-</sup> cattle at 10 or 12 months of age, but further study will be necessary to determine whether the slight differences observed in the knockout cattle might be attributed to disruption of *PRNP* gene function or the presence of the knockout cassettes.

The normal prion protein, PrP<sup>C</sup>, is most abundantly expressed in the central nervous system (CNS) and lymphoid cells, and the propagation and accumulation of PrP<sup>BSE</sup> in the CNS leads to neurodegeneration and prion disease<sup>10,11</sup>. To evaluate the impact of PrP<sup>C</sup> deletion on calf development, we carried out extensive gross and histopathological analyses on two *PRNP*<sup>-/-</sup> and two age-, sex- and breed-matched wild-type cattle at 14 months of age. Representative samples of tissues were evaluated by gross and microscopic examinations. The two groups of cattle were of approximately similar body weights, their carcasses were in good nutritional condition, and no significant lesions were observed on gross examination of organs. At least 14 sections of various areas of the brain (including obex, pons, colliculi, cerebellum, hippocampus, thalamus and cerebral cortex) of each animal were examined by light microscopy. Two sections of spinal cord at cervical, thoracic and lumbar regions were also evaluated by light microscopy. No obvious abnormalities or significant lesions were observed in any tissues in either of the two groups. In particular, no plaques of spongiform tissue or neurodegeneration were detected in the obex and cerebellum of *PRNP*<sup>-/-</sup> cattle (Fig. 2). The cerebellar Purkinje cells of the *PRNP*<sup>-/-</sup> animals at 14 months of age showed no evidence of cell loss (Fig. 2d).

Cells of the immune system play an important role in the pathogenesis of prion diseases, and PrP<sup>C</sup> expression is readily detected in immune cells<sup>12,13</sup>. Therefore, we examined the effects of PrP<sup>C</sup> deficiency on the immune system of *PRNP*<sup>-/-</sup> cattle at 12–13 months of age. We evaluated B-cell and T-cell populations in peripheral blood lymphocytes (PBLs) of *PRNP*<sup>-/-</sup> cattle by flow cytometry. No significant differences were observed in any of these cell subsets between *PRNP*<sup>-/-</sup> and age-, sex- and breed-matched wild-type cattle (Fig. 3a–d; statistical comparison is provided in Supplementary Table 3).

In *Prnp*<sup>-/-</sup> mice, T-cell proliferation and cytokine production induced by T-cell mitogens are significantly affected, suggesting a role of PrP<sup>C</sup> in T-cell function<sup>14,15</sup>. Therefore, PBLs were isolated from *PRNP*<sup>-/-</sup> cattle and stimulated with anti-CD3 antibody, concanavalin A and phytohemagglutinin (PHA). In contrast to *Prnp*<sup>-/-</sup> mice, no significant difference in T-cell proliferation after T-cell mitogen stimulation was observed for *PRNP*<sup>-/-</sup> cattle as compared to similarly treated cells from wild-type cattle (statistical analysis using Student's *t*-test: anti-CD3, *P* = 0.9; Con A, *P* = 0.4; PHA, *P* = 0.7) (Fig. 3f). In addition, no obvious difference between the two groups of cattle for intracellular and secreted interferon-γ (IFNγ) production was observed after T-cell mitogen stimulation (Fig. 3g,h). Finally, to address immune competence of *PRNP*<sup>-/-</sup> cattle *in vivo*, we immunized them with ovalbumin, a T cell–dependent antigen. The ovalbumin-specific humoral immune response in *PRNP*<sup>-/-</sup> cattle was similar to that of the controls (Fig. 3i). Collectively, these data indicate that ablation of PrP<sup>C</sup> expression does not appear to have deleterious effects on the immune systems of cattle.

The *PRNP*<sup>-/-</sup> bulls reached sexual maturity at a normal age and semen was collected from two knockout animals at 16 months of age. Sperm appeared morphologically normal (Supplementary Fig. 1a) and were capable of generating normal-appearing blastocysts (Supplementary Fig. 1b) by *in vitro* fertilization (IVF) with oocytes derived from wild-type cows at an efficiency similar to that of control IVF (Supplementary Table 4). Twelve blastocysts were implanted and eight cows were pregnant at 40 d of gestation. This result indicates that *PRNP*<sup>-/-</sup> sperm appears to be reproductively normal. Future studies will determine whether the blastocysts can produce normal offspring.

To determine whether the absence of endogenous bovine PrP<sup>C</sup> indeed prevents PrP<sup>BSE</sup> propagation *in vitro*, we collected two brain regions (cortex and hypothalamus) from 10-month-old *PRNP*<sup>-/-</sup> cattle for a protein misfolding cyclic amplification (PMCA) assay<sup>7,16-18</sup>. As control substrate for the PMCA assay, CNS tissues from the identical anatomic sites were obtained from an age-, sex- and breed-matched wild-type calf. PMCA was carried out with brain homogenates derived from either *PRNP*<sup>-/-</sup> or wild-type cattle as 'PMCA substrates'; a brain homogenate from a BSE-infected cow was used as the PrP<sup>BSE</sup>-containing inoculum. No propagation of proteinase K (PK)-resistant PrP<sup>BSE</sup> was detected by western blot analysis when brain homogenates from the *PRNP*<sup>-/-</sup> cattle were used as substrates (Fig. 4a,b). In contrast, PrP<sup>BSE</sup> was readily amplified and detected in western blot analysis when brain homogenates from the wild-type cattle were used as substrates (Fig. 4a,b). These results indicate that the presence of endogenous bovine PrP<sup>C</sup> is essential for PrP<sup>BSE</sup> propagation *in vitro* and that any other host-derived cellular cofactors included in the brain homogenates, such as RNA<sup>19</sup> and sulfated glycosaminoglycan<sup>20</sup>, cannot support the *in vitro* PrP<sup>BSE</sup> propagation in the absence of endogenous bovine PrP<sup>C</sup>. We also performed a similar PMCA assay using a brain homogenate from cattle infected with transmissible mink encephalopathy (TME), another prion strain infectious to cattle, as inoculum and detected no propagation of the PrP<sup>TME</sup> (Fig. 4c). This indicates that *PRNP*<sup>-/-</sup> cattle could be resistant to propagation of various prion strains.

In summary, we have demonstrated the usefulness of the sequential gene targeting system to efficiently produce *PRNP*<sup>-/-</sup> cattle. Excluding three animals sacrificed for the histopathological analysis and PMCA assay, all the nine *PRNP*<sup>-/-</sup> cattle have remained healthy for at least 20 months after birth without showing obvious clinical abnormalities. This indicates that 'loss of function' of bovine PrP<sup>C</sup> itself does not cause BSE and that ablation of the normal cellular prion protein PrP<sup>C</sup> function does not adversely affect normal bovine development. It has been reported that evolution has exerted very intense purifying selection on exon 3 of bovine *PRNP*; the *PRNP* gene should have some indispensable function in bovine development because such strong purifying selection is usually seen only for proteins essential to eukaryotic life<sup>21</sup>. Therefore, our findings appear to be of particular interest in supporting a general hypothesis that PrP<sup>C</sup> function is dispensable for normal animal development.

Moreover, brain homogenates from *PRNP*<sup>-/-</sup> cattle were resistant to the *in vitro* propagation of at least two different prion strains, PrP<sup>BSE</sup> and PrP<sup>TME</sup>, by the PMCA method. PMCA has been shown to closely mimic *in vitro* the prion propagation process that occurs *in vivo*, leading to the formation of high quantities of misfolded prion protein that are infectious to wild-type animals<sup>16</sup>. PMCA is described to be at least as sensitive to prion propagation as *in vivo* infection<sup>22</sup> and reproduces the species barrier and prion strain phenomenon typical of the prion infectious agent (J.C. and C.S., unpublished data).

In *Prnp*<sup>-/-</sup> mice, phenotypes vary depending on how the *Prnp* gene locus is disrupted. For example, ataxia and loss of Purkinje cells in aged mice have been observed in some *Prnp*<sup>-/-</sup> mouse strains where, in addition to the *Prnp* open reading frame (ORF), 5' flanking genomic sequences were also deleted, which results in exon skipping between *Prnp* and *Prnd* (located 16 kb downstream of the murine gene *Prnp* and encoding Dpl protein)<sup>23-27</sup>. The cerebellar symptoms in these *Prnp*<sup>-/-</sup> mouse strains were suggested to be caused by ectopic expression of Dpl protein in brain<sup>28</sup>. In the *PRNP*<sup>-/-</sup> cattle described here, only the ORF of the bovine *PRNP* gene was disrupted by insertion of the *neo* and *puro* cassettes without any deletion of *PRNP* genomic sequences, so that any splicing donor/acceptor sites remain intact<sup>6</sup>. It remains to be determined whether or not the bovine *PRND* locus might be affected by the disruption of *PRNP*, but if it occurs, its effect would appear to be minimal because obvious abnormalities, such as ataxia and Purkinje cell loss, were not found in *PRNP*<sup>-/-</sup> cattle up to 20 months of age.

Consistent with our observations in the *PRNP*<sup>-/-</sup> cattle, *Prnp*<sup>-/-</sup> mice with exclusive disruption on the *Prnp* ORF remained healthy<sup>2,3</sup> and showed only slightly abnormal phenotypes, such as altered synaptic function<sup>29,30</sup> and sleep-wake circadian rhythms<sup>31,32</sup>. However, the phenotype in the synaptic function appears to be normal in other murine genetic backgrounds<sup>33</sup>. We have monitored sleep-wake activity in the knockout cattle, along with age-, sex- and breed-matched wild-type controls, at frequent intervals throughout the day and night for one week, but did not observe any obvious alterations. Some other *Prnp*<sup>-/-</sup> mouse models show subtle abnormalities, such as learning differences<sup>34</sup>, and deletion of parts of the murine *Prnp* ORF have more severe effects<sup>35</sup>, suggesting that complete ablation of PrP<sup>C</sup> function as done in this study may have less detrimental phenotypes than the partial manipulation or rearrangement of the *Prnp* ORF.

*PRNP*<sup>-/-</sup> cattle are likely a more relevant model for elucidating PrP<sup>C</sup> function and the basic mechanisms of prion pathogenesis than mice, as cattle are a natural host of prion diseases. In particular, *PRNP*<sup>-/-</sup> cattle allow *in vivo* tests of resistance to prion propagation. We have undertaken such tests, which will require at least 3 years to complete.

*PRNP*<sup>-/-</sup> cattle could be a preferred source of a wide variety of bovine-derived products that have been extensively used in biotechnology, such as milk, gelatin, collagen, serum and plasma. In addition to the *PRNP*<sup>-/-</sup> cattle described here, we have also generated healthy *PRNP*<sup>-/-</sup>*IGHM*<sup>-/-</sup> (ref. 6) double-knockout cattle (Supplementary Fig. 2). This indicates that other genetic modifications can be added to the *PRNP*<sup>-/-</sup> background by means of consecutive rounds of gene modification and recloning, alleviating time-consuming breeding of livestock. Additional genetic modifications to *PRNP*<sup>-/-</sup> background could be useful for production of prion protein-free therapeutic recombinant human proteins, tissue and organs in transgenic livestock for biomedical applications.

Although a ban on feeding cattle ruminant-derived meat-bone meal has greatly reduced BSE infections in cattle, the possibility cannot be completely excluded that some PrP<sup>BSE</sup> strains might have originated from ‘spontaneous’ misfolding of the endogenous PrP<sup>C</sup> protein. This view is supported by recent reports suggesting the presence of atypical PrP<sup>BSE</sup> strains<sup>36–38</sup>. Two cattle recently identified in the United States (Texas and Alabama) appear to show an atypical PrP<sup>BSE</sup> pattern, and one animal with atypical PrP<sup>BSE</sup> characteristics was born after the feed ban<sup>37</sup>. Thus, the ban may not completely alleviate concerns about BSE. The *PRNP*<sup>-/-</sup> cattle produced in this study would prevent BSE due to spontaneous misfolding because of a complete lack of endogenous PrP<sup>C</sup>.

## METHODS

### Embryonic cloning

Cloned fetuses and calves were produced using the chromatin transfer procedure as described<sup>6</sup>. *In vitro* embryo development rate is provided in Supplementary Table 5. All animal work described in this section was done following a protocol approved by the Transova Genetics Institutional Animal Care and Use Committee.

### Generation of *PRNP*<sup>-/-</sup> fetal cell lines

Sequential gene targeting was carried out as described previously<sup>6</sup>. Two types of knockout vectors were used (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors) to sequentially disrupt both alleles of *PRNP*. To provide more specific information on the bovine *PRNP* genomic DNA, we used ~8.3 kb of *Bam*HI-*Bam*HI region (base position 65605–73896 of GenBank accession no. AJ298878) for the 3' homologous arm and ~1.2 kb of *Bgl*III-*Bam*HI region (base position 64494–65604 of AJ298878) for the 5' homologous arm. Male Holstein primary fetal fibroblast line 6594 was electroporated at 550 V and 50 μF with the first knockout vector



(pBPrP(H)KOneo). We screened 94 colonies resistant to G418 (500 µg/ml) by PCR to identify homologous recombinants (primer pair; neoF7 × neoR7) and then homologous recombinants were identified (40/94; 43%). Based on their morphology, we selected seven colonies and cloned embryos to generate fetuses. At 40–60 d of gestation, five fetuses were collected and three of them (2180, 3560-1 and 3560-2) were confirmed to be *PRNP*<sup>+/-</sup> (primer pair; neoF7 × neoR7). The heterozygous *PRNP*<sup>+/-</sup> cell line, 3560-2, was electroporated with the second knockout vector (pBPrP(H)KOpuro), and 182 colonies resistant to puromycin (1 µg/ml) were screened by PCR (primer pair; puroF14 × puroR14) to identify homozygously targeted colonies. Six colonies were identified to be *PRNP*<sup>-/-</sup>, four of which were used for embryonic cloning to generate recloned fetuses. At 40–75 d of gestation, ten fetuses were collected and then fibroblast cell lines were established. All of them were confirmed to be homozygous *PRNP*<sup>-/-</sup> by the targeting event-specific (puroF14 × puroR14 and neoF7 × neoR7) and negative (BPrPex3-F × BPrPex3-R) PCR analyses. We also performed Southern hybridization analysis on *SphI* and *BamHI*-double digested genomic DNA extracted from *PRNP*<sup>-/-</sup> fibroblast cell lines using the coding region of the *neo* or *puro* gene as a probe, which showed the expected band size and a single-site integration of the knockout cassettes.

### RT-PCR

RNA was extracted from *PRNP*<sup>+/-</sup> and *PRNP*<sup>-/-</sup> calf fibroblasts by using RNeasy mini kit (Qiagen), and first strand cDNA synthesis was done by using superscript first-strand synthesis system for RT-PCR (Invitrogen). PCR was done as previously described<sup>6</sup>.

### Western blotting

Protein was extracted from *PRNP*<sup>+/-</sup> and *PRNP*<sup>-/-</sup> calf fibroblasts, peripheral blood lymphocytes and brain stem. The protein content was quantified with Bio-Rad protein assay reagent. Western blot analysis was carried out by running ~75 µg of protein sample on a 12% SDS-PAGE gel under nonreducing conditions. The proteins were transferred to a nitrocellulose membrane and the membrane was stained by the mouse anti-bovine prion protein monoclonal antibody (F 89/160.1.5 from Alexis Biochemicals) as a primary antibody and, second, stained with peroxidase-labeled affinity-purified antibody to mouse IgG (H+L). The stained membrane was developed by ECL plus western blot analysis detection system (Amersham Bioscience) and exposed to Biomax light film by film developer. Detection limit for PrP<sup>C</sup> protein in this western blot analysis was estimated to be ~1.2 µg of brain homogenate from the wild type, which was ~60-fold less than the protein amount (75 µg total brain protein) used in Figure 1.

### Ovalbumin immunization

Four *PRNP*<sup>-/-</sup> and four control wild-type calves 12–13 months old were immunized with ovalbumin antigen (Sigma) at 1 mg/dose formulated with Montanide ISA 25 adjuvant (Seppic) as water-in-oil emulsion. The calves were immunized twice at 3-week intervals (primary immunization followed by first booster after 3 weeks). Vaccine was administered by intramuscular injection (2-ml dose containing 1 mg/ml ovalbumin plus 1 ml of ISA-25 adjuvant) in the neck region. Serum samples were collected before each immunization (V1 and V2) and 7 d and 14 d after each immunization for antibody titer analysis. Blood was drawn into serum separator tubes (tiger-top), allowed to clot and serum was separated by centrifugation. Serum was then divided into 0.5- to 1-ml aliquots and stored frozen until assays were performed. Anti-ovalbumin antibody titers were determined by ovalbumin-specific IgG enzyme-linked immunosorbent assay (ELISA).

### Flow cytometry

Peripheral blood was collected from four *PRNP*<sup>-/-</sup> and four control wild-type calves 12–13 months old by jugular venipuncture into heparinized tubes. Whole white blood cells

(leukocytes) were isolated from heparinized blood using red blood cell lysis (RBC-lysis buffer from Sigma) followed by two washes with PBS. Sheep anti-bovine IgM-FITC (Bethyl Laboratories) was used to label bovine surface IgM (sIgM) on the B cells. Mouse anti-bovine CD21 (Clone MCA1424 from Serotec) antibody or anti-bovine lambda light chain (Clone: BIG501E, VMRD) followed by anti-mouse IgG1-PE secondary antibody (Caltag Laboratories) was used to label surface CD21 marker on bovine B cells. For T-cell analysis, anti-CD3 (Clone MM1A, VMRD), anti-CD4 (Clone IL-A11, VMRD), anti-CD8 (Clone BAQ 111A, VMRD) and anti- $\gamma\delta$  TCR (Clone GB21A, VMRD) monoclonal antibodies were used, followed by fluorochrome labeled isotype-specific secondary antibodies (IgG1-PE; IgG2a-FITC; IgM-PE; IgG2b-TC, purchased from Caltag). Staining was done by a standard protocol and ~10,000 gated lymphocytes were analyzed by FACScan flow cytometer (BD Biosciences).

### ***In vitro* T-cell responses to mitogen stimulation**

Heparinized blood was collected from four *PRNP*<sup>-/-</sup> and four age-, breed- and sex-matched wild-type control calves 12–13 months old, and PBLs were isolated using Ficoll gradient centrifugation. After three washes with sterile HBSS, cells were resuspended in complete RPMI medium (Sigma) with 10% FBS (Hyclone) and cultured with medium only (control), 5  $\mu$ g/ml Con A (Sigma), 2.5  $\mu$ g/ml PHA (Sigma) or 5  $\mu$ g/ml purified anti-CD3 mAb (GB21-A from VMRD) immobilized on culture wells. Separate cultures were set up in triplicate microtiter wells or 48-well plate for proliferation and cytokine assays. For proliferation assays, cultures were pulsed at 48 h and 72 h with 0.5  $\mu$ Ci of <sup>3</sup>H thymidine (Amersham BioSciences) for 4 h and incorporation of <sup>3</sup>H thymidine (proliferation) was measured by liquid scintillation counter as c.p.m. units. For IFN- $\gamma$  ELISA, cultures were left for 72 h and culture supernatant was analyzed for secreted IFN- $\gamma$  protein using calibrated Bovine IFN- $\gamma$  ELISA Kit (MabTech). Intracellular IFN- $\gamma$  production in T cells was measured by dual-color intracellular immunofluorescent staining. Brefeldin and Monensin (Sigma) were added to 48-well cultures at 66 h to stop secretion of cytokines and cells were cultured for a further 6–8 h. Cells were harvested and stained for surface CD3 marker using anti-CD3 monoclonal antibody (GB21-A from VMRD). Cells were then fixed, permeabilized and stained by anti-IFN- $\gamma$ -Biotin antibody and Streptavidin-Tricolor for intracellular IFN- $\gamma$  protein (MabTech). Approximately 10,000 gated lymphocytes were analyzed by FACScan cell sorter (Becton Dickinson) and WinMDI software (Scripps Research Institute).

### **Gross and microscopic examinations**

Four calves (2 wild-type and 2 *PRNP*<sup>-/-</sup> calves) were killed with pentobarbital and were subjected to complete necropsy examinations. Representative samples of skin, nasal turbinate, lung, liver, kidney, spleen, salivary gland, thyroid gland, tonsils (pharyngeal, palatine), thymus, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), aorta, striated muscles (heart, tongue, masseter, diaphragm, triceps, psoas major, biceps femoris), testicle (from two animals), nictitating membrane, sciatic nerve, both trigeminal nerves and ganglia, pituitary gland, spinal cord (cervical, thoracic, lumbar), one eye with its optic nerve and the whole brain were evaluated by gross and microscopic examinations. The calves were of similar body weights and the mean ratio of brain weight (g)/body weight (kg) was 1.10 and 1.25 for *PRNP*<sup>-/-</sup> and the control cattle, respectively. The gyri of the knockout calves were slightly narrower when compared to the controls. The samples were immersion-fixed in 10% neutral buffered formalin. One eye with its optic nerve was immersion-fixed in 2% Bouin's fluid. The fixed brain was cut into 2- to 4-mm wide coronal sections for examination. The fixed tissues were processed for routine histopathology, embedded in paraffin wax, sectioned at 5  $\mu$ m and stained with hematoxylin and eosin for examination by light microscopy. The examination was done by a board-certified, experienced bovine pathologist (A.N.H.) in side-by-side comparisons of the tissues using multiple levels of magnification.

### ***In vitro* fertilization**

Bovine cumulus oocyte complexes (COCs) collected from slaughter house ovaries and matured for approximately 24 h were fertilized in fertilization medium. Fresh semen collected from *PRNP*<sup>-/-</sup> bulls were prepared using Percoll gradient separation method and COCs were cultured with sperm ( $2 \times 10^6$  motile sperm/ml) for 18 h at 39 °C in an atmosphere of 5% CO<sub>2</sub> in air, after which they were stripped by vortexing in TL-HEPES for 2 min to remove the cumulus cells. Fertilized oocytes were cultured similarly to cloned embryos.

### **PMCA procedure**

Ten percent brain homogenates (wt/vol) were prepared from the cortex or hypothalamus of either BSE or TME-affected animals, wild-type or *PRNP*<sup>-/-</sup> cattle. The homogenates were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA and the complete protease inhibitor cocktail from Boehringer Mannheim). The samples were clarified by a brief, low-speed centrifugation (1,500 rpm for 30 s) using an Eppendorf centrifuge, model 5414. Dilutions of this brain homogenate were done in conversion buffer and they are expressed in relation to the brain, for example a 100-fold dilution is equivalent to a 1% brain homogenate. Aliquots of wild-type, *PRNP*<sup>-/-</sup> and BSE or TME brain homogenate prepared in conversion buffer were mixed and either immediately frozen or subjected to 48 cycles of PMCA. For PMCA, tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000) and programmed to perform cycles of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at 60% potency. The detailed protocol, including troubleshooting, has been recently published elsewhere<sup>7,16-18,22</sup>. Samples were incubated with 50 µg/ml of proteinase K (PK) for 60 min at 45 °C. The digestion was stopped by adding electrophoresis sample buffer. Proteins were fractionated by SDS-PAGE under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 antibody (Prionics) diluted 1:5,000 in PBS. The immunoreactive bands were visualized by enhanced chemiluminescence assay (Amersham).

### **Statistical analysis**

Statistical analysis was performed by Student's *t*-test using both confidence interval estimate analysis and *t* score probability hypothesis testing method for two independent sample groups. Both methods of analysis showed that there were no significant differences between *PRNP*<sup>-/-</sup> and WT control cattle groups at  $\alpha$  level of 0.05 and  $P > 0.2$  or 0.1. However, there is a considerable probability of statistical error due to the limited numbers of subjects in this study.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **Acknowledgments**

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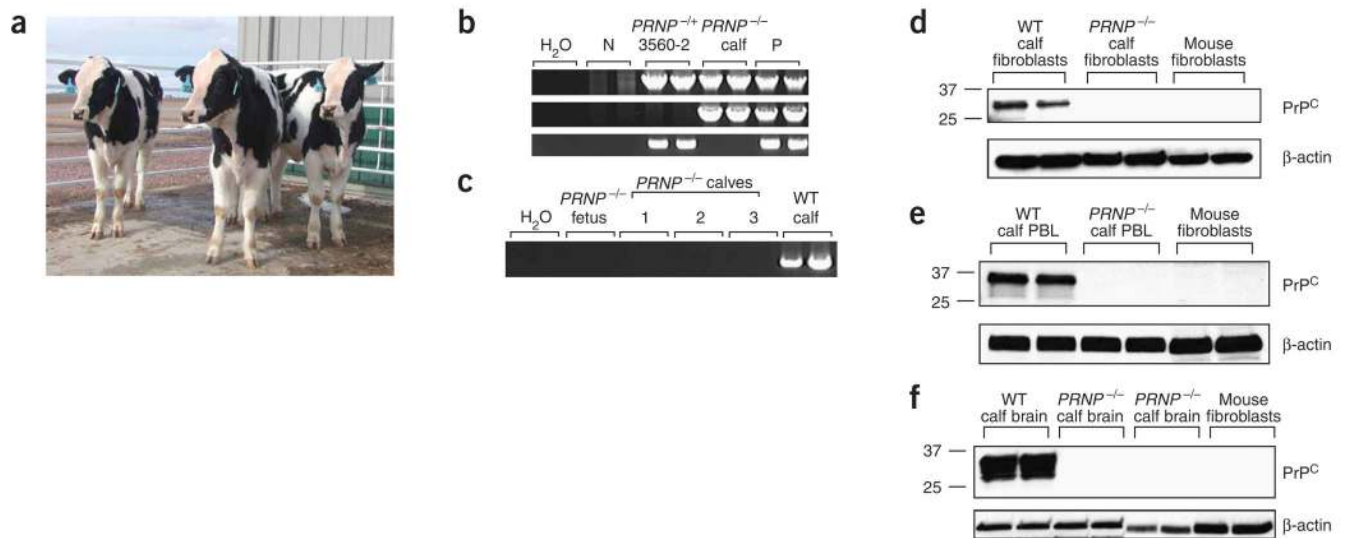
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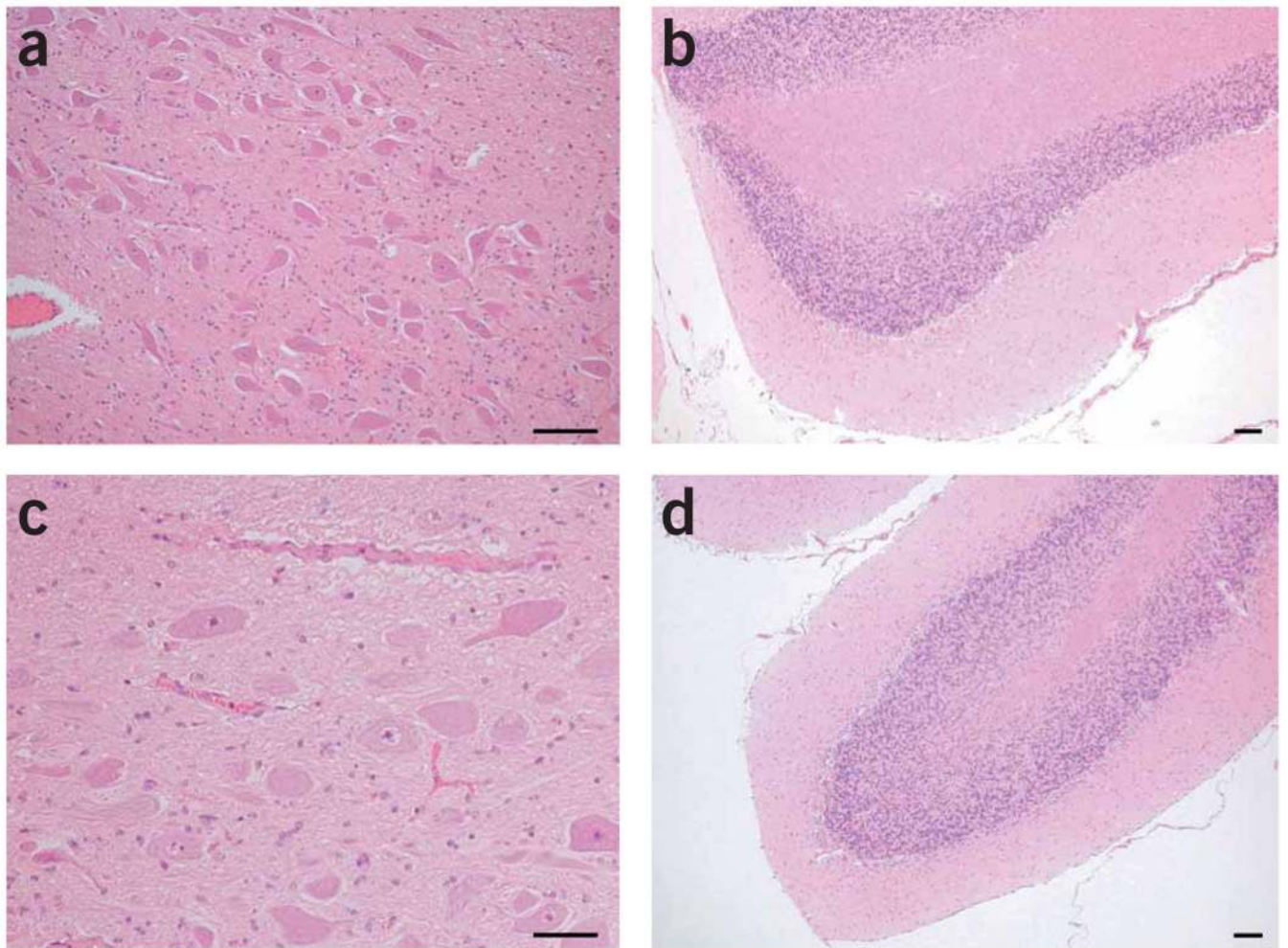
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**Figure 1.**

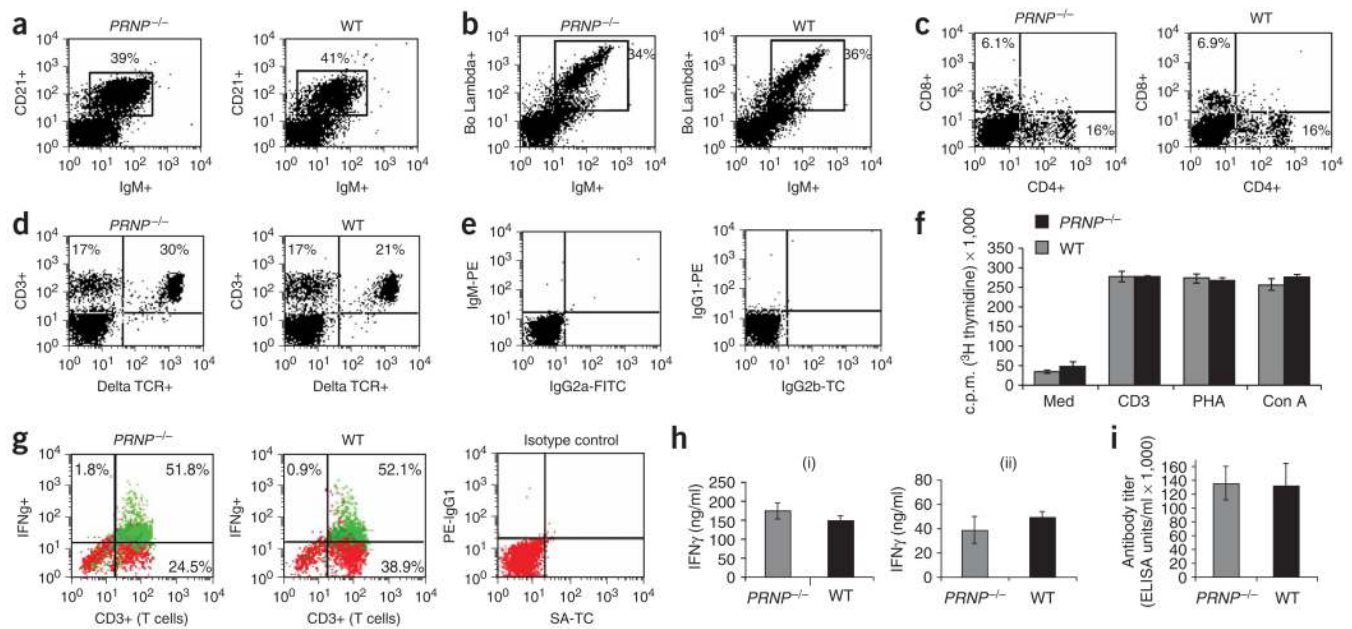
Generation of *PRNP*<sup>-/-</sup> cattle. (a) *PRNP*<sup>-/-</sup> cattle at 13 months of age. (b) Verification of *PRNP*<sup>-/-</sup> genotype in the ear biopsy fibroblasts by genomic PCR. P, positive control<sup>6</sup>; N, negative control<sup>6</sup>. The *PRNP*<sup>-/-</sup> calf is positive with puroF14 × puroR14 (middle) and neoF7 × neoR7 (top) primers, which are specific to the targeting events at both alleles of *PRNP*<sup>6</sup>. The calf is negative with wild-type alleles of *PRNP* amplified with BPrPex3F × BPrPex3R (bottom) primers<sup>6</sup>. (c) RT-PCR analysis on the *PRNP*<sup>-/-</sup> calves shows disruption of mRNA expression in fibroblasts of *PRNP*<sup>-/-</sup> calves. Primers: PrPmF3 × PrPmR3 (ref. 6). (d) Western blot analysis on the *PRNP*<sup>-/-</sup> calf shows absence of PrP<sup>C</sup> protein in fibroblasts. As a positive control, a wild-type (WT) calf was analyzed. As a negative control, protein extracts from mouse fibroblasts were used because the monoclonal antibody used is claimed to be specific to bovine PrP<sup>C</sup> protein. Protein extracts from wild-type calf show the presence of 33–35 kDa of bovine PrP<sup>C</sup> protein in size, but no positive band from the *PRNP*<sup>-/-</sup> calf. Its replica blot was probed with anti-β actin antibody and served as an internal positive control. (e) Absence of PrP<sup>C</sup> protein in peripheral blood lymphocytes (PBLs) of *PRNP*<sup>-/-</sup> calf by western blot analysis. (f) Absence of PrP<sup>C</sup> protein in brain stem of *PRNP*<sup>-/-</sup> calves by western blot analysis.



**Figure 2.**

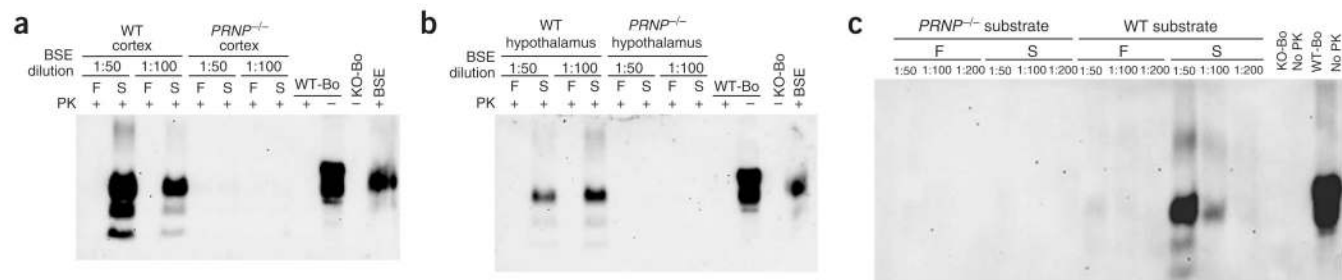
Histopathological analysis of obex and cerebellum of 14-month-old cattle. (a,b) *PRNP*<sup>+/+</sup> dorsal motor nucleus of vagus (a) and molecular layer, granular layer and white matter (b). (c,d) *PRNP*<sup>-/-</sup> dorsal motor nucleus of vagus (c) and molecular layer, granular layer and white matter (d). There are neither plaques of spongiform tissues nor apparent neurodegeneration in the tissues. H & E stain. Scale bars, 100  $\mu$ m.



**Figure 3.**

Comparative analysis of immune system of *PRNP*<sup>-/-</sup> and wild-type (WT) control cattle at 12–13 months old. **(a)** Flow cytometry in peripheral blood lymphocyte (PBL), stained with anti-IgM and anti-CD21 antibodies. **(b)** PBLs stained with anti-IgM and anti-lambda light-chain antibodies. **(c)** PBLs stained with anti-CD4 and anti-CD8 antibodies. **(d)** PBLs stained with anti-CD3 and anti-γδ T cell-receptor antibodies. **(e)** Secondary antibody isotype control staining. **(f)** *In vitro* mitogenic response of T cells in *PRNP*<sup>-/-</sup> and WT cattle. PBLs from four *PRNP*<sup>-/-</sup> and four WT cattle were cultured with medium only (Med) or stimulated with immobilized anti-CD3 monoclonal antibody (CD3), Con A (concanavalin A) or PHA (phytohemagglutinin) mitogens for 48 h and proliferation was measured by <sup>3</sup>H thymidine incorporation. Mean of T-cell response of *PRNP*<sup>-/-</sup> group and WT group cattle and their s.e.m. are shown. No significant differences were found. **(g)** Intracellular cytokine analysis of IFNγ expression in *PRNP*<sup>-/-</sup> and WT control cattle by dual-color flow cytometry. PBLs were stimulated by immobilized anti-CD3 monoclonal antibody for 72 h and intracellular IFNγ production was analyzed by surface CD3 and intracellular IFNγ (positive, green; negative, red) dual-color immunofluorescent staining. Percentage of IFNγ<sup>+</sup> T cells are shown in the upper right quadrant. **(h)** *In vitro* IFNγ production by PBLs in *PRNP*<sup>-/-</sup> and WT cattle. PBLs isolated from four *PRNP*<sup>-/-</sup> and four WT cattle were stimulated by (i) immobilized anti-CD3 monoclonal antibody or (ii) Con A for 72 h and secreted IFNγ in the culture supernatant was analyzed by calibrated bovine IFNγ ELISA. Mean of the IFNγ production (ng/ml) in *PRNP*<sup>-/-</sup> group and WT control cattle and their s.e.m. are shown. Statistical analysis using Student's *t*-test showed no significant difference between *PRNP*<sup>-/-</sup> and WT cattle (*P* = 0.5). **(i)** Humoral immune response to ovalbumin protein antigen in *PRNP*<sup>-/-</sup> and WT cattle. Four *PRNP*<sup>-/-</sup> and four WT cattle were immunized with ovalbumin twice at day 0 (V1) and day 21 (V2) and ovalbumin-specific IgG antibody titers at 7 d after V2 were determined. Mean antibody titers of *PRNP*<sup>-/-</sup> group and WT group cattle and their s.e.m. are shown. Statistical analysis using Student's *t*-test showed no significant difference between *PRNP*<sup>-/-</sup> and WT cattle (*P* = 0.9).





**Figure 4.**  
*In vitro* propagation of PrP<sup>BSE</sup> and PrP<sup>TME</sup> in *PRNP*<sup>-/-</sup> and *PRNP*<sup>+/+</sup> wild-type (WT) cattle brain homogenates. **(a,b)** *In vitro* propagation of PrP<sup>BSE</sup> in 10% homogenates from cortex **(a)** or hypothalamus **(b)**. **(c)** *In vitro* propagation of PrP<sup>TME</sup> in 10% homogenates from cortex **(c)**. The pathological form of the prion protein, PrP<sup>BSE</sup> or PrP<sup>TME</sup>, in the inoculum, was derived from BSE- or TME-affected cattle, respectively. We used 1:50, 1:100 and 1:200 dilutions of the infectious material. Samples were either frozen immediately after mixture (F) or subjected to 48 PMCA amplification cycles (S). The appearance of PrP<sup>BSE</sup> or PrP<sup>TME</sup> was assessed by western blot analysis after proteinase K (PK) digestion. Samples from *PRNP*<sup>+/+</sup> wild-type (WT-Bo), *PRNP*<sup>-/-</sup> (KO-Bo) cattle (substrates) and the BSE-positive control brain homogenate (inoculum) are shown for comparison with and without PK treatment.

Table 1

Production of cloned calves from *PRNP*<sup>-/-</sup> fibroblast cell lines

Cell line ID	Embryos implanted	Recipients	Pregnant at (%) <sup>a</sup>			Live animals at 6 months (%) <sup>a</sup>
			40 d	90 d	150 d	270 d
5211	45	30	17 (38)	7 (16)	7 (16)	6 (13)
5232	21	21	7 (33)	4 (19)	4 (19)	3 (14)
4296	19	19	9 (47)	7 (37)	6 (32)	5 (26)
Total	85	70	33 (47)	18 (26)	17 (24)	14 (20)

<sup>a</sup> Percentages were calculated by dividing the number of fetuses or calves by that of embryos implanted.