Overexpression of chimaeric murine/ovine PrP (A$_{136}$H$_{154}$Q$_{171}$) in transgenic mice facilitates transmission and differentiation of ruminant prions

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Development of transgenic mouse models expressing heterologous prion protein (PrP) has facilitated and advanced in vivo studies of prion diseases affecting humans and animals. Here, novel transgenic mouse lines expressing a chimaeric murine/ovine (Mu/Ov) PrP transgene, including amino acid residues alanine, histidine and glutamine at ovine polymorphic codons 136, 154 and 171 (A$_{136}$H$_{154}$Q$_{171}$), were generated to provide a means of assessing the susceptibility of the ovine AHQ allele to ruminant prion diseases in an in vivo model. Transmission studies showed that the highest level of transgene overexpression, in Tg(Mu/OvPrP AHQ )EM16 (EM16) mice, conferred high susceptibility to ruminant prions. Highly efficient primary transmission of atypical scrapie from sheep was shown, irrespective of donor sheep PrP genotype, with mean incubation periods (IPs) of 154–178 days post-inoculation (p.i.), 100% disease penetrance and early Western blot detection of protease-resistant fragments (PrP$_{res}$) of the disease-associated isoform, PrP$_{Sc}$, in EM16 brain from 110 days p.i. onwards. EM16 mice were also highly susceptible to classical scrapie and bovine spongiform encephalopathy (BSE), with mean IPs 320 and 246 days faster, respectively, than WT mice. Primary passage of atypical scrapie, classical scrapie and BSE showed that the PrP$_{res}$ profiles associated with disease in the natural host were faithfully maintained in EM16 mice, and were distinguishable based on molecular masses, antibody reactivities and glycoform percentages. Immunohistochemistry was used to confirm PrP$_{Sc}$ deposition in brain sections from terminal phase transmissible spongiform encephalopathy-challenged EM16 mice. The findings indicate that EM16 mice represent a suitable bioassay model for detection of atypical scrapie infectivity and offer the prospect of differentiation of ruminant prions.

INTRODUCTION

Prion or transmissible spongiform encephalopathy (TSE) diseases are a group of fatal neurodegenerative diseases affecting man and animals, and include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans. A fundamental feature of TSE pathogenesis, as proposed in the prion hypothesis (Prusiner, 1982, 1991), is the post-translational conformational change of the normal, host-encoded cellular prion protein (PrP$^{C}$) (Oesch et al., 1985) to an abnormal, disease-associated isoform (PrP$^{Sc}$) (Prusiner, 1989), which accumulates in the central nervous system of affected hosts causing spongiform changes in the brain, eventually leading to death.

In sheep, genetic susceptibility to naturally occurring scrapie is linked to at least three polymorphisms in the ovine PrP-encoding gene (PrP, Prnp) (Goldmann et al., 1990; Hunter et al., 1997). Typically, valine (V) or alanine (A) at codon 136, together with arginine (R) at 154 and glutamine (Q) at 171, confer greater susceptibility to classical scrapie, as in PrP$^{VQR}$ and PrP$^{ARQ}$, whereas AHQ (H, histidine) and ARR residues at the three codons are associated with relative
resistance (Dawson et al., 1998). Nor98/atypical scrapie is a recently identified prion disease of small ruminants (Benestad et al., 2003) occurring in several countries (Benestad et al., 2008), predominantly in sheep. PrP genotypes associated with relative resistance to classical scrapie. Ovine PrP alleles AHQ and AF141RQ (F, phenylalanine) are strongly associated with atypical scrapie susceptibility (Moum et al., 2005; Saunders et al., 2006; Arsac et al., 2007; Lühken et al., 2007). There is also a strong association of H154 with the disease in goats (Colussi et al., 2008). The origin of atypical scrapie and whether it is infectious or spontaneous under natural conditions is unknown.

Rapid and confirmatory diagnosis of TSEs utilizes detection of PrPSc or protease-resistant PrPSc fragments (PrPres) by biochemical or immunohistochemical methods. However, bioassays in animal hosts are required to show prion infectivity and determine infectious titres by endpoint titration. The disadvantages of bioassays in natural hosts are that they are expensive and time-consuming. Standard bioassay models such as WT mice are inefficient and affected by transmission barriers due, at least in part, to a lack of homology between donor PrPSc and recipient PrPC (Prusiner et al., 1990), leading to prolonged incubation periods (IPs) and incomplete disease persistence. Failure of transmission may occur, as exemplified by inoculation of WT mice with atypical scrapie (Le Dur et al., 2005; Arsac et al., 2009; Griffiths et al., 2010). However, transgenic expression of heterologous PrPC can reduce or overcome transmission barriers and provide improved prion disease models, as shown in mice overexpressing hamster PrPC, whereby IPs were inversely proportional to transgene expression levels in brain (Prusiner et al., 1990). Transgenic models expressing other mammalian PrPs have been produced (Groschup & Buschmann, 2008), offering the possibility of faster, more economical bioassays, which may be more sensitive to prion detection than natural hosts.

Transgenic mice expressing ovine PrPVRQ (tg338, Laude et al., 2002) or PrPRM3 (TgOvPrP4, Crozet et al., 2001; TgshpXI, Kupfer et al., 2007) proteins on murine PrP-null backgrounds showed markedly reduced IPs with sheep classical scrapie compared with WT mice (Crozet et al., 2001; Vilotte et al., 2001; Arsac et al., 2009) and enhanced transmissions of atypical scrapie (Le Dur et al., 2005; Arsac et al., 2009; Griffiths et al., 2010). Compared with the ancestral ARQ allele, the AHQ allele occurs at a low frequency in UK sheep (Goldmann et al., 2005). The influence of the AHQ allele on scrapie susceptibility or resistance varies among breeds (Billinis et al., 2004). Although susceptible to experimental scrapie (Vaccari et al., 2007) and BSE (Foster et al., 2001), the risk of natural scrapie in AHQ/AHQ sheep in the UK has been reported as low/moderate (Baylis et al., 2004; Tongue et al., 2006). Naturally occurring cases (Benestad et al., 2003) and experimental transmission (Simmons et al., 2007) of atypical scrapie have been identified in AHQ/AHQ homozygotes. Here, we report the generation of transgenic mice overexpressing murine/ovine PrP^AHQ [Tg(Mu/OvPrP^AHQ)] and their assessment as a novel bioassay model for improved detection and differentiation of ruminant prions.

RESULTS

Generation and characterization of transgenic PrP mice

Microinjection of FVB/N embryos with the chimeraic PrP transgene (Fig. 1) produced 15 founder mice. Following transgene introgression onto a PrP-null mouse background to facilitate prion challenges, three lines producing stable transgene transmission were selected: Tg(Mu/OvPrP^AHQ)EM16 (EM16) and Tg(Mu/OvPrP^AHQ)EF50 (EF50) mice were bred to transgene homozygosity, whilst Tg(Mu/OvPrP^AHQ)EM52 (EM52) mice could only be bred as hemizygotes. Western blotting was used to estimate Mu/OvPrP^AHQ protein expression levels in the brains of 12-week-old mice. Homozygous EM16 and EF50 mice, and hemizygous EM52 mice, showed estimated expression levels of 8–16-fold, 2–4-fold and 0.5–1.0-fold, respectively, compared with normal ovine brainstem (Fig. S1a–c, available in JGV Online). Southern blot hybridization confirmed the presence of transgenic Prnp in transgene PCR-positive mice, and was used to estimate transgene copy numbers as 19 ± 1, 18.5 ± 0.5 and 2.5 ± 0.5 for EM16, EF50 and EM52 mice, respectively, compared with PrP-null mouse DNA spiked with known copies of the ovine/murine insert (Fig. S1d). Mice of the three established lines showed apparently normal behaviour and breeding, with no signs of spontaneous disease. In a group of nine uninfected EM16 mice aged 1–2 years, which remained healthy, six mice were examined by Western blot (WB) and showed an absence of PrPres (Fig. S1e).

TSE transmissions to Tg(Mu/OvPrP^AHQ) mice

Primary transmission of atypical scrapie, classical scrapie and BSE produced a progressive neurological disease in affected Tg(Mu/OvPrP^AHQ) mice, including clinical signs of nervousness, rough coat, weight loss and affected gait, leading in the terminal phase to hunched posture, severe weight loss and a prominent hind-limb ataxia. The development of clinical signs in affected scrapie- and BSE-challenged C57BL/6 mice was similar to that in EM16 mice but occurred at a slower rate. Survival curves showed transmission of five atypical scrapie isolates to EM16 mice (Fig. 2a) with relatively uniform mean IPs of 154–178 days post-inoculation (p.i.) and 100 % disease penetrance (Table 1), highlighting the susceptibility of this line to atypical scrapie irrespective of donor sheep PrP genotype. This could suggest similar levels of prion infectivity in the five inocula; however, ELISA results indicated variations in PrPres content (Table S1). As noted by Andréoletti et al. (2011), discrepancies between infectivity and ELISA titres could result from inefficient PrPres recovery during sample processing. Comparison of survival curves of atypical
scrapie-infected EM16 mice revealed no significant difference between four isolates (AHQ/AHQ-10, AHQ/AHQ-14, AHQ/AHQ-22 and ARR/ARR-11) \( (P = 0.29) \), with higher (varying) ELISA values (Table S1) and shorter IPs (Table 1), whereas isolate ARR/ARR-13, which showed low ELISA and WB signals (Fig. S2), transmitted with a significantly longer IP \( (P = 0.0061) \), which was most likely due to lower prion titre than to genotype effect. Isolate ARR/ARR-15, which produced higher ELISA and WB signals (Fig. S2), transmitted to lower-expression EF50 mice with a comparatively fast IP (Table S2).

The effects of transgene expression level were shown in the transmission of atypical scrapie ARR/ARR-11 to homozygous and hemizygous EM16 mice (Fig. 2b). A significant difference in mean IPs at 154 and 379 days p.i., respectively, was seen \( (P = 0.0034) \), with no effect on susceptibility (100 % in both groups). Primary and secondary passage of AHQ/AHQ-10 in EM16 mice produced similar mean IPs (Table 1), with similar onset of clinical signs, suggesting the lack of a transmission barrier to sheep atypical scrapie on primary isolation. However, potential differences in prion infectivity titres between the sheep and mouse-passaged inocula, not quantified here, could be contributory. In the higher-expression EM16 and EF50 lines, challenge with the same atypical scrapie isolates (AHQ/AHQ-10 and AHQ/AHQ-14; Tables 1 and S2) revealed a greater influence of brain expression level on IP (faster by 14–27 % in EM16) than on susceptibility (no difference, both at 100 %), whereas lower-expression-level EM52 mice challenged with atypical scrapie isolates (ARR/ARR-13 and AHQ/AHQ-14) did not succumb to disease (Table S2).

WT C57BL/6 mice were challenged with scrapie and BSE but not atypical scrapie, as the latter does not transmit to conventional mice (Le Dur et al., 2005; Arsac et al., 2009; Griffiths et al., 2010). The scrapie pool (derived from multiple donor sheep PrP genotypes; Table S1) transmitted to all three Tg(Mu/OvPrP AHQ) lines with faster mean IPs than to C57BL/6 mice (Fig. 2c, Tables 1 and S2), with a clear ordered trend of survival of EM16, EF50, EM52, C57BL/6 mice \( (P < 0.0001) \). The higher expression level in EM16 mice compared with EF50 mice showed a greater effect on scrapie IP [faster by 104 days (27 %) in EM16] than on susceptibility (no difference, both at 100 %). BSE transmitted efficiently to EM16 mice compared with prolonged, less-efficient transmissions to EM52 and C57BL/6 mice (Fig. 2d, Tables 1 and S2); the survival curves revealed a significant difference \( (P = 0.0002) \). As indicated by SEM values (Table 1), EM16 mice showed less variability in IP ranges with atypical scrapie than with scrapie or BSE.

**WB of TSE-challenged EM16 mice**

Following transmission of five atypical scrapie isolates, PrP\(^{res}\) was detected in the brains of 100 % of EM16 mice by

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Fig. 1. Schematic representation of the Mu/OvPrP\(^{AHQ}\) transgene. (a–e) The Prnp coding sequence is shown for mouse (Mu; shaded box) and sheep (Ov; hatched box). I1 and I2 are introns 1 and 2. The relative positions of specific restriction sites (Smal, SalI, NotI and PstI) are shown. (a) WT mouse Prnp gene sequence. (b) In the transgene, exons 2 and 3 are fused; E3 is chimaeric and composed of mouse (nt 1–117) and sheep (nt 118–762, including the stop codon; transgene numbering) sequences. Relative positions of amino acid residues A, H and Q (at codons 136, 154 and 171; sheep numbering) in the sheep PrP ORF. (c) Overlapping PCR product. (d) The sheep DNA probe specific for sheep E3 sequence. (e) In PrP-null mice, E3 was disrupted by homologous recombination with a neomycin-resistance cassette (TK/neo; dotted box) (Büeler et al., 1992). 3′UTR, 3′untranslated region.
WB. PrP\textsuperscript{res} profiles detected by mAb Sha31 were similar, irrespective of donor sheep PrP genotype, consisting of five bands of estimated molecular masses of ~31, 28, 24, 18 and 8–11 kDa, as shown for nine mice inoculated with ARR/ARR-13 (Fig. 3a), which was consistent with reports of atypical scrapie in sheep (Gretzschel et al., 2006; Klingeborn et al., 2006; Arsac et al., 2007) and on transmission to transgenic mice (Le Dur et al., 2005; Arsac et al., 2009; Griffiths et al., 2010). Masses similar to the prominent 8–11 kDa fragment (which possibly consists of two separate unglycosylated peptides at 5 and 8 kDa, as described by Götte et al., 2011) were absent from the scrapie and BSE PrP\textsuperscript{res} profiles (Fig. 3a). In a time-course infection of AHQ/AHQ-22 in EM16 mice, the first detection of PrP\textsuperscript{res} by WB in subclinical phase mouse brain was at 110 days p.i. (Fig. S3), 44 days before the mean IP of terminal disease.

Primary transmission of atypical scrapie, classical scrapie and BSE to EM16 mice produced distinct PrP\textsuperscript{res} profiles allowing differentiation of the three classes of TSE agent (Fig. 3). PNGase treatment of transmitted atypical scrapie (AHQ/AHQ-10 and ARR/ARR-11) PrP\textsuperscript{res} (Fig. 3b, c, lanes 2 and 4, respectively) revealed similar, distinct deglycosylated bands at 18 and 8–11 kDa (faintly at 23 kDa), as reported previously (Arsac et al., 2007), whereas removal of carbohydrate residues produced dominant bands at 21 kDa with scrapie (Fig. 3b, c, lane 6) and 19 kDa with BSE (Fig. 3b, lane 8). Mab Sha31 (Fig. 3b) and P4 (Fig. 3c) reactivities to PrP\textsuperscript{res} confirmed the differences between the TSEs transmitted to EM16, suggesting distinct strains. P4 reactivity to the scrapie PrP\textsuperscript{res} triplet (Fig. 3c, lane 11) and to the unglycosylated 21 kDa band (Fig. 3c, lane 12) but not to the BSE triplet or unglycosylated band (Fig. 3c, lanes 9–10) in the natural host cases was reproduced in the transgenic model (Fig. 3c, lanes 5–6 and 7–8). Glycoform quantification revealed that the mean percentages of PrP\textsuperscript{res} fragments produced by the three classes of TSE agent passaged in EM16 mice were similar to natural cases of each (Table S3) and were distinct.

**Neuropathology of TSE-challenged EM16 mice**

A subset of brains from terminal phase EM16 mice inoculated with atypical scrapie AHQ/AHQ-10 (n=6) and ARR/ARR-13 (n=7), classical scrapie (n=3) and BSE (n=3) were examined by histopathology and immunohistochemistry (IHC) as further confirmation of prion transmission. TSE-related vacuolation was identified in...
Haematoxylin and eosin (H&E)-stained sections derived from all of the examined brains, which were also analysed by IHC to detect PrPSc deposition. EM16 mice inoculated with atypical scrapie demonstrated prominent vacuolation in the cerebral cortex grey matter and in the white matter, which was distinguishable from classical scrapie- or BSE-inoculated EM16 mice. However, based on the small number of mice examined here (and on results from ongoing projects; unpublished observations), it was not possible to distinguish scrapie and BSE based on the intensity and distribution of vacuolation. Atypical scrapie-challenged EM16 mice displayed granular deposits and small aggregates distributed in the cerebral cortex grey matter, mainly along layers V and VI. The thalamus and basal ganglia were also affected. In the hippocampus, PrPSc deposits appeared more aggregated and were delineated along the hippocampal fissure and corpus callosum (Fig. 4a, b). PrPSc was deposited along white matter structures, such as the corpus callosum, alveus and external capsule. In cerebellar cortex, only the molecular layer was affected by dense, granular, multifocal deposits; cerebellar nuclei were unaffected. No deposits were detected in the midbrain and medulla, with the possible exception of the locus coeruleus.

The classical scrapie pool produced fine granular, punctate, intraglial and intraneuronal PrPSc deposits in the medulla, midbrain, thalamus and basal ganglia of EM16 mice. PrPSc deposits were also prominent in the hippocampus, mainly in the CA2 area (Fig. 4c, d). The cerebral cortex was also affected, albeit to a lesser extent. In the cerebellar cortex, granular deposits were detected scantily in the granular layer and white matter; cerebellar nuclei were also affected. In BSE-inoculated EM16 mice, granular deposits, PrPSc aggregates and plaques were prominent in the medulla and midbrain, with increasing intensity in the tegmentum and thalamus. The cerebral cortex and hippocampus were also affected by similar PrPSc types, albeit with decreased intensity.

### Table 1. Transmission of TSE agents to EM16 and C57BL/6 mice

<table>
<thead>
<tr>
<th>TSE inoculum/genotype</th>
<th>Transgenic EM16</th>
<th>WT C57BL/6</th>
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<tbody>
<tr>
<td></td>
<td>IP ± SEM</td>
<td>n/total*</td>
</tr>
<tr>
<td><strong>Atypical scrapie</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHQ/AHQ-10</td>
<td>164 ± 5</td>
<td>8/8</td>
</tr>
<tr>
<td>AHQ/AHQ-10 (2nd)‡</td>
<td>162 ± 3</td>
<td>5/5</td>
</tr>
<tr>
<td>AHQ/AHQ-14</td>
<td>166 ± 7</td>
<td>3/3</td>
</tr>
<tr>
<td>AHQ/AHQ-22</td>
<td>154 ± 4</td>
<td>11/11</td>
</tr>
<tr>
<td>ARR/ARR-11</td>
<td>154 ± 2</td>
<td>5/5</td>
</tr>
<tr>
<td>ARR/ARR-11§</td>
<td>379 ± 25</td>
<td>5/5</td>
</tr>
<tr>
<td>ARR/ARR-13</td>
<td>178 ± 3</td>
<td>9/9</td>
</tr>
<tr>
<td><strong>Classical scrapie</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scapie pool-06</td>
<td>296 ± 21</td>
<td>9/9</td>
</tr>
<tr>
<td>Scapie pool-06l</td>
<td>282 ± 12</td>
<td>8/8</td>
</tr>
<tr>
<td>BSE</td>
<td>363 ± 20</td>
<td>10/10</td>
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</table>

* n/total, number of mice with confirmed TSE disease (WB PrPres positive)/number of mice inoculated.
† Data from Griffiths et al. (2010); cross-reference to inocula codes given in Table S1.
‡ 2nd, Secondary passage (AHQ/AHQ-10 in EM16 only).
§ EM16 mice inoculated in this group were transgene hemizygous; all other EM16 mice inoculated in the study were transgene homozygous. || Mice inoculated by combined i.c. and i.p. routes (scapie pool-06 only); all other mice were inoculated by the i.c. route.

### Fig. 3. WB of TSE-challenged EM16 mice. (a) PrPres detection in nine of nine EM16 mice challenged with atypical scrapie ARR/ARR-13 at 167–186 days p.i. (lanes 1–9), uninfected EM16 brain (lanes 10 and 11), and sheep atypical scrapie, cattle BSE, sheep classical scrapie (lanes 12–14; TSE PrPres controls). (b, c) Differentiation of prion agents: atypical scrapie AHQ/AHQ-10 (lanes 1 and 2) and ARR/ARR-11 (lanes 3 and 4), classical scrapie (lanes 5 and 6) and BSE (lanes 7 and 8) in EM16 mice by reactivity with mAb Sha31 (b) or P4 (c). Controls were bovine BSE (lanes 9 and 10), sheep scrapie (lanes 11 and 12) and uninfected EM16 brain (lanes 13–16). PK, proteinase K; PNG, peptide N-glycosidase; M, molecular mass marker (kDa).
intensity (Fig. 4e, f). In the cerebellar cortex, granular deposits were detected in the granular layer and to a lesser extent in white matter; cerebellar nuclei were also affected. Intraneuronal deposits were detected in large neurons, mainly in the medulla.

**DISCUSSION**

Historically, infectivity studies in WT mouse lines have revealed variable transmission efficiencies for TSE agents such as classical scrapie and BSE, often producing prolonged IPs and low attack rates, as well as failure of transmissibility of atypical or certain classical scrapie strains (Griffiths et al., 2010; Thackray et al., 2012). We generated transgenic mice expressing chimaeric Mu/ OvPrP<sup>AHQ</sup> protein on a mouse PrP-null background and found that mice expressing the highest level (EM16) showed 100% disease susceptibility with the three classes of TSE, producing shorter IPs (± SEM) compared with lower expression or WT mice. The absence of spontaneous disease in uninfected transgenic mice indicated that expression of the mature chimaeric PrP produced no adverse effects.

**Fig. 4.** IHC of brain sections from TSE-challenged EM16 mice. The sections (left panels – a, c and e) and corresponding insets (right panels – b, d, and f) show detection of PrP<sup>Sc</sup> deposits in the hippocampus region of mice challenged with: atypical scrapie AHQ/AHQ-10 (a, b); classical scrapie (c, d); and BSE (e, f). The scale bar represents 200 μm in panels (a), (c) and (e), and 100 μm in panels (b), (d) and (f). cc, corpus callosum; Ctx, cerebral cortex; hf, hippocampal fissure; Hip, hippocampus; Th, thalamus.
High susceptibility and reduced IPs with atypical scrapie

Mu/OvPrPAHQ protein overexpression, particularly in EM16 mice, facilitated the highly efficient transmission of atypical scrapie from sheep of different PrP genotypes, including early detection in subclinical infection. The effects of expression level on susceptibility to atypical scrapie were shown: lower expression EM52 mice were not susceptible to atypical scrapie, but were susceptible to scrapie and BSE. This finding is in agreement with the demonstration that PrP expression levels in TgOvPrP4 mice exhibited a greater influence on transmission and survival periods in atypical scrapie infection than with scrapie or BSE (Arsac et al., 2009). The inverse correlation between brain expression level and IP was shown by rapid transmission of atypical scrapie to EM16 homozygotes, with longer IPs in hemizygotes. Primary passage of atypical scrapie isolates in EM16 mice revealed similar, if not identical, PrPres profiles, and no clear differences by IHC.

We found that four atypical scrapie isolates (AHQ/AHQ-10, AHQ/AHQ-14, ARR/ARR-11 and ARR/ARR-13) transmitted to EM16 mice with significantly lower mean IPs by 45–54 days (~25 % reduction) compared with tg338 mice, although attack rates were similar; with three of the inocula, EM16 mice revealed an ~50 % reduction in mean IPs and an overall higher attack rate compared with TgshpXI mice (Table S4; Griffiths et al., 2010). Whether abbreviated IPs reflect a higher sensitivity of EM16 mice to atypical scrapie, which could be due to the polymorphisms at PrP codons 136 and/or 154, is unknown. The rapid response and high susceptibility of EM16, which may not be explained by PrP homology alone, suggests that the line could be a useful bioassay model for detection of atypical scrapie infectivity.

Improved bioassay performance compared with WT mice

Higher expression levels in Tg(Mu/OvPrPAHQ) mice also correlated with improved responses to scrapie and BSE compared with C57BL/6 mice. EM16 mice exhibited greater susceptibility (100 % for both TSEs) and reductions in mean IPs of 54 % with scrapie and 40 % with BSE. It is unknown whether differences in the IPs of the two inocula in EM16 were due to differences in prion titres (Table S1) or other factors, including sensitivity of the mice and/or species-specific effects, as in potentially less-efficient BSE transmission to a new host (transgenic Mu/OvPrPAHQ).

Interestingly, heterozygosity for the AHQ allele in sheep combined with more susceptible alleles produces a reduced risk for scrapie (Baylis et al., 2004; Billinis et al., 2004). The scrapie pool transmitted efficiently to Tg(Mu/OvPrPAHQ) mice, which could be due to strain-dependent susceptibility of the AHQ allele or to the complex effects of PrP polymorphisms and polygenic modulation of the PrP gene on host susceptibility (Diaz et al., 2005). Reported variations in the susceptibility and IP of tg338 and TgOvPrP4 mice to scrapie strains could be influenced by PrP genotype, and/or reflect differences in expression levels (Arsac et al., 2009), promoter effects and prion titres. The response of the EM16 model to a wider range of scrapie strains from sheep of different PrP genotypes will require further investigation. High susceptibility of EM16 mice to BSE indicates the potential use of this surrogate OvPrPAHQ model to investigate the characteristics of other BSE-related prions such as ovine BSE, atypical BSE and variant CJD.

Previously, brain lesion profiling of WT and transgenic mice has been used to strain type TSEs (Thackray et al., 2008; Beck et al., 2010); however, IHC may be more informative, as prion strains can be discriminated at the individual mouse level rather than as the mean of more than five clinically positive mice required for lesion profiling (Beck et al., 2010). As the focus here was to establish primary transmission of TSEs, rather than strain typing, IHC was used to confirm prion infection in the brains of selected clinically affected EM16 mice. Early observations suggested that different TSE sources can induce different PrP deposition patterns in EM16 mice, which could be used to differentiate TSE strains, as shown in other transgenic and WT mice (Thackray et al., 2011; Beck et al., 2012). Distinction of the three classes of TSE was also shown by differences in PrP profiles, antibody reactivities and glycoform percentages, following passage in EM16 mice, which were similar to natural cases of each TSE.

Whereas atypical scrapie (Le Dur et al., 2005; Arsac et al., 2007; Griffiths et al., 2010; Gotte et al., 2011) and BSE (Collinge et al., 1996; Bruce et al., 1997, 2002; Green et al., 2005; Ritchie et al., 2009) appear each to be composed of a single strain of TSE agent, at least several different classical scrapie strains have been identified in WT mouse bioassays (Bruce et al., 1994, 2002). EM16 mice challenged with the scrapie pool produced a single IHC pattern in the brains examined, which could have represented the emergence of a predominant strain facilitated by expression of Mu/OvPrPAHQ protein. It is also feasible that only one scrapie strain may have been present in the inoculum, despite its mixed sheep brain origin.

Overexpression of Mu/OvPrPAHQ in EM16 mice has produced a bioassay model demonstrating a range of susceptibility, reduced IPs and differentiation of ruminant prions. These mice offer the prospect of improved detection and typing of natural and experimental prions, and are likely to be better suited than WT mice to investigate the complexities of susceptibility/resistance to atypical and classical scrapie in small ruminants. The model could be used to replace WT mouse bioassays and reduce the need for large-scale studies in the natural host. Development and assessment of transgenic PrP models, such as EM16, in the transmissibility and pathogenesis of TSEs remain crucial to the identification of new prion risk materials for human and animal health, and to reduce the spread of ruminant prion diseases.
METHODS

Construction of a chimaeric Mu/OvPrPhG transgene. The strategy, based on previous approaches (Buschmann et al., 2000; Viňot et al., 2001), utilized Smal and SalI restriction sites in the pgPrP vector (Fischer et al., 1996) and insertion of an ovine/murine overlapping DNA fragment (Fig. 1a–e). A portion of the ovine Prnp gene ORF was amplified from DNA extracted (DNeasy kit; Qiagen) from an AHQ/AHQ Cheviot sheep blood sample using PCR primers 5'-GAGCCGGATCCGCGGCAAGGACAC-3' and 5'-GATCCG- CACGATGAGAAAATGAGG-3' (Viňot et al., 2001), which introduced a Smal site into the 5' end, and four murine bases into the 3' end (silent changes in both). Using primer pair 5'-CCTCATTTTCTCTATCGGGATGA-3' and 5'-CGAGGTCTGGAC-TGACCTGAGCTACT-3', the murine Prnp 3' UTR was PCR-amplified. An overlapping (ovinePrPhG–murine 3' UTR) insert was generated from both PCR products using the ovine 5' and murine 3' primers. Following Smal/SalI restriction digestion, the insert (Fig. 1c) was ligated into digested pgPrP to replace the corresponding sequence and recombinant clones were produced. Recombinant DNA was digested with NotI and SalI to release the 12 kb Mu/OvPrPhG transgene encoding chimaeric PrP consisting of 253 aa (aa 1–39 of MuPrP and aa 40–253 OvPrPhG), with expression driven by the pgPrP mouse promoter, and containing the murine 3' UTR sequence (Fig. 1b). In the mature protein, MuPrP aa sequence (residues 23–29) is identical to that of ovine PrP, except for the absence of a single glycine residue equivalent to sheep codon 33; in addition, the murine PrP base sequence in the same region shows a number of silent changes compared with ovine PrP. The transgene was gel extracted (Strataprep; Stratagen), resuspended in micro-injection buffer (Sigma) and quantified by spectrophotometry.

Generation and characterization of transgenic PrP mice. Purified transgene (0.5–4.0 ng DNA µl⁻¹) was microinjected into 200 pronuclear FVB/N embryos (Nuclei). DNA from the generated mice was PCR screened to detect the transgenic sequence (A. Buschmann, personal communication). Founder mice were crossed with PrP-null mice (Prnp−/−/ Zurich I’ mice; Fig. 1e) (Büeler et al., 1992) to establish expression on a mouse PrP-null background. Progeny of founder x PrP-null crosses, and subsequent crosses to PrP-null mice, were PCR screened for the transgene, PrP-null alleles (Büeler et al., 1992) and absence of WT mouse Prnp (using primers 5’-CGTTAACCACTCTACGGTGGACCT-3’ and 5’-GGTGCTGCT-GTGATCTCTCCTCCGTTGA-3’). Mice were observed daily for signs of intercurrent or spontaneous disease and euthanized based on welfare considerations.

PrPC expression was estimated by WB of brain from transgenic mice and normal sheep. Immediately after collection, brain samples were prepared as 10% (w/v) homogenates in homogenization buffer (0.5% NP-40, 1% sodium deoxycholate, 10 mM EDTA, in PBS, pH 7.4) containing protease inhibitors (Roche) and stored at −80°C. The next day, thawed aliquots were serially diluted in homogenization buffer containing centrifuged (2000 g, 5 min), resuspended in reducing loading buffer (Bio-Rad), heated (100°C, 5 min), separated by SDS-PAGE on 12% Bis-Tris gels (Criterion XT; Bio-Rad) in 3-(N-morpholino)propanesulfonic acid buffer (200 V, 40 min) and transferred to PVDF membranes (Bio-Rad) in Tris/N-cyclohexyl-3-aminopropanesulfonic acid buffer (115 V, 60 min). PrPC was immunodetected by a TeSeE WB kit (Bio-Rad), visualized by enhanced chemiluminescence (Abersham, GE Healthcare) and autoradiography (Abersham Hyperfilm), and quantified by densitometry (Quantity One; Bio-Rad).

Transgene copy number was estimated by Southern blot hybridization (Viňot et al., 2001) of Sgt-digested DNA from the three transgenic lines compared with PrP-null mouse DNA spiked with 0, 1, 5 or 10 copies of Smal–SalI insert and with sheep DNA. Similar amounts (10 µg) of each were digested, separated by electrophoresis in 1% agarose gels and blotted onto nylon membranes (Hybond-N+; Amersham). Using a 658 bp psoralen–biotin-labelled ovine Prnp gene probe (Fig. 1d), signals were visualized by enhanced chemiluminescence (BrightStar; Ambion) and autoradiography, and quantified as above.

TSE challenge of Tg(Mu/OvPrPhG) mice. TSE sources from sheep and cattle (Tables 1 and S1) were prepared as 10% (w/v) brain homogenates by extrusion in Ca²⁺ - or Mg²⁺-free saline, clarified (1000 g, 10 min, 4°C), sterility checked and stored at −80°C. Bacterial contamination was treated as described previously (Griffiths et al., 2010). Atypical scrapie inocula were quantified previously for PrP Kab using ELISA (TeSeE; Bio-Rad) (Table S1) and shown to be transmissible to tg338 and/or TgshpX1 mice (Griffiths et al., 2010). Classical scrapie pool and BSE pool inocula were previously titrated in WT mice and ELISA tested (Table S1). Aliquots of stored inocula were retested prior to use by WB (Fig. S2). Mice (6–12 weeks old) were inoculated under general anaesthesia by intracerebral (i.c.) (20 µl) or i.c. and intraperitoneal (i.p.) (100 µl) routes. Mice were monitored weekly for clinical signs of TSEs according to established guidelines. Following progression of adverse neurological signs, mice were euthanized and their brains collected: one-third parasagittal portions were stored freshly frozen at −80°C for WB and two-thirds portions were used for IHC of selected cases. Mouse studies were conducted within the legal and ethical framework of UK Home Office regulations.

WB of TSE-challenged Tg(Mu/OvPrPhG) mice. PrPrams detection in brain confirmed prion transmission to mice. Frozen brain tissue (50–100 mg) was processed and treated with proteinase K according to the manufacturer’s recommendations (TeSeE; Bio-Rad), and immunoblotted (as above) using mAbs Sha31 (Bio-Rad) and P4 (R-Biopharm AG) (recognizing OvPrP148YEDRYYRE155 and 93WGQGGSH99, respectively). For comparison, sheep atypical scrapie, scrapie scrapie, cattle BSE and uninfected transgenic brain were treated similarly. Deglycosylation of homogenized, proteinase K-treated extracts of transgenic mouse brains was carried out using peptide N-glycosidase (New England Biolabs), as described previously (Griffiths et al., 2010).

Neuropathology. Brain samples from selected, clinically affected TSE-challenged EM16 mice were fixed in 10% neutral-buffered formalin and processed for histopathology. Paraffin-embedded sections (3 µm thick) from pre-defined coronal levels were stained with H&E and examined by microscopy to assess TSE-associated vacuolation. PrPrams deposits were detected by IHC using polyclonal anti-PrP rabbit antiserum Rb486, as described by Beck et al. (2010).

Statistics. TSE transmissions were expressed as mean IP (the time between inoculation and death for WB TSE-confirmed mice) or as the longest surviving mouse in a group with unconfirmed transmission. Kaplan–Meier survival curves were plotted for TSE-confirmed mice; TSE-negative mice with/without intercurrent disease were censored. The log-rank (Mantel–Cox) test was used to assess differences in survival characteristics between groups (GraphPad Prism, v5.0); P values <0.05 were considered statistically significant.

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