Silent Propagation of Classical Scrapie Prions in Homozygous K_{222} Transgenic Mice

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Classical scrapie affects sheep and goats. To control prevalence in sheep, the European Union initiated breeding programs targeting resilient genotypes. Although certain goat polymorphisms, such as $Q_{222}K$, are linked to resistance, specific breeding programs have not been implemented. Hemizygous transgenic mice carrying the goat K_{222} cellular prion protein (PrP) allele (K_{222}-Tg516) exhibited resistance to several classical scrapie isolates. We inoculated homozygous K_{222}-Tg516 and Q_{222}-Tg501 mice with various scrapie isolates. Homozygous K_{222}-Tg516 mice reached the end of their lifespan without exhibiting clinical signs; we observed brain proteinase K–resistant PrP accumulation in those mice that was lower than in Q_{222}-Tg501 mice. Histologically, K_{222}-Tg516 brains lacked prion-related lesions, except for the presence of few isolated scrapie PrP plaques in cases of isolates highly adapted to the K_{222}-PrP^C environment. Our findings caution against including that polymorphism in breeding programs, because it could lead to emergence of asymptomatic silent prion carriers of classical scrapie among goat populations.

Scrapie is a fatal infectious neurodegenerative disease inherent to sheep and goats that falls within the spectrum of transmissible spongiform encephalopathies (TSEs) or prion diseases. Of note, various mammals, including cattle with bovine spongiform encephalopathy (BSE), mink with transmissible mink encephalopathy, cervids with chronic wasting disease, and humans with Creutzfeldt-Jakob disease, can also succumb to TSEs. The hallmark of those diseases is posttranslational conversion of the host cellular prion protein (PrP), PrP^C, into a misfolded pathologic isoform causing scrapie, $PrPS^C$, which accumulates within the central nervous system of affected individuals (1).

Infection with TSEs in an organism is influenced by 2 main factors: the similarity between the primary PrP sequence of the host (recipient) and the donor (inoculum), and the prion strain (*2*). Together, those factors define the concept of the transmission barrier. Sheep and goats share the same PrP primary sequence, although polymorphisms differ between the animals. In sheep, high susceptibility to classical scrapie is associated with the $V_{136}R_{154}Q_{171}$ and $A_{136}R_{154}Q_{171}$ alleles, whereas the $A_{136}R_{154}R_{171}$ genotype is linked to resistance (*3*–*8*). To control and decrease classical scrapie in sheep, European Union member states have established breeding programs on the basis of the selection of the resistant $A_{136}R_{154}R_{171}$ allele, although the variant does not confer resistance against the atypical/Nor98 scrapie strain (*9*). In goats, some polymorphisms, such as $I_{142}M$ (*10*–*13*) and $N_{146}S$ (*14*), have been associated with resistance to scrapie infection.

The most promising results of studies were in regard to goat-resistant polymorphisms for the goat $Q_{222}K$ polymorphism. The lysine allele (K_{222}) was first reported to confer resistance in Italy (*15*,*16*), and similar results were later found in France (*10*) and Greece (*17*,*18*). Cell-free conversion assays also indicated that K_{222} provides protection against the ME7 scrapie strain (*19*). Experimental studies in goats found that heterozygous Q/K_{222} and homozygous K_{222} goats either showed resistance to classical scrapie or exhibited clear delays in incubation times after intracerebral or oral inoculation (*20*–*23*) and reduced contribution of K_{222} to proteinase K–resistant PrP (PrP^{res}) formation in Q/K_{222} heterozygous goats infected with scrapie (*24*). In addition, Q/K_{222} heterozygous goats were

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found to harbor a relative abundance of the natural α-cleaved PrP^C fragment C1, which has also been detected in classical scrapie-resistant R_{171} sheep (*25*). Furthermore, Q/K_{222} heterozygous goats inoculated with goat BSE showed neither evidence of clinical prion disease nor PrP^{Sc} accumulation in the brain or peripheral tissues (*26*,*27*), but low infectivity was detected after long postinoculation times (*26*). Finally, 1 goat harboring the K_{222}-PrP^C variant tested positive for atypical/Nor98 scrapie, indicating that the genotype may still be susceptible to this scrapie strain (*28*). All those results were replicated using a hemizygous transgenic mouse line expressing the K_{222}-PrP^C allele, which was found to be resistant to several classical scrapie isolates and cattle BSE, while susceptible to goat or sheep BSE and atypical scrapie (*29*,*30*).

We conducted our study on the transgenic homozygous mouse line, along with its control counterpart harboring the wild-type glutamine allele (Q_{222}). We intracranially inoculated the mice with several isolates representative of different categories of classical scrapie strains to test whether animals still remained uninfected, as previously reported (*29*), or if they mimicked the results found in homozygous goats (*22*).

Methods

Ethics Considerations

We performed animal experiments in strict accordance with the recommendations included in the guidelines of European Community Council 2010/63/UE and made all efforts to minimize animal suffering. The Committee on the Ethics of Animal Experiments of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria and the General Directorate of the Madrid Community Government approved the study (permit nos. CEEA 2011–050, PROEX 263/15).

Prion Transmission Studies

We intracranially inoculated 20 μL of 10% (wt/vol) brain homogenate from previously characterized classical scrapie isolates (Table 1) into the right parietal lobe of 5–7 transgenic mice (6–7 weeks old), which expressed either the wild-type goat PrP^C (Q_{222}-Tg501) or the K_{222}-PrP^C variant (K_{222}-Tg516) (*29*,*30*) in homozygosity. PrP^C expression levels of both mice lines were 2- to 4-fold the physiologic levels found in goat brain (*29*). We used a 25-gauge disposable hypodermic needle to inoculate animals while they were anesthetized with isoflurane.

After inoculation, we monitored mice daily and assessed their neurologic status twice a week. We euthanized animals when the progression of prion disease was evident, at the end of their lifespan (around 650 days postinoculation), or at previously established endpoints as part of a kinetic study. We harvested mouse brains and sliced them sagittally. We fixed half of each brain in 10% buffered formalin for histopathologic analysis and homogenized the remaining portion as 10% (wt/vol) in 5% glucose to detect PrP^{res} by Western blot.

We calculated survival time as the mean number days postinoculation for all mice that tested positive for PrP^{res} in the brain, with the SD included. We expressed attack rate as the proportion of PrP^{res}-positive mice among all the inoculated mice.

Western Blotting

We homogenized mouse brain tissue in 5% glucose solution in distilled water using grinding tubes (Bio-Rad Laboratories, https://www.bio-rad.com) and adjusted to 10% (wt/vol) using a TeSeE Precess 48TM homogenizer (Bio-Rad) according to the manufacturer's instructions. We determined PrP^{res} presence in transgenic mouse brains by Western blot analysis of 10–100 μL of 10% (wt/vol) brain homogenate, as previously described (*32*). We incubated membranes with the Sha31 monoclonal antibody (mAb) (*33*), which recognizes the $_{148}$YEDRYYRE$_{155}$ epitope of the goat PrP sequence. We detected immunocomplexes with horseradish peroxidase-conjugated mouse IgG (GE HealthCare, https://www.gehealthcare.com) after 1 hour of incubation. We visualized immunoreactivity by chemiluminescence with ECL Select (GE HealthCare). We captured images using ChemiDoc XRS + System (Bio-Rad) and processed them using Image Lab 5.2.1 software (Bio-Rad).

Histologic Analysis

To analyze brain tissue, we trimmed and dehydrated formalin-fixed brains, embedded them in paraffin wax, and cut 4-μm slices. We dewaxed and rehydrated the specimens by standard procedures. We established the vacuolar lesion profile of the brains in accordance with published standard methods and semiquantitatively scored vacuolation on a scale of 0–5 in different brain areas (*34*,*35*).

For immunohistochemical (IHC) demonstration of PrP^{Sc} accumulation, tissue sections underwent antigen retrieval and hydrogen peroxide quenching as previously described (*36*). We incubated the sections with 2A11 mAb (*37*), which recognizes the $_{163}$QVYYRPVDQ$_{171}$ epitope of the goat PrP sequence. Subsequently, we subjected the sections to antigen retrieval and inactivation of endogenous peroxidase activity

before incubating them with the 2A11 mAb. We used a commercial immunoperoxidase technique (VECTASTAIN Elite ABC Kit; Vector Laboratories, https://vectorlabs.com), according to the manufacturer's instructions. Finally, we counterstained the sections with Mayer's hematoxylin. We used the Sha31 mAb (*33*) for paraffin-embedded tissue blotting, as previously described (*38*,*39*).

Results

Homozygous K_{222}-Tg516 Mice and Resistance to Classical Scrapie PrP^{Sc}

We intracranially inoculated homozygous K_{222}-Tg516 with classical scrapie isolates (Table 1) previously characterized as representative of different prion strains circulating in Europe (*31, 40*). Although all mice expressing the wild-type goat PrP (Q_{222}-Tg501) developed recognizable prion disease, K_{222}-Tg516 mice reached the end of their lifespan without showing clinical signs indicative of prion disease (Table 2). After second passage, survival times were still prolonged, even reaching the end of the mice's lifespan again (Table 2). However, in both first and second passages, Western blot analysis showed the presence of PrP^{res} in the brains of K_{222}-Tg516 animals inoculated with the different classical scrapie isolates (Figure 1, panel A). For the 198/9 and S2 isolate, the percentage of PrP^{res}-positive animals in the first passage was not 100% of the inoculated animals (Tables 2, 3). At least for the S2 isolate, 100% of the inoculated mice were PrP^{res}-positive by the completion of the second passage (Tables 2, 3). Comparison between the PrP^{res} signature of the original inoculum and the PrP^{res} obtained molecular mass for the nonglycosylated band, depending on the individual (Figure 1, panel A). In addition, brain PrP^{res} accumulation in K_{222}-Tg516 mice was remarkably reduced compared with that in Q_{222}-Tg501 mice for most of the inoculated isolates, with the exception of F14 and F10 (Figure 1, panel A).

K_{222}-Tg516 PrP^{res}-positive animals exhibited only a few vacuolations that were difficult to distinguish from those resulting from the physiologic aging process (Figure 2). Immunohistochemistry of K_{222}-Tg516 mice inoculated with CP060146/K_{222} goat and F10/K_{222}-Tg516 inocula revealed only a few large and focalized PrP^{Sc} plaques and lacked any other type of deposits affecting neurons or microglia cells (Figures 3, 4). Those PrP^{Sc} deposits were restricted to the mesencephalon, thalamus, and hypothalamus areas (Figures 3, 4). We detected no deposits for the remaining inoculations (data not shown). Consistent with our findings, paraffin-embedded tissue blotting showed clear PrP^{res} deposition only in K_{222}-Tg516 mice inoculated with CP060146/K_{222} goat (Figure 5) and F10/K_{222}-Tg516 inocula (data not shown), with deposition to the exact same brain areas affected by IHC (Figures 3, 4). We detected no deposits for the remaining inoculations (data not shown).

Proteinase K Studies in K_{222}-Tg516 Mice

The differential brain PrP^{res} accumulation observed between K_{222}-Tg516 and Q_{222}-Tg501 mice (Figure 1,

Table 1. Isolates used in study of classical scrapie prions in homozygous K_{222} transgenic mice*

Category	Isolate	Species	Origin	Goat PrP genotype†	Description	Supplier
I	198/9	Sheep	Italy	wt; $S_{240}S$	Classical scrapie isolate from a naturally infected sheep	ISS
II	S2	Goat	Spain	wt; $S_{240}P$	Classical scrapie isolate from a naturally infected goat	UNIZAR
	CP060146 (*22*)	Goat	France	wt	Classical scrapie isolate from an experimentally infected goat	ENVT
	CP060146/K_{222} (*22*)	Goat	France	K_{222}	Classical scrapie isolate from an experimentally infected goat	ENVT
II + III	UKA2	Goat	United Kingdom	wt; $S_{240}P$	Classical scrapie isolate from a naturally infected goat	APHA
	F14	Goat	France	wt; $I_{142}M$, $S_{240}P$	Classical scrapie isolate from a naturally infected goat	INRA
IV	F10	Goat	France	wt; $S_{240}P$	Classical scrapie isolate from a naturally infected goat	INRA
	C1	Goat	Cyprus	wt	Classical scrapie isolate from a naturally infected goat	VS
Negative control	Healthy goat brain	Goat	France	wt	Brain from a noninfected goat	INRA

*Isolates were classified as previously described (*31*) on the basis of prion biochemical features when transmitted in transgenic mice expressing the bovine PrP (Bo-Tg110) and biologic features transmitted in mice expressing the ovine PrP (Q_{222}-Tg501). APHA, Animal and Plant Health Agency, Surrey, United Kingdom; ENVT, École Nationale Vétérinaire de Toulouse, Toulouse, France; INRA, French National Institute for Agricultural Research, Nouzilly, France; ISS, Istituto Superiore di Sanitá Animal, Rome, Italy; UNIZAR, Universidad de Zaragoza, Spain; VS, Veterinary Services, Nicosia, Cyprus; wt, wild-type.

†The wt goat prion protein genotype is $A_{136}R_{154}P_{240}/A_{136}R_{154}P_{240}$. S240S, S240P and I142M refer to polymorphisms at specific codons of the PRNP gene.

Table 2. Transmission of classical scrapie isolates to Q_{222}-Tg501 homozygous mice and survival of mice in study of classical scrapie prions in homozygous K_{222} transgenic mice*

		1st passage		2nd passage	
Category	Isolate	Mean survival time ± SD, d	No. diseased and PrP^{res}-positive/no. inoculated	Mean survival time ± SD, d	No. diseased and PrP^{res}-positive/no. inoculated
I	198/9	592 ± 13	6/6	536 ± 46	5/5
II	S2	228 ± 15	6/6	233 ± 4	6/6
	CP060146	379 ± 31	5/5	ND	NA
	CP060146/K_{222} goat	415 ± 40	6/6	ND	NA
II + III	UKA2	245 ± 36	5/5	252 ± 8	6/6
	F14	526 ± 46	4/4	241 ± 22	4/4
IV	F10	449 ± 19	5/5	372 ± 14	6/6
	F10/K_{222}-Tg516	495 ± 26	3/3	ND	NA
	C1	483 ± 15	4/4	301 ± 10	4/4
Negative control	Healthy goat brain	>650	0/6†	>650	0/6†

*NA, not available; ND, not done; PrP^{res}, proteinase K–resistant PrP.
†Animals were found dead or were euthanized at the end of their lifespan without showing clinical signs of classical scrapie.

panel A) can be attributed to 2 alternative hypotheses. There could be a genuine reduction in PrP^{res} accumulation for these classical scrapie isolates in K_{222}-Tg516 mice. Alternatively, the produced PrP^{res} might be more susceptible to proteinase K treatment, resulting in a weaker Western-blotting signal. To distinguish between those 2 possibilities, we performed proteinase K resistance analyses using different enzyme concentrations in both Q_{222}-Tg501 and K_{222}-Tg516 mice inoculated with F10 (which exhibited similar PrP^{res} accumulation between K_{222}-Tg516 and Q_{222}-Tg501 mice) and CP060146 (which showed reduced PrP^{res} accumulation in K_{222}-Tg516 mice compared with Q_{222}-Tg501 mice) isolates. In all cases, proteinase K consistently acted at a concentration of 50 μg/mL (which falls within the normal proteinase K concentration range for routine Western blotting); we observed the same pattern and signal intensity at a concentration of 500 μg/mL (Figure 6). However, protease action did not achieve proper PrP^{res} resolution at concentrations of 1 μg/mL and 0.1 μg/mL (Figure 6). Those results suggest that both isolates, when replicating in either Q_{222}-PrP^C or K_{222}-PrP^C contexts, retain the same proteinase K sensitivity. Thus, the differences in Western blotting signals detected previously (Figure 1, panel A) truly account for reduced brain PrP^{res} accumulation in K_{222}-Tg516 mice.

Transmission in K_{222}-Tg516 Mice and Host-Induced Reversible Strain Adaptations

After the second passage in K_{222}-Tg516 mice or adaptation in a K_{222} homozygous goat, F10 and CP060146 isolates were transmitted back into Q_{222}-Tg501 mice (Table 3). The purpose of those inoculations was to determine whether replication in the K_{222} context resulted in host-induced reversible adaptations of the

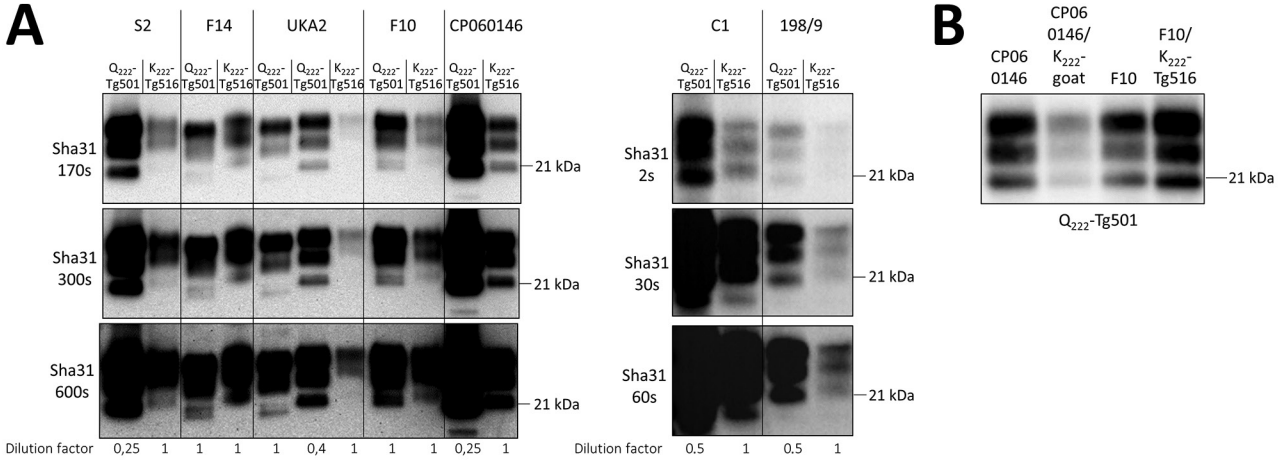

Figure 1. Proteinase K–resistant PrP (PrP^{res}) accumulation in brains of K_{222}-Tg516 and Q_{222}-Tg501 homozygous mice in study of propagation of classical scrapie prions. A) Comparison of the biochemical profile of brain PrP^{res} from classical scrapie isolates in K_{222}-Tg516 mice with that in Q_{222}-Tg501 mice using Sha31 monoclonal antibody. Exposure time and dilution factor are specified. B) Comparison of the biochemical profile of brain PrP^{res} of CP060146 and F10 isolates of classical scrapie, before (left) and after (right) adaptation to the K_{222}-PrP^C context, in Q_{222}-Tg501 mice, using the Sha31 monoclonal antibody. Molecular weight markers are indicated on the right side of each band.

Table 3. Transmission of classical scrapie isolates to K_{222}-Tg516 homozygous mice in study of classical scrapie prions in homozygous K_{222} transgenic mice*

Category	Isolate	1st passage		2nd passage	
		Mean survival time ± SD, d	No. diseased and PrP^{res}-positive/no. inoculated	Mean survival time ± SD, d	No. diseased and PrP^{res}-positive/no. inoculated
I	198/9	>650	1/6†	ND	NA
II	S2	>650	3/4†	>650	7/7†
	CP060146	>650	5/5†	>650	5/5†
	CP060146/K_{222} goat	>650	4/4†	>650	6/6†
II + III	UKA2	>650	4/4†	>650	5/5†
	F14	>650	4/4†	>650	5/5†
IV	F10	>650	6/6†	>650	5/5†
	F10/K_{222}-Tg516	>650	5/5†	>650	6/6†
	C1	>650	7/7†	ND	NA
Negative control	Healthy goat brain	>650	0/6†	>650	0/6†

*NA, not available; ND, not done; PrP^{res}, proteinase K–resistant PrP.
†Animals were found dead or euthanized at the end of their lifespan without showing clinical signs of classical scrapie.

strain, including changes in prion strain characteristics such as biologic properties (mean survival time and proportion of PrP^{res}-positive animals) and biochemical properties (brain PrP^{res} accumulation and PrP^{res} glycosylation pattern). In both cases, the survival times were comparable to those observed for the primary transmission of the same inocula in Q222-Tg501 mice. Specifically, survival time was 449 ± 19 days (5/5) for the original F10 inoculum from a wild-type goat versus 495 ± 26 days (3/3) after adaptation in K_{222}-Tg516 mice and 379 ± 31 days (5/5) for the original CP060146 inoculum from a wild-type goat versus 415 ± 40 days (6/6) after adaptation in a K_{222} goat (Table 2). The PrP^{res} signatures obtained were identical to those observed after the primary transmission of these isolates in Q_{222}-Tg501 mice (Figure 1, panel B).

Differences between K_{222} and Q_{222} PrP^{res} Formation Kinetics

Once we confirmed the lower brain PrP^{res} accumulation in K_{222}-Tg516 mice compared with the Q_{222}-Tg501 control counterparts, we conducted kinetic studies on PrP^{res} formation in both transgenic lines using the goat isolates F10 and CP060146, which had been previously adapted for propagation in a K_{222}-PrP^{C} context. Of interest, both K_{222} and Q_{222}-PrP^{res} appeared at equal levels by 300 days postinoculation (Figure 7). However, Q_{222}-PrP^{res} accumulation continued to increase steadily until the time of death, whereas K_{222}-PrP^{res} remained at low levels throughout the lifespan of the mice (Figure 7).

Discussion

Previous studies conducted in heterozygous Q/K_{222} and homozygous K_{222} goats (*20*–*23*), as well as in hemizygous K_{222}-Tg516 mice (*29*), have highlighted the $Q_{222}K$ polymorphism as one of the most promising candidates for reducing prion disease transmission in goats. Although the K_{222} allele has been consistently reported in certain countries in Europe, such as Italy (*15*,*16*), France (*10*), and Greece (*17*,*42*), in other countries, such as the United Kingdom, the polymorphism has been reported as infrequent (*43*). However, once

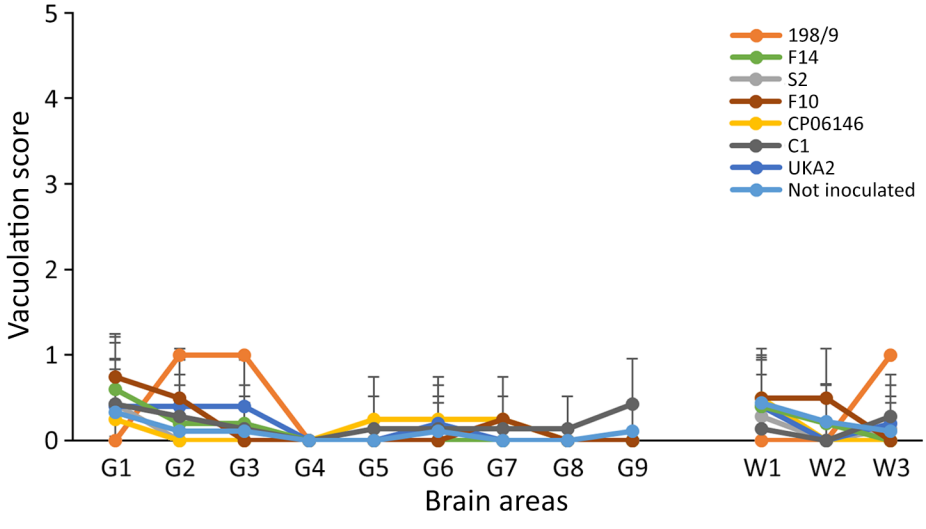

Figure 2. Histologic analysis of brain tissue from K_{222}-Tg516 homozygous mice inoculated with classical scrapie in study of propagation of classical scrapie prions. Comparative analysis shows the vacuolar lesion profile in homozygous K_{222}-Tg516 mice inoculated with different scrapie isolates compared with noninoculated mice. G, gray matter; W, white matter.

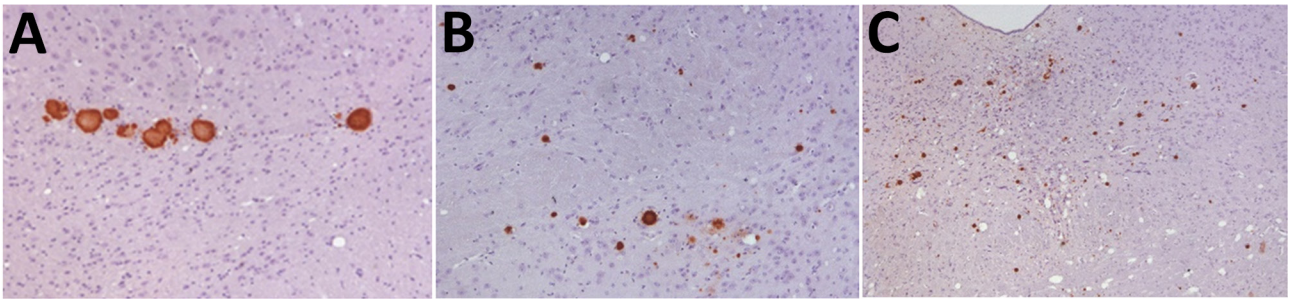

Figure 3. Immunohistochemistry results of brain tissue in study of propagation of classical scrapie prions. Images are of tissue specimens from K_{222}-Tg516 mice inoculated with F10 goat scrapie isolate at second passage. Results are visualized using the Sha31 monoclonal antibody. A) Thalamus specimen. B) Hippocampus specimen. C) Midbrain specimen. Original magnification ×40.

the supposed protective effect against prion diseases is confirmed, the frequency of the K_{222} allele could increase across different countries through selective breeding programs.

Transgenic mice expressing K_{222}-PrP^C in homozygosity emerge as the optimal tool for definitively testing the susceptibility or resistance that allele confers to prions. Our model enables the testing of multiple prion strains more rapidly and cost-effectively than the model using goats. In our study, classical scrapie isolates representing different classical scrapie strains circulating within Europe (*40*–*42*) were selected and used to challenge homozygous K_{222}-Tg516 mice.

Once the expression level is increased, homozygous K_{222}-Tg516 mice become susceptible to all tested classical scrapie isolates (Table 3). The K_{222}-PrP^C variant is capable of sustaining PrP^{Sc} replication even in the absence of the Q_{222}-PrP^C variant, which was identified as responsible for most accumulated brain PrP^{res} in $Q_{222}K$ heterozygous goats (*24*). Furthermore, K_{222}-Tg516 mice exhibit consistently lower brain PrP^{res} accumulation than Q_{222}-Tg501 mice (Figure 1). The explanation that K_{222}-PrP^{res} is more sensitive to proteinase K treatment and so reduced detection of brain PrP^{res} accumulation has been ruled out (Figure 6). Therefore, we recommend careful analysis of the general features and behavior of classical scrapie K_{222}-PrP^{res}.

K_{222}-Tg516 mice inoculated with classical scrapie did not develop typical prion pathology and showed no clinical signs of prion disease, which suggests that classical scrapie K_{222}-PrP^{res} might not be toxic or might not induce the signaling pathways leading to neuronal death. Those conclusions are not only caused by insufficient time for the onset of neuronal death pathways within the animal lifespan; second passages in K_{222}-Tg516 yielded identical results to the first ones.

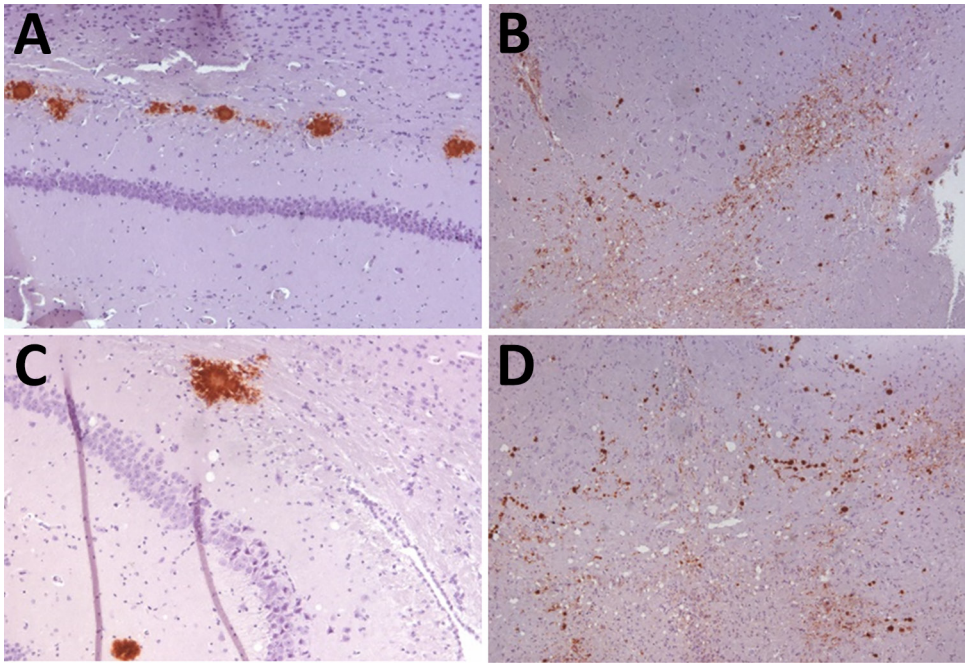

Figure 4. Immunohistochemistry results of brain tissues in study of propagation of classical scrapie prions. Images are of tissue specimens from K_{222}-Tg516 mice inoculated with CP060146/K_{222} goat isolate. Results are visualized using the Sha31 monoclonal antibody. A) Hippocampus specimen tested at first passage. B) Midbrain specimen tested at first passage. C) Hippocampus specimen tested at second passage. D) Midbrain specimen tested at second passage. Original magnification ×40.

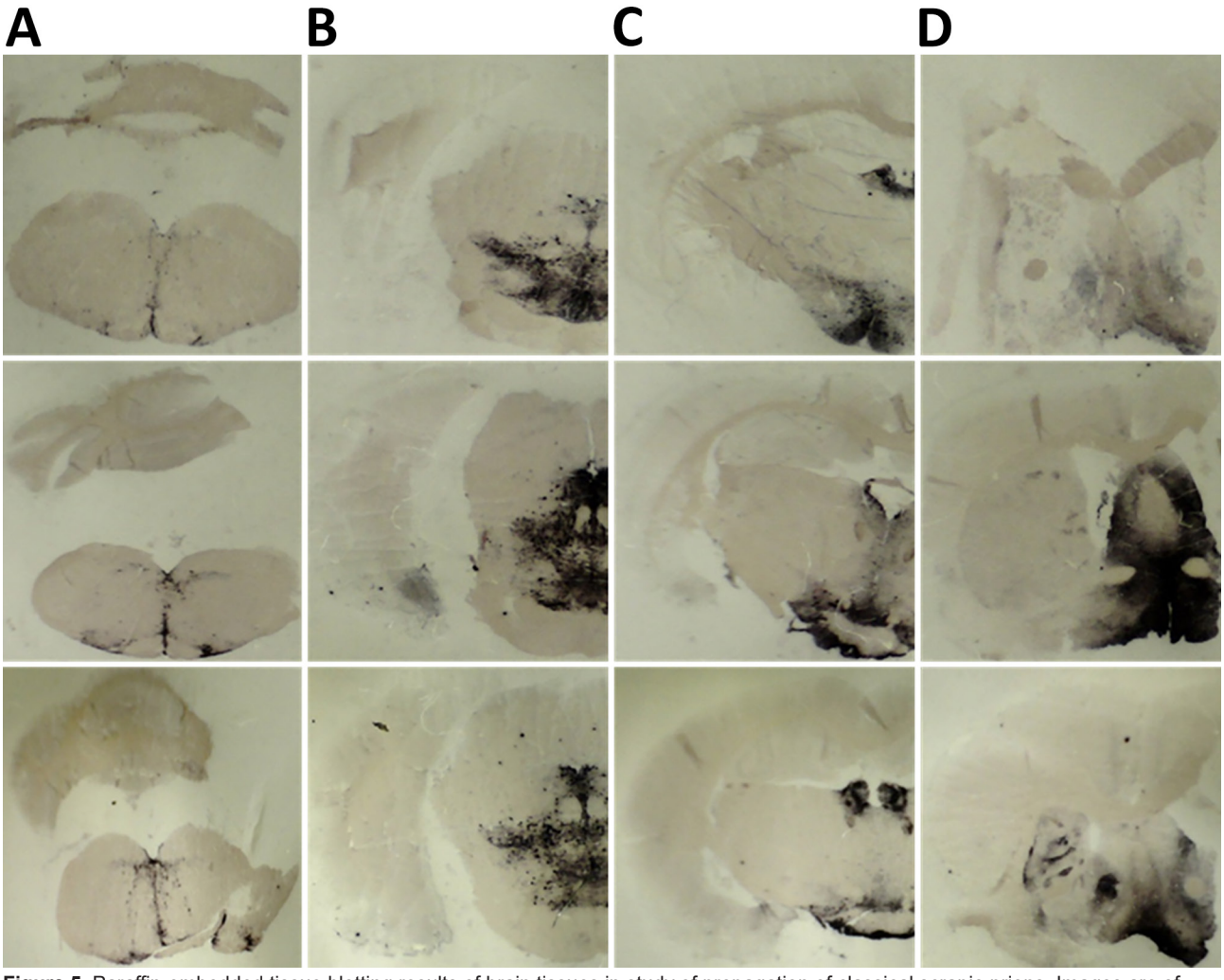

Figure 5. Paraffin-embedded tissue blotting results of brain tissues in study of propagation of classical scrapie prions. Images are of brain specimens from 3 distinct K_{222}-Tg516 mice inoculated with CP060146/K_{222} goat isolate. Results are visualized with the Sha31 monoclonal antibody. A) Cerebellum specimens. B) Thalamus specimens. C) Hippocampus specimens. D) Cerebral cortex specimens. Proteinase K–resistant prion protein is visible as dark staining in similar brain regions in the 3 mice. Original magnification ×20.

However, we noted that the lower brain PrP^{res} accumulation in K_{222}-Tg516 animals could lead to a misinterpretation of those results. The reduced accumulation might reflect insufficient replication within the animal's lifespan, possibly caused by consistently low replication rates, as suggested by our kinetic experiments, or by more efficient clearance of PrP^{res} aggregates. Those factors could explain why transmission does not necessarily result in prion disease, highlighting a dissociation between infectivity and toxicity of classical scrapie K_{222}-PrP^{res}.

All circulating prion strains must be considered in the design of breeding selection programs. Programs aimed at controlling and reducing classical scrapie in sheep, implemented by EU member states, have identified sheep herds that are more susceptible to atypical/Nor98 scrapie (*44*). In our study, K_{222}-Tg516 mice died without exhibiting overt clinical signs after inoculation with different classical scrapie isolates; we found that PrP^{res} accumulated in their brains (Table 1). Of note, K_{222}-derived PrP^{res} retained infectivity when transmitted back to Q_{222}-Tg501 mice, recovering the strain characteristics observed in the original inocula. Our findings suggest that, under the experimental conditions we established, the K_{222} allele does not confer full resistance to classical scrapie agents.

Of interest, the reversibility of strain features observed upon reinoculation of K_{222}-derived PrP^{res} into Q_{222}-Tg501 mice is reminiscent of the phenomenon of nonadaptive prion amplification as described previously (*45*). In that model, PrP^{Sc} can replicate transiently in a nonpermissive host without inducing a permanent

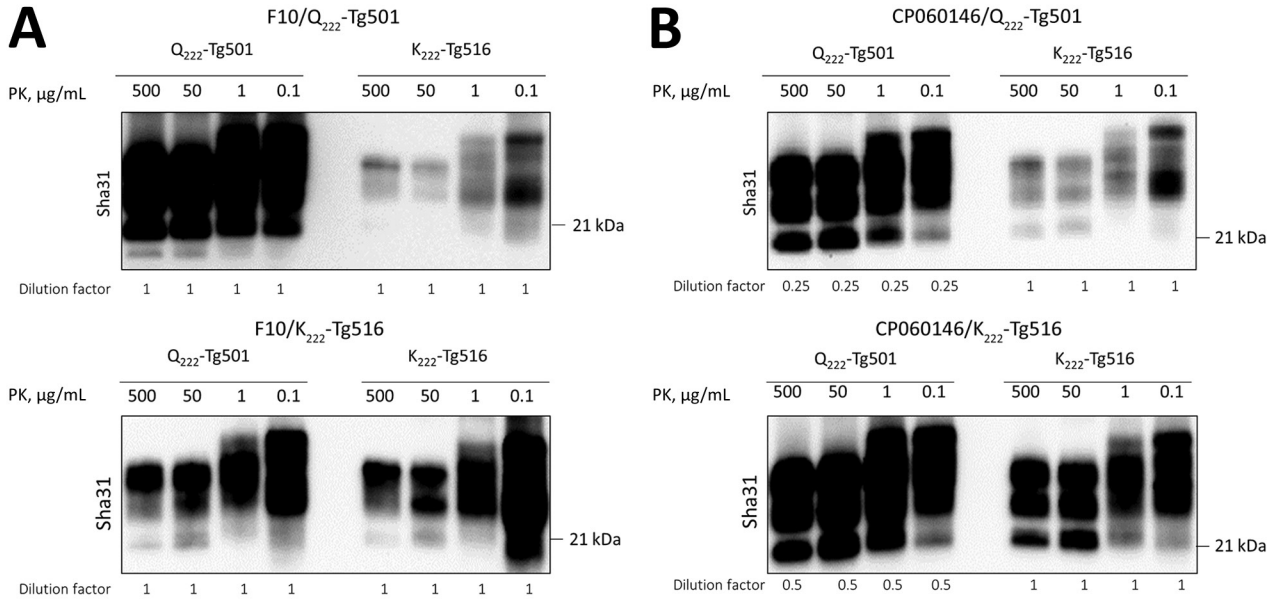

Figure 6. Proteinase K digestion studies conducted as part of study of propagation of classical scrapie prions. K_{222}-Tg516 and Q_{222}-Tg501 homozygous mice were inoculated with classical scrapie. A) Proteinase K–resistant prion protein (PrP^{res}) sensitivity in the brains of Q_{222}-Tg501 and K_{222}-Tg516 mice initially inoculated with F10 scrapie isolate and subsequently reinoculated into both the original model and its counterpart. B) PrP^{res} sensitivity in the brains of Q_{222}-Tg501 and K_{222}-Tg516 mice initially inoculated with CP060146 scrapie isolate and subsequently reinoculated into both the original model and its counterpart. In both cases, proteinase K concentrations of 500, 50, 1 and 0.1 µg/mL were tested. Western blot visualizations were done using the Sha31 monoclonal antibody. Molecular weight markers are indicated on the right side of each band.

adaptation of the strain. Our data are consistent with that concept; the classical scrapie agents replicated in K_{222}-Tg516 mice but reverted to their original biochemical and biologic properties upon passage back into a permissive Q_{222} context. That interpretation reinforces the view that the K_{222} allele may enable subclinical or low-efficiency replication of classical scrapie agents without supporting stable strain selection or adaptation.

It is important to note that the use of transgenic models with PrP overexpression may enhance prion replication efficiency, potentially uncovering low-level or subclinical conversion events that might not occur under physiologic PrP expression in goats. In addition, all animals were inoculated intracerebrally; that route does not mimic natural exposure and bypasses key peripheral barriers such as the gut and

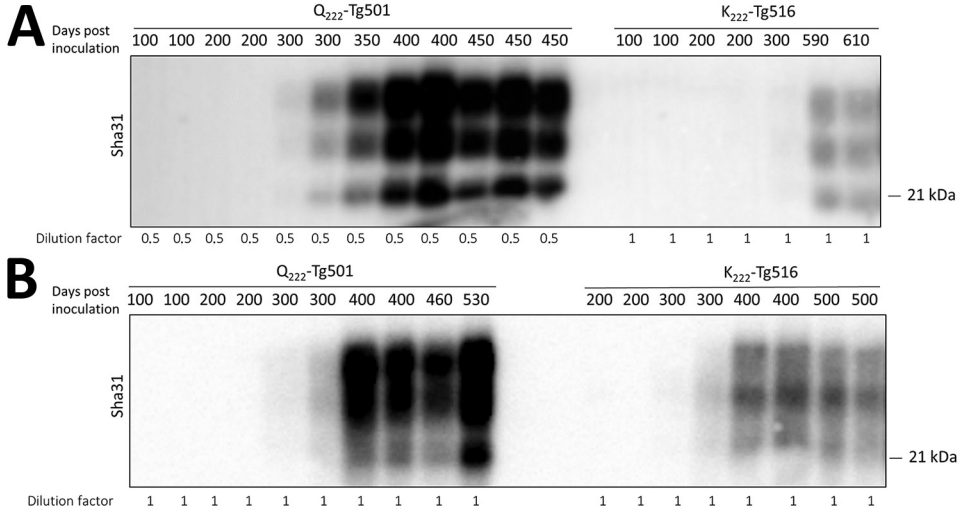

Figure 7. Kinetic studies of proteinase K–resistant prion protein (PrP^{res}) detection in K_{222}-Tg516 and Q_{222}-Tg501 homozygous mice inoculated with classical scrapie in study of propagation of classical scrapie prions. Brain PrP^{res} from mice euthanized at various time points postinoculation were analyzed by Western blotting and visualized using the Sha31 monoclonal antibody. A) Q_{222}-Tg501 and K_{222}-Tg516 mice inoculated with the CP060146 classical scrapie isolate adapted to the K_{222} cellular prion protein (PrP^{C}) context (CP060146/K_{222}-goat). B) Q_{222}-Tg501 and K_{222}-Tg516 mice inoculated with the F10 classical scrapie isolate adapted to the K_{222}-PrP^{C} context (F10/K_{222}-Tg516). Molecular weight markers are indicated on the right side of each band.

associated lymphoid tissues, which play a critical role in determining prion susceptibility and pathogenesis under field conditions. Therefore, although our results highlight the potential for silent propagation of classical scrapie strains in the context of the K_{222} variant, extrapolation to the natural host should be made with caution.

Interest has grown for in-depth characterization of the strains of Q/K_{222} heterozygous goats affected with scrapie, which are abundant in various regions of Greece. The interest lies in determining whether prions propagated under the K_{222} allele can act as potential silent carriers of the disease, as shown in previous studies. Furthermore, understanding whether the presence of the K_{222} allele induces a change in the biologic properties of the strains and their potential transmission to other animal species is crucial.

Overall, our results underscore the need for further in vivo studies using physiologically relevant models or natural hosts to fully evaluate the protective efficacy of the K_{222} allele. Until such evidence becomes available, the inclusion of the K_{222} polymorphism in breeding selection programs should be critically considered, especially in regions where classical scrapie strains with known zoonotic potential remain present. Furthermore, experiments conducted in classical BSE-inoculated Q/K_{222} heterozygous goats have shown at least low infectivity in goat tissues after long postinoculation periods (*26*), whereas heterozygous K_{222}-Tg516 mice were already fully susceptible to goat BSE (*29*). In addition, at least 1 Q/K_{222} heterozygous goat tested positive for atypical/Nor98 scrapie (*28*), and homozygous K_{222}-Tg516 mice were found to be completely susceptible to atypical/Nor98 scrapie (*30*). Taken together, those data suggest that the protective effect of the $Q_{222}K$ polymorphism may be limited, and its use in breeding programs should be carefully evaluated.

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