PrP\textsuperscript{Sc} detection and infectivity in semen from scrapie-infected sheep

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A scrapie-positive ewe was found in a flock that had been scrapie-free for 13 years, but housed adjacent to scrapie-positive animals, separated by a wire fence. Live animal testing of the entire flock of 24 animals revealed seven more subclinical scrapie-positive ewes. We hypothesized that they may have contracted the disease from scrapie-positive rams used for breeding 4 months prior, possibly through the semen. The genotypes of the ewe flock were highly scrapie-susceptible and the rams were infected with the ‘Caine’ scrapie strain having a short incubation time of 4.3–14.6 months in sheep with 136/171 VQ/VQ and AQ/VQ genotypes. PrP\textsuperscript{Sc} accumulates in a variety of tissues in addition to the central nervous system. Although transmission of prion diseases, or transmissible spongiform encephalopathies, has been achieved via peripheral organ or tissue homogenates as well as by blood transfusion, neither infectivity nor PrP\textsuperscript{Sc} have been found in semen from scrapie-infected animals. Using serial protein misfolding cyclic amplification followed by a surround optical fibre immunoassay, we demonstrate that semen from rams infected with a short-incubation-time scrapie strain contains prion disease-associated-seeding activity that generated PrP\textsuperscript{Sc} in sPMCA (serial protein misfolding cyclic amplification). Injection of the ovinized transgenic mouse line TgSShpPrP with semen from scrapie-infected sheep resulted in PrP\textsuperscript{Sc}-seeding activity in clinical and, probably as a result of the low titre, non-clinical mouse brain. These results suggest that the transmissible agent, or at least the seeding activity, for sheep scrapie is present in semen. This may be a strain-specific phenomenon.

INTRODUCTION

Scrapie in sheep and goats is a transmissible spongiform encephalopathy (TSE), or prion disease, a category that also includes Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cattle (Detwiler & Baylis, 2003; Collinge, 2001). A hallmark of TSEs is the accumulation, most prominently in the central nervous system, of an abnormally folded prion protein isoform, PrP\textsuperscript{Sc}, which is derived from the host-encoded cellular form (PrP\textsuperscript{c}). PrP\textsuperscript{Sc} is the only known component of infectious prions and is also assumed to be responsible for the neuropathology caused by these agents (Prusiner, 1982; Weissmann, 2004). Diagnosis of TSE infection largely relies on the detection of the pathological isoform, which differs from PrP\textsuperscript{c} by various properties, most notably an increased resistance to proteolysis (Bolton et al., 1982).

In sheep scrapie, TSE susceptibility (incidence and age of onset of natural and experimental disease) is mainly controlled by polymorphisms at three codons in the ovine PRNP: aa 136 (A or V), 154 (R or H) and 171 (R, Q or H) (Hunter, 1996). The genotypes V\textsubscript{136}R\textsubscript{154}Q/VRQ, ARQ/VRQ, ARQ/ARQ and VRQ/ARQ PrP are most susceptible to scrapie, while the ARR/ARR and AHR/AHR alleles are associated with natural resistance to the clinical disease (Goldmann et al., 1994; Westaway et al., 1994; Belt et al., 1995; Ikeda et al., 1995; Elsen et al., 1999; Baylis et al., 2002, 2004). Incubation time in sheep has been shown to rely on the strain of ovine scrapie as well as the genotype of the recipient sheep (Hunter et al., 1993; Goldmann et al., 1994; Bulgin et al., 2006; Hamir et al., 2009). ‘V’-dependent...
strains such as the SSSP/1 strain reported by Hunter and the X124 (‘Caine’) strain characterized by Hamir et al. (2009) have much shorter incubation times in VRQ/VRQ and ARQ/VRQ sheep, while showing more traditional incubation times in sheep with ARQ/ARQ genotypes.

Expression of PrP<sup>C</sup> is not restricted to nervous tissues; PrP<sup>C</sup> is present in virtually all tissues in the body (Bendheim et al., 1992; Moudjou et al., 2001). Gatti et al. (2002) demonstrated the presence of PrP<sup>C</sup> in sperm and demonstrated that it is synthesized in the genital tract where it is released in large quantities as a soluble form in the epididymal fluid and seminal plasma of the ram. Proteolytic processing of the prion protein also appears to occur at this point. However, Gatti et al. (2002) did not detect proteinase K (PK)-resistant PrP<sup>Sc</sup> in the seminal plasma from scrapie-infected animals.

The risk of TSE infectivity in semen from sheep, goats and cattle is assumed to be extremely low. Bioassays in mice have failed to detect infectivity in testis, seminal vesicles and semen of scrapie-affected rams, or in testis, epididymis, prostate, seminal vesicles and semen of BSE-affected bulls. Wrathall et al. (2002) reported that the use of semen from bulls clinically affected with BSE to inseminate clinically affected cows did not lead to BSE infection of the resulting embryos. This is not surprising due to the lack of infectivity and PrP<sup>Sc</sup> in peripheral cattle tissue from BSE-infected cattle. In contrast, sheep are known to contain both infectivity and pathology in peripheral tissues. In an early experiment, semen from a scrapie-affected ram was inoculated into 20 1-day-old lambs (Palmer, 1959) and there was no disease transmission. However, the experiment was stopped only 30 months after inoculation and no genotype information is available limiting the interpretation of the study. Wang et al. (2001, 2002) mated a scrapie-positive ram with one scrapie-positive and one scrapie-negative ewe. Embryos collected from these two ewes yielded seven and four offspring, respectively, which were scrapie-free at 60 months of age, suggesting that scrapie was not transmitted via the semen. However, this was not statistically significant based on the number of samples.

Sarradin et al. (2008) examined semen from scrapie-infected rams at different stages of incubation, including the clinical phase of the disease, for prion infectivity. Bioassays performed with scrapie-susceptible transgenic mice overexpressing the V<sub>136R154Q171</sub> allele of the ovine PRNP did not result in clinical disease or PrP<sup>Sc</sup> in the brains of these mice.

Detection of PrP<sup>Sc</sup> by a laser-based assay, surround optical fibre immunoassay (SOFIA), has recently been reported to provide increased sensitivity for animal prion diseases (Chang et al., 2009; Rubenstein et al., 2010, 2011). This assay is not dependent on PK treatment for the detection of PrP<sup>Sc</sup>, which eliminates PrP<sup>C</sup>, but does require serial protein misfolding cyclic amplification (sPMCA) for detection of prion-associated-seeding activity from extremely low titre sources; the prion disease-associated-seeding activity (PASA) may not be PrP<sup>Sc</sup>. Here, we report detection of PASA in semen by using a combination of sPMCA followed by SOFIA. We also demonstrate the presence of prion infectivity in the original semen samples by bioassays in a newly generated sheep scrapie-susceptible transgenic mouse line, TgSShpPrP. Semen tested came from a flock originating in Idaho that were naturally scrapie infected, condemned and donated by the State to the University of Idaho, Caine Veterinary Teaching Center (CVTC) for scrapie research. The flock was closed to new additions in 1999. These sheep have been bred annually to maintain the disease and animal numbers are limited by the disease and selective euthanasia. Approximately 20 % of the flock succumbs to scrapie every year. This flock is distinctive in that it harbours a unique US strain of scrapie causing a rapid disease in ARQ/VRQ and VRQ/VRQ genotype sheep, while the disease in ARQ/ARQ sheep exhibit more traditional signs of scrapie with an incubation period averaging greater than 37–42 months (Bulgin et al., 2006).

Since 1996, a small group dubbed the ‘sentinel flock’ had been kept separate but in close proximity to the above flock. The original sheep in the sentinel flock were 136/171 ARQ/ARQ kept expressly to monitor the risk of having susceptible sheep in close proximity, but not intermingled with scrapie-exposed and scrapie-positive animals. They were bred annually to ensure availability of lambs with a susceptible genotype. Breeding and selecting for the more susceptible ARV/VRQ and VRQ/VRQ sheep was started in 2004 using subclinical 136AV and VV genotype rams (Tables 1 and S1, available in JGV Online) born in the scrapie-positive flock in order to provide the most genetically susceptible sheep in both the scrapie-positive and the sentinel flock. The cross breeding and selection programme, using VRQ/VRQ and ARQ/VRQ rams and the original sentinel ARQ/ARQ Targhee ewes resulted in a flock composed of 5.3 % ARR/VRQ, 10.5 % ARQ/ARQ, 26.3 % VRQ/VRQ and 57.9 % ARV/VRQ crossbred ewes totalling 24 animals by year 2009.

In spite of the close proximity of the two flocks and susceptible genotypes, there had been no evidence of transmission of scrapie to this flock for more than 13 years. However, in the spring of 2009, 4 months after exposure to subclinical scrapie-positive rams for breeding purposes, a ewe in the sentinel flock developed a slight ear tip tremor. This ewe tested positive for scrapie utilizing immunohistochemistry (IHC) on biopsy samples. Further testing of the flock revealed additional positive ewes.

The objective of this study was to determine whether semen from clinical and subclinical scrapie-positive rams with highly susceptible genotypes (Tables 1 and Table S1) and a unique US strain of scrapie might contain the scrapie-transmissible agent, or PASA, and be detectable by sPMCA and SOFIA.

**RESULTS**

The use of SOFIA alone to detect PrP<sup>Sc</sup> in semen samples from scrapie-infected sheep was unsuccessful (data not
shown). In previous studies (Rubenstein et al., 2010, 2011), we also reported that SOFIA alone was unable to detect PrPSc in blood and urine from scrapie-infected sheep and cervids with chronic wasting disease. However, in combination with sPMCA, which utilizes PASA, PrPSc in blood and urine was detectable. The level of PrPSc detected was dependent on the number of serial cycles of PMCA. Furthermore, amplification is efficient independent of the genotypes of the PrPC and PrPSc (Rubenstein et al., 2010, 2011). The level of PrPSc measured by SOFIA following sPMCA80 and sPMCA120 (Fig. 1) was similar to that found in urine (Rubenstein et al., 2011) and significantly less than that found in blood (Rubenstein et al., 2010) from scrapie-infected sheep. This suggests that the PASA found in the semen of these animals is similar to that for urine from infected animals, approximately 1 attogram ml$^{-1}$. However, it is not possible to determine whether prion-seeding activity is actually PrPSc (Rubenstein et al., 2011).

The ovinized PrP transgenic mouse line, TgSShpPrP, was analysed for ovine PrPC expression. Serial twofold dilutions of brain homogenates from TgSShpPrP mice and wild-type

**Table 1.** Ram genotypes

<table>
<thead>
<tr>
<th>Ram</th>
<th>Genotype</th>
<th>IHC*</th>
<th>Clinical</th>
<th>Semen collection</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>3272</td>
<td>$A_{136}$R$<em>{154}$Q$</em>{171}$/$V_{136}$R$<em>{154}$Q$</em>{171}$</td>
<td>33† (+)‡</td>
<td>33†</td>
<td>43†</td>
<td>43†</td>
</tr>
<tr>
<td>7031</td>
<td>ARQ/VRQ</td>
<td>11 (+)</td>
<td>26</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>7035</td>
<td>ARQ/VRQ</td>
<td>11 (+)</td>
<td>13</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>8001</td>
<td>VRQ/VRQ§</td>
<td>10 (+)</td>
<td>20</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>8031</td>
<td>ARQ/ARQ</td>
<td>10 (−)¶</td>
<td>NA¶</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>531#</td>
<td>ARR/ARR**</td>
<td>ND††</td>
<td>NA‡‡</td>
<td>15</td>
<td>U§§</td>
</tr>
<tr>
<td>539#</td>
<td>ARR/ARR</td>
<td>ND</td>
<td>NA</td>
<td>19</td>
<td>U</td>
</tr>
<tr>
<td>560#</td>
<td>ARR/ARR</td>
<td>ND</td>
<td>NA</td>
<td>U</td>
<td>U</td>
</tr>
</tbody>
</table>

*Immunohistochemistry of third eyelid or rectal tissue.
†Age of animal (months).
‡PrPSc positive.
§Most susceptible genotype.
¶PrPSc negative by IHC but positive in brain by SOFIA following sPMCA$_{40}$.
¶Not clinical.
#Certified scrapie-free flock.
**Most resistant genotype.
††Not done.
‡‡Not applicable.
§§Uncertain.

![Fig. 1. SOFIA detection of PASA product following PMCA in semen. Semen samples from three uninfected (Un) sheep and five scrapie-infected (Sc) sheep were subjected to serial PMCA for 80 (sPMCA$_{80}$) and 120 cycles (sPMCA$_{120}$) followed by SOFIA for the analysis of PASA and the detection of PrPSc. All PMCA products were subjected to IP with mAb 8E9 followed by SOFIA. Values represent the mean of triplicate assays ± SD.](http://vir.sgmjournals.org/10.1109/377)
(WT) mice on the same FVB genetic background were analysed by Western blotting. PrP\(^{\text{C}}\) was immunostained using the PrP-specific mAb, 08-6/2F7-2F11. The dilutions producing equivalent PrP\(^{\text{C}}\) immunostaining between the two mouse lines indicate that the level of expression of sheep PrP\(^{\text{C}}\) in the brains of TgSShpPrP mice is approximately fourfold greater than mouse PrP\(^{\text{C}}\) in the WT mice (Fig. 2).

The ability of TgSShpPrP mice to support replication of the sheep prion agent was analysed and is summarized in Table 2. Brain homogenate from scrapie-infected ram #8001, which was positive for PK-resistant PrP\(^{\text{Sc}}\) by Western blotting (Fig. 3a, lanes 3–4), was used to infect TgSShpPrP mice. Injection of nine TgSShpPrP mice by the intracerebral (IC) route with a 10 % brain homogenate from ram #8001 had a mean incubation period of 167 ± 7 days, indicating that these mice support replication of the ovine prion agent. Western blotting of brain from clinical TgSShpPrP mice demonstrated the presence of PK-resistant PrP\(^{\text{Sc}}\) (Fig. 3a, lane 8). In contrast, TgSShpPrP mice showed no clinical signs or PrP\(^{\text{Sc}}\) after over 520 days following IC injection of either a 10 % normal sheep brain (NSB) homogenate or a 10 % ME7 mouse-adapted scrapie strain brain homogenate (Table 2).

Semen samples from scrapie-positive rams (# 3272, 7031, 7035 and 8001) were injected IC into TgSShpPrP mice (five mice per semen sample) to bioassay for prion infectivity (Table 2). One TgSShpPrP mouse from the ram #7031 semen sample group and one from the ram #3272 semen-injected group showed clinical signs after an incubation period of 310 and 655 days, respectively. Western blotting of the brain from these mice demonstrated PK-resistant PrP\(^{\text{Sc}}\) (Fig. 3a and b). Interestingly, the mouse from the ram #3272 group did not display any clinical signs at 648 days post-injection (p.i.), but by 655 days p.i. the animal was at the end stage of clinical disease. This is about half the time it typically takes mice to proceed through the same clinical disease course. The remaining mice from these groups, as well as all the mice from the other ram semen-injected groups did not display any clinical disease throughout the entire experiment (>670 days p.i.).

Animals were randomly selected from the groups of non-clinical TgSShpPrP mice injected with semen samples from ram #3272 (385 days p.i.), 7031 (385 days p.i.), 7035 (350 days p.i.) and 8001 (335 days p.i.). TgSShpPrP mouse brain homogenates were prepared and analysed by Western blotting for the presence of PrP\(^{\text{Sc}}\). PK-resistant PrP\(^{\text{Sc}}\), indicative of prion disease, was detected in the brain homogenates of all the semen-injected non-clinical TgSShpPrP mice (Fig. 3b, lane 2; Fig. 4 lanes 6, 8, 10 and 12). Interestingly, all of the PK-resistant PrP\(^{\text{Sc}}\) profiles from the non-clinical mice injected with different semen samples were similar with a prominent protein band at 27–30 kDa, representing the truncated diglycosylated PrP, a less intense immunostained band at 23–25 kDa indicative of the

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**Table 2. Overview of injected TgSShpPrP mice**

<table>
<thead>
<tr>
<th>Sample</th>
<th>#TgSShpPrP injected</th>
<th>#Clinical (#tested/#PrP(^{\text{Sc}}) pos.)</th>
<th>#Non-clinical (#tested/#PrP(^{\text{Sc}}) pos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSB*</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (5/0)</td>
</tr>
<tr>
<td>ME7†</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (5/0)</td>
</tr>
<tr>
<td>#8001</td>
<td>9</td>
<td>9 (5/5)</td>
<td>0 (0/0)</td>
</tr>
<tr>
<td>semen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3272</td>
<td>5</td>
<td>1 (1/1)</td>
<td>4 (2/2)</td>
</tr>
<tr>
<td>#7031</td>
<td>5</td>
<td>1 (1/1)</td>
<td>4 (2/2)</td>
</tr>
<tr>
<td>#7035</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (1/1)</td>
</tr>
<tr>
<td>#8001</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (1/1)</td>
</tr>
<tr>
<td>#531</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (5/0)</td>
</tr>
<tr>
<td>#539</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (5/0)</td>
</tr>
<tr>
<td>#560</td>
<td>5</td>
<td>0 (0/0)</td>
<td>5 (5/0)</td>
</tr>
</tbody>
</table>

*Normal sheep brain.
†ME7 mouse-adapted scrapie strain.
truncated monoglycosylated form of PrP and a lighter, truncated unglycosylated PrP form at 19–20 kDa. Additional analysis of random, non-clinical TgSShpPrP mice selected from the semen-injected groups at 523 days p.i. produced similar positive Western blot results and the same PrPSc profile (data not shown). In contrast, and as expected, clinical TgSShpPrP mice injected with either ram #8001 brain homogenate (Fig. 3a, lane 8; Fig. 4, lane 2), or semen from ram #7031 (Fig. 3a, lane 10) and 3272 (Fig. 3b, lane 6) had a PK-resistant PrPSc pattern consisting of three intensely immunostained bands. A similar pattern of three intensely immunostained bands was also seen in a non-clinical mouse from ram #3272 semen-injected group at 648 days p.i. (Fig. 3b, lane 4), just 7 days before another mouse from this group displayed extensive clinical disease. None of the five TgSShpPrP mice/group injected with semen from the uninfected rams displayed any clinical signs or PK-resistant PrPSc by 650 days p.i. (data not shown).

**Fig. 3.** (a) Western blot of brains from uninfected and infected sheep and TgSShpPrP mice. Ten per cent brain homogenates were prepared from normal (lanes 1–2) sheep brain, scrapie-infected (lanes 3–4) sheep brain (#8001), uninfected TgSShpPrP mouse (lanes 5–6), clinical TgSShpPrP mouse (167 days p.i.) infected with #8001 sheep brain (lanes 7–8) and clinical TgSShpPrP mouse (310 days p.i.) infected with #7031 sheep semen (lanes 9–10). Brain homogenates were untreated (lanes 1, 3, 5, 7 and 9) or PK-treated (lanes 2, 4, 6, 8 and 10) followed by Western blotting and immunodetection with biotinylated mAb 08-6/2F7-2F11. (b) Western blot of brains from non-clinical and clinical TgSShpPrP mice infected with #3272 semen. Ten per cent brain homogenates were prepared from non-clinical TgSShpPrP mice at 385 (lanes 1–2) and 648 (lanes 3–4) days p.i. Brain homogenates were also prepared from clinical TgSShpPrP mice at 655 days p.i. Samples were either untreated (lanes 1, 3 and 5) or PK-treated (lanes 2, 4 and 6) prior to Western blotting with mAb 08-6/2F7-2F11.

The IHCs performed on all reproductive tissues (prostate, epididymis, vesicular glands and testes) of the above rams were negative for PrPSc (Table S2). Ram #8031 (ARQ/ARQ) also tested negative by IHC on RAMALT and third eyelid and his brain and all other tissues tested were IHC negative for PrPSc when euthanized following semen collection (Table S2). Western blot analysis of brain tissue from ram #8031 was negative for PK-resistant PrPSc. Western blotting of the samples following 40 cycles of serial PMCA (sPMCA40) using ram #8031 brain tissue as a seed was also negative for PK-resistant PrPSc (data not shown) presumably due to the limitations of assay sensitivity. However, using sPMCA40 PASA in ram #8031 brain tissue could be detected and the product (i.e. PrPSc) measured by SOFIA with signal intensities (sample/background) of 39.8 ± 2.1 compared with 1.01 ± 0.02 for uninfected control sheep brain.

**DISCUSSION**

The decision to use rams from the scrapie-positive flock to breed ewes in the sentinel flock resulted from the scarcity and difficulty of finding 136V/V and A/V rams in private certified scrapie-free flocks and the prevalent belief at that time that rams do not transmit scrapie as indicated by the National Scrapie Surveillance Plan (2010). Current USDA Scrapie Eradication Uniform Methods and Rules (2005) do not require the quarantining of flocks following the introduction of exposed rams. Furthermore, the European Food Safety Authority recently reached the same conclusion that the risk of TSE transmission associated with semen and embryos collected from classical scrapie incubating sheep and goats ranges from negligible to low [EFSA Panel on Biological Hazards (BIOHAZ), 2010].

Despite extensive research, the natural routes of TSE transmission are still rather poorly understood. Alimentary
excretion and oral ingestion contributes to horizontal transmission, but long incubation periods make it difficult to link clinical cases to the original source of infection. Host genetic factors, especially in sheep scrapie, make epidemiological interpretations even more difficult, and the existence of different TSE strains brings further complexity. Understanding the risks of transmission, especially those posed by reproductive technologies, requires information on the presence and levels of infectivity in various body tissues, which typically entails detection of PrPSc by immunohistochemical or immunoblotting assays. However, since the ability to detect PrPSc does not necessarily equate with the amount of infectivity, bioassays in mice are also required.

The general conclusion from research to date is that the risk of TSE infection by semen from sheep, goats and cattle is extremely low. Cross-contamination of the semen samples used in this study is one explanation for the infectivity detected in the semen samples from the infected animals. However, TgSShpPrP mice injected with semen from the uninfected rams did not show any sign of disease or the presence of PrPSc in their brains. The results of the current study demonstrate detection of PASA in semen as well as the presence of PrPSc in transgenic mice following IC injection. The detection of PASA was performed by using normal brain homogenate as a PrPSc source from sheep with a genotype that, in some cases, did not match the genotype of the ram semen samples. It is doubtful that this had any negative influence on PASA amplification of the semen samples since previous studies using mismatched genotypes similar to that for semen did not hinder amplification in blood and urine (Rubenstein et al., 2011, 2011). The detection of the infectious agent in semen by bioassays in ovinized transgenic mice contrasts with the previous report by Sarradin et al. (2008). Sarradin et al. (2008) examined the role of semen in sheep scrapie transmission by analysing whether semen (i.e. seminal plasma and spermatozoa) could infect and cause disease in the tg338 transgenic mouse line, which overexpresses the permisive V136R154Q171 allele of the ovine prion protein. None of the tg338 mice injected with semen from the VRQ/VRQ genotype or from the ARR/ARR control ram showed any clinical signs during the period of the experiment. The semen samples differed between the two studies, as did the transgenic mouse lines, and most likely the scrapie strain. While both the VRQ and ARQ genotypes are more sensitive to scrapie infection than the ARR genotype, it is conceivable that in the transgenic mouse bioassay the ARQ protein confers greater susceptibility to semen-derived PrPSc. PrP in sperm is known to undergo different processing than that in brain (Shaked et al., 1999; Ecroyd et al., 2005). Further, the impact of alanine substitutions can be substantial since the threonine to alanine mutation at position 183 of the human prion protein is linked to a familial disease and results in retention of the prion protein in the endoplasmic reticulum due to structural changes in the protein (Capellari et al., 2000). Whether or not these factors were responsible for the differences in the final outcomes of these studies remains to be determined. However, it seems premature to rule out the potential of semen-associated-seeding activity as a factor in the transmission of scrapie.

Ram #8031 is of particular interest. He was euthanized due to an overabundance of ARQ/ARQ and showed no clinical signs at the time of death at age 16 months. All post-mortem tissues were IHC negative (Table S2) and yet sPMCA80 and sPMCA120 followed by SOFIA showed that PASA was present in the semen of this ram. No doubt this ram was infected and subclinical. He had been born and raised in a highly contaminated environment, but possessed a long incubation time genotype. Typical of this strain, he would not have shown clinical signs or positive testing utilizing IHC for 3–5 years. This supports and strengthens our view that the assay used in this study is more sensitive than the presently accepted IHC for early detection of scrapie and could be of value as a live animal test.

We tend to believe that a positive ram was the likely source of the scrapie-infected ewes, if not from semen, then by another type of contact. All but one of the affected ewes were VRQ/VRQ; the incubation time of the ‘Caine’ strain of scrapie normally in these genotypes is 4–7 months (Bulgin et al., 2006; Hamir et al., 2009), suggesting that the exposure occurred during the period when the rams were placed with the ewes for breeding. However, the presence of the infectious agent in the semen of the 136AV and VV rams infected with the ‘Caine’ strain of scrapie, adds another intriguing possibility.

An interesting question is why it took so long for the disease to present itself when the first exposure of the flock during breeding to positive rams occurred 4 years previously. During the preceding 7 years, even though the flock was genotypically susceptible, there was no scrapie transmission. The disease onslaught definitely appears to be promoted by the change in genetic composition of the sentinel flock and their degree of susceptibility. When the 136VV and AV rams were first introduced to the flock in 2004, the ewes were all 136AA. Thus, the population slowly shifted to become more susceptible as the 136V genotype was selected for. It is noteworthy that every one of the scrapie-infected ewes, if not from semen, then by another type of contact. All but one of the affected ewes were VRQ/VRQ; the incubation time of the ‘Caine’ strain of scrapie normally in these genotypes is 4–7 months (Bulgin et al., 2006; Hamir et al., 2009), suggesting that the exposure occurred during the period when the rams were placed with the ewes for breeding. However, the presence of the infectious agent in the semen of the 136AV and VV rams infected with the ‘Caine’ strain of scrapie, adds another intriguing possibility.

In summary, it seems premature to rule out the potential of semen-associated-seeding activity as a factor in transmission of scrapie. In that regard, experiments are currently under way to assess the transmissibility of the PrPSc from the clinically normal mice that had been infected with sheep semen as well as the material produced by PMCA using a semen seed. These results will demonstrate whether the seeding activity found in semen is competent to promote scrapie transmission. Also, demonstration of natural transmission of scrapie in sheep by semen will have to be
confirmed by inoculation of highly susceptible oestrous ewes via artificial insemination using semen collected from positive ‘Caine’ strain scrapie-infected rams since this may be a strain-specific phenomenon.

**METHODS**

**Sheep care.** Facilities, care and procedures met Federal and University of Idaho Animal Care and Use specifications (IACUC protocol #2006-50). Each sheep was uniquely identified by an ear tag and records were kept for each animal.

**Scrapie testing.** Unusual behaviour in sentinel animals initiated RAMALT and third eyelid biopsies. Animals dying naturally or euthanized also underwent necropsies and IHC testing (Bulgin et al., 2006) (Supplementary File S1, Table S2).

**Generation of ovinized transgenic mice.** The ovinized transgenic mouse line carrying the Suffolk sheep PrP gene (TgSShpPrP) containing a Kozak ribosome-binding sequence was generated from a sheep PrP cosmid clone in the pWE15 vector (Westaway et al., 1994). The sheep PrP gene ORF was amplified by PCR using a Sall-containing (lower case italics) upstream primer (5’-TACTG-GCggcACATcGTTGAAAAGCCACATAG-3’) containing the Kozak sequence (bold) and a Sall-containing downstream primer (5’-ACACGCGctgacCTATCTCTATGAGAAA-3’). The PCR product was cloned into the pCR2.1-TOPO TA vector (Invitrogen) and sequenced to confirm the fidelity of the amplified material (GenBank accession #U67922) as described previously (Lee et al., 1998) for the Suffolk sheep PrP, which contained the A136R154Q171/ARQ allele. The sequenced DNA was cloned into the Sall sites of the cosSHa.Tet expression vector (Scott et al., 1988) and used for generating the transgenic mouse line TgSShpPrP.

**Semen collection and infection of mice.** Treatment of animals and sample collection followed an IACUC-approved protocol (protocol #2010-53). Semen was collected from control rams (Table 1) by holding a clean small plastic bag over the end of the penis, while stimulating the ram with an hand-held ram electro-ejaculator.

Semen from scrapie-affected rams (Table 1) was collected by anaesthetizing the rams using intravenous xylazine (0.1 mg per lb body weight) and 5 ml ketamine HCl. They were placed left-side down on a hydraulic table and raised to a comfortable working height. The wool from around the prepuce and adjacent area was shaved and skin scrubbed as for aseptic surgery. The penis was exteriorized and rinsed several times with sterile saline, then catheterized using an appropriate sized sterile polypropylene urinary catheter. The exterior end of the catheter was held over the lip of a sterile conical 15 ml centrifuge tube, while the ram was ejaculated using a hand-held ram electroejaculator. As much semen as possible was collected in this manner, ejaculating the rams several times (collected into a new tube each time) allowing a 3–4 min rest between ejaculations. The semen was centrifuged at 3000 g for 30 min; the supernatant fluid was separated using sterile technique and frozen in 0.5 ml aliquots. The spermatozoa-containing fractions were also frozen and saved.

For injection (Table 2), TgSShpPrP mice were inoculated IC with 25 μl of a 10% brain homogenate prepared from normal or scrapie-infected sheep in PBS. For semen supernatant, samples were diluted 1 : 1 with PBS and 25 μl was injected IC. All mice were monitored a minimum of three times a week until termination of the experiments. Signs of clinical disease included initial hypersensitivity followed shortly thereafter by hypersensitivity and lethargy, ataxia, altered gait, disorientation, ruffled coat and hunched back. The incubation period is defined as the number of days p.i. to the time of termination. Mice were sacrificed using isoflurane and their brains immediately removed and stored at -80 °C.

**PMCA.** A 1% NSB homogenate was used as a source of PrPSc for PMCA. Sheep with PRNP genotypes A136R154R171/ARR and A136R154Q171/ARQ were used as a source of brain material for the homogenates. The homogenates, prepared in PBS containing 150 mM NaCl, 1% Triton X-100, 4 mM EDTA and complete protease inhibitor cocktail (Calbiochem), were centrifuged (400 g, 2 min) and the resulting supernatant was stored at -80 °C. Previous studies on PrPSc detection in body fluids demonstrated that genotype matching of the brain material used as a source of PrPSc with the source of the infected sample does not affect the extent or efficiency of PMCA (Rubenstein et al., 2010, 2011).

For sPMCA, semen samples (100 μl) were mixed with 10 μl of 1% NSB homogenate and incubated for 1 h at 37 °C with shaking followed by a 90 s pulse with a model Q-705A sonicator (500 W power output, 40000 J) (Qsonica). This is defined as one PMCA cycle. Prior to the start of each subsequent cycle, an additional 10 μl of 1% NSB homogenate was added. At the completion of 10 cycles, 100 μl of the contents was transferred to a new tube and the amplification cycles continued (including the addition of 10 μl of 1% NSB homogenate). At the completion of 80 serial PMCA cycles (sPMCA80) and 120 serial PMCA cycles (sPMCA120), a 200 μl aliquot of the PMCA products was removed for PK digestion, immunoprecipitation with mAb 8E9 and analysis by SOFIA.

**Immunoprecipitation.** One hundred microlitres of MagnaBind protein G beads (5 mg ml⁻¹) (Pierce) was washed three times with PBS containing 0.2% Tween 20 (PBST), resuspended in 1 ml PBS, and mixed with 100 μg mAb 8E9 in a final volume of 5 ml PBS. After constant shaking at room temperature for 1 h, the beads were magnetically separated, washed three times with PBST, resuspended in 5 ml PBS and then mixed with a 500 μl sample of the PMCA product. Following incubation on a rotator for 1 h at room temperature, the beads were magnetically separated, washed three times with PBST, resuspended in 500 μl PBS, and heated 100 °C for 15 min. After centrifugation at 16000 g for 2 min in a microcentrifuge, the supernatant was analysed by SOFIA.

**SOFIA.** Two hundred microlitres of mAb 11F12 (5 mg ml⁻¹) (Pierce) was washed three times with PBST containing 0.2% Tween 20 (PBST), resuspended in 1 ml PBS, and mixed with 100 μg mAb 8E9 in a final volume of 5 ml PBS. After constant shaking at room temperature for 1 h, the beads were magnetically separated, washed three times with PBST, resuspended in 500 μl PBS, and heated 100 °C for 15 min. After centrifugation at 16000 g for 2 min in a microcentrifuge, the supernatant was analysed by SOFIA.

**Western blot analysis.** Ten per cent sheep brain or TgSShpPrP mouse brain homogenates were prepared in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA; pH 8.0. A 100 μl sample of each homogenate was digested with 100 μg PK ml⁻¹ at 50 °C for 30 min followed by the addition of 1% complete protease inhibitor cocktail (Calbiochem). After the addition of 1 x Laemmli sample buffer, samples were heated at 100 °C for 10 min.
and the denatured proteins were separated by SDS PAGE. The proteins are transferred to a nitrocellulose membrane and the membrane was blocked overnight in 30 ml PBS containing 5% non-fat dry milk and 0.1% Tween 20. The proteins are immunode-
tected by incubation with 2 μg biotinylated mAb 08-6/2F7-2F11 ml⁻¹, washed three times for 10 min each in 50 ml 1× PBS containing 0.5% Tween 20 at room temperature, followed by incubation with streptavidin-HRP conjugate (1:5000; Invitrogen) for 60 min at room temperature in 1× PBS containing 0.5% Tween 20, and three washes for 10 min each in 1× PBS containing 0.5% Tween 20 at room temperature. Following a wash with distilled H₂O, proteins were detected using the ECL Supersignal West Dura kit following the manufacturer’s instructions (Thermo Scientific).

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