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Correlation of Cellular Factors and Differential Scrapie Prion Permissiveness in Ovine Microglia

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Abstract

Prion diseases are fatal neurodegenerative disorders by which the native cellular prion protein (PrP^C) is misfolded into an accumulating, disease-associated isoform (PrP^D). To improve the understanding of prion pathogenesis and develop effective treatments, it is essential to elucidate factors contributing to cellular permissiveness. We previously isolated five clones from an immortalized subline of ovine microglia, two of which had demonstrated differential permissiveness to a natural isolate of sheep scrapie and distinct transcriptomic profiles. To more robustly identify factors contributing to this activity, relative permissiveness, cell proliferation, selected gene transcript level, and matrix metalloproteinase 2 (MMP2) activity were compared amongst all five clones. Differences in cell proliferation were not detected between clones; however, significant correlations were identified between relative permissiveness and genes associated with cell growth (i.e., *RARRES1* and *PTN*), protein degradation (i.e., *CTSB* and *SQSTM1*), and heparin binding (i.e., *SEPP1*). MMP2 activity varied amongst clones, but did not correlate with permissiveness. These associations support the contribution of cell division and protein degradation on the permissiveness of cultured ovine microglia to PrP^D.

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Keywords

Scrapie; ovine microglia; permissiveness; extracellular matrix; matrix metalloproteinase; cell proliferation

1. Introduction

Prion diseases are fatal neurodegenerative disorders that affect humans (e.g., Creutzfeldt-Jakob disease) and several animal species (e.g., scrapie in sheep) (Aguzzi and Calella, 2009). A key event in pathogenesis is the conversion of the host-encoded, cellular form of the prion protein (PrP^C; C superscript for “cellular”) into misfolded isoforms (PrP^D; D superscript for “disease-associated”) (Prusiner, 1998). PrP^C is a cell-surface protein encoded by the prion gene (*PRNP*) and is highly expressed in multiple cell types, notably those of the central nervous system (CNS) (e.g., neurons and microglia) (Aguzzi and Calella, 2009; Prusiner, 1998). Accumulation of PrP^D within the CNS leads to slowly progressive neurodegeneration and death (Aguzzi and Calella, 2009; Prusiner, 1998). The development of therapeutics is in part hampered by incomplete knowledge about cellular pathogenesis, including mechanisms underlying cellular permissiveness to prions.

While it is well established that cellular expression of PrP^C is required for infection (Bueler et al., 1993; Vilette et al., 2001), expression level alone is insufficient to explain variation in permissibility. Certain changes to the amino acid sequence of PrP^C dramatically affect relative susceptibility to infection and disease incubation time (Belt et al., 1995; Goldmann et al., 1994). However, other factors must also play a role given the differences in susceptibility and disease observed between mice bearing the same *PRNP* genotype (Lloyd et al., 2001) and the differential permissiveness observed between clones of cultured cell lines similarly expressing sequence-matched PrP^C (Klohn et al., 2003; Munoz-Gutierrez et al., 2015; Neale et al., 2010; Polymenidou et al., 2008). Such observations clearly suggest the existence of non-PrP^C factors that affect cellular permissiveness to prions.

Factors that impact PrP conversion, localization, and degradation may affect cellular permissiveness to prion infection (Ghaemmaghami et al., 2007; Grassmann et al., 2013). Cell division (Ghaemmaghami et al., 2007) and the expression of extracellular matrix (ECM) components (Marbiah et al., 2014) are specifically known to influence prion propagation in a murine neuroblastoma cell line. For example, prion permissiveness of this model system was related to a gene network that in part regulates homeostasis of the ECM (Marbiah et al., 2014). Functions of the identified genes were dependent on cellular differentiation and included activation of matrix metalloproteinase 2 (MMP2) (e.g., through *Fnl1* expression), sulfation of glycosaminoglycans (e.g., through *Papss2* expression), and regulation of cell shape and motility (e.g., through *NCKAP1L* expression (Weiner et al., 2007)). These studies did not, however, determine if there were key hyposulfated proteoglycans nor relate variation of ECM components with the mitotic rate. Furthermore, the above studies were limited to murine neuroblastoma cells.

We have similarly investigated an immortalized ovine microglia subline for factors that affect permissiveness to a *PRNP*-homologous source of sheep brain-derived scrapie prions.

In an initial study comparing two clones of this subline (Munoz-Gutierrez et al., 2016), we demonstrated that differential permissiveness was not due to differences in PrP^C expression and, by analysis of the transcriptome, revealed an association with differential regulation of numerous genes, including those with roles specific to heparin binding (e.g., *SEPP1*), cell proliferation (e.g., *RARRES1*), protein degradation (e.g., *SQSTM1*), and the ECM (e.g., *MMP14*).

In the study described herein, these and other factors were more closely and robustly evaluated by comparing the capacity of prion propagation in five clones of the immortalized ovine microglia subline described above. Permissibility was measured using the Standard Scrapie Cell Assay (SSCA) and tested for correlation with cell proliferation rate, MMP2 activity, and transcript levels from a selected subset of genes previously correlated with permissiveness to prions (Munoz-Gutierrez et al., 2016).

2. Materials and methods

2.1 Cell lines and inoculation

The ovine cell line used in this study was originally isolated, immortalized, cloned, and characterized in previous studies (Munoz-Gutierrez et al., 2016; Munoz-Gutierrez et al., 2015), which were approved by the Institutional Animal Care and Use Committees of Washington State University (ASAF04575). For these experiments, five clones (434, 438, 439, 440, and 441) previously generated from human telomerase (hTERT) immortalized ovine microglia subline H were utilized (Munoz-Gutierrez et al., 2015). Naïve clones were maintained in culture at 37 °C in OMEM (Opti-MEM [Gibco] medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 IU of penicillin, and 10 mg streptomycin ml⁻¹) and passaged every 5 days. For passaging, cells were lifted with 1× trypsin-EDTA in 1× PBS (Gibco).

The clones were inoculated with a sheep-derived scrapie isolate (Utah isolate) as previously described (Munoz-Gutierrez et al., 2015). Briefly, cells were plated at a density of approximately 1×10⁵ cells per well into a twelve-well tissue culture plate. After overnight incubation, media was exchanged for 500 µl of 2.5% (w/v) scrapie (Utah) brain homogenate in OMEM. Mock-inoculated (i.e., negative control) cells received 500 µl of OMEM. Cells were incubated for six hours after which 500 µl of OMEM were added to each well. Cells were incubated for 5 additional days prior to the replacement of inoculum with fresh OMEM. Following 9 additional days of incubation, cells were expanded to 25-cm² culture flasks and split 1/5 every 2 weeks with fresh OMEM added weekly.

2.2 Quantification of PrP^{res} accumulation by SSCA

To measure the permissiveness of clones to scrapie prion infection, the capacity of PrP^{res} propagation (res superscript for proteinase K [PK]-resistant PrP^D) was evaluated at post-inoculation passages 3 (P-3), 5 (P-5), and 8 (P-8) using the Standard Scrapie Cell Assay (SSCA) (Mahal et al., 2008) as adapted to sheep microglia (Dinkel et al., 2016). The SSCA was selected as the measure of prion permissibility since it measures the percent of a cell population that has detectable levels of PrP^{res}. Thus, a higher percentage of positive cells

within a sample indicates that the cells are more permissive to prion propagation. This is in contrast to assays such as immunoblots, which measure PrP^{res} in a pooled sample, and thus may have the same number of positive cells, but with an increased amount of PrP^{res} in those positive cells. Cells were collected and plated at an estimated density of 20,000 cells/well into a 96-well ELISpot plate (MultiScreen-IP 0.45- μ M filter plate, Millipore) (Mahal et al., 2008). For each clone, eight Utah-inoculated (i.e., scrapie-inoculated cells) and eight mock-inoculated (i.e., OMEM-treated cells) wells were plated. Cells were treated with 0.6 μ g PK ml⁻¹ (2.5 units mg⁻¹, Roche Applied Science, Indianapolis, IN, USA) in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 150 mM NaCl) for 90 minutes shaking at 37 °C. Cells were subsequently labeled with the anti-PrP mAb 6H4 (0.5 μ g ml⁻¹; Prionics, Zurich, Switzerland) and an alkaline-phosphatase-conjugated anti-mouse IgG₁ antibody (1:2000; Southern Biotechnology Associates, Birmingham, AL, USA) (Dinkel et al., 2016). A cutoff value representing the 95% upper confidence limit of the average spot number detected in all mock-inoculated wells (i.e., background spot number) was calculated (Frey et al., 1998) to objectively categorize clones as PrP^{res}-positive or -negative. Permissiveness was expressed as the average spot number determined from at least 3 independent replicate assays and statistically compared as described in subsection 2.7.

2.3 PrP^{res} evaluation by immunoblot

PrP^{res} accumulation was also evaluated by immunoblot, to confirm the molecular weight and glycoform pattern. At P-6, cells were collected by lifting and centrifugation at 1,000 $\times g$ (JA50.5 rotor) for 7 minutes at room temperature (RT). Cells were washed with 1 $\times$ Dulbecco's-PBS (D-PBS) and pellets were frozen at -80 °C until testing. Cell pellets were lysed and lysates were electrophoresed, electroblotted, and immunoblotted as previously described (Stanton et al., 2008). Briefly, lysates were measured for total protein by the bicinchoninic acid protein assay (BCA kit; Thermo Scientific) and diluted to equivalent protein concentrations prior to proteinase K (50 μ g μ l⁻¹, 2.5 units mg⁻¹, Roche Applied Science) digestion and phosphotungstic acid (PTA) precipitation; both steps included a 1-hour incubation at 37 °C. PrP^{res} bands were labeled using 3.5 μ g ml⁻¹ of the mAb F99/97.6.1 (O'Rourke et al., 2000) and a goat anti-mouse IgG₁ antibody conjugated to HRP (1:5000; Southern Biotechnology Associates). Membranes were incubated with chemiluminescent substrate (LuminataTM Forte Western HRP Substrate; Millipore) for 3 minutes and signals were detected by capture onto autoradiography film (GeneMate, Kaysville, UT, USA).

2.4 Measurement of cell proliferation

The BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, MA, USA) was used to measure the 24-hour incorporation of BrdU into dividing cells in two experiments. First, cellular proliferation of naïve clones was measured by plating clones in duplicate into a 96-well plate at a density of approximately 11,000 cells per well and incubating with bromodeoxyuridine (BrdU) for 24 hours. The cells were then fixed and BrdU content was assessed by spectrophotometry (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) per manufacturer's instructions. Cell proliferation was expressed as the 24-hour incorporation of BrdU (OD_{450–550}). To correct for variation between replicate assays,

absorbance values (OD_{450–550}) for each clone were normalized to the scrapie negative clone 441 (interassay standard). In similar fashion, cell proliferation was measured for clones at P-8 following Utah- or mock-inoculation. For both naïve and inoculated clones, cell proliferation was expressed as the average normalized absorbance values from at least 3 independent replicate assays.

2.5 Determination of transcript levels by RT-qPCR

Reverse transcription, quantitative PCR (RT-qPCR) was used to measure transcript levels of genes of interest in naïve and inoculated (P-5, Utah and mock inoculated) clones. Following passage, cells were collected and washed as described in subsection 2.3, and then lysed and total RNA collected using QIAshredder spin columns (Qiagen, Valencia, CA, USA) and the RNeasy Mini Kit per manufacturer's instructions (Qiagen). RNA was quantified by spectrophotometry (ND-1000, Thermo Scientific, Wilmington, DE, USA). Ten micrograms of each sample were treated with DNase using the DNA-free™ DNA Removal Kit (Ambion, Austin, TX, USA). One microgram of RNA was reverse transcribed into cDNA using the SuperScript® III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, Carlsbad, CA, USA). The transcripts of 11 genes were quantified by real-time PCR using the CFX96 detection system (Bio-Rad, Hercules, CA, USA) in 20 µl reactions containing 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 0.2 µM of each gene-specific primer, and 2 µl of cDNA diluted 1:100 into water. All primers used in this study were described previously (Budhia et al., 2006; Munoz-Gutierrez et al., 2016; Stanton et al., 2012), except those for *NCKAPIL*, and are listed in Table 1. *NCKAPIL* primers were designed based on the *Ovis aries* NCK-associated protein 1-like mRNA reference sequence (XM_004006271.3) using the primer-blast tool (Ye et al., 2012). The PCR product generated from this primer set was of the expected molecular weight and sequence for ovine *NCKAPIL*. Run conditions for RT-qPCR consisted of 95 °C for 30 s, 35 cycles of 95 °C for 15 s and 60 °C for 10 s, followed by a melt curve at 0.5 °C increments from 60 °C to 95 °C. A standard curve for each primer set was included in each replicate plate to measure primer efficiency per assay. Transcripts were normalized to *GAPDH* (Budhia et al., 2006) and fold-change values in transcript levels were calculated by REST 2009 (V2.0.13, Qiagen). Target transcripts were also calculated relative to *PRNP* (Stanton et al., 2012) to determine if permissiveness is related to the ratio of target gene to *PRNP*. Fold-changes were mean-centered and autoscaled as previously described (Willems et al., 2008). Fold-changes in the naïve clones were expressed as the change in transcript relative to the least permissive clone. Fold-changes in the inoculated clones were expressed within each clone as the transcript level after Utah- versus mock-inoculation. For both experiments, fold-change was expressed as the average value from at least 3 independent replicate assays.

2.6 Zymogram analysis of MMP2

MMP zymography was used to quantify MMP2 levels in the naïve and inoculated (P-3, Utah- and mock- inoculated) clones. Prior to passage, conditioned media was collected and centrifuged at 500 × *g* (JA50.5 rotor) for 10 minutes at 4 °C. Supernatant was collected and stored at –80 °C until testing. Supernatants were diluted to 3000 µg ml^{–1} in 1 × PBS before being mixed 1:1 with Tris-glycine SDS sample buffer (Novex, Invitrogen). After 15 minutes of incubation at RT, the samples were electrophoresed through 10% gelatin zymogram gels

(Novex, Invitrogen) at 125 volts for 2 hours. Gels were renatured in Zymogram Renaturing buffer (Novex, Invitrogen) for 30 minutes and incubated in Zymogram Developing buffer (Novex, Invitrogen) by shaking at 50 rpm overnight at 37 °C. Gels were then washed with Type I ultrapure water, stained for 1 hour using SimplyBlue SafeStain (Invitrogen), and finally washed twice with water. Digital images of stained gels were acquired using a gel doc imaging system (Molecular Imager ChemiDoc XRS System and Quantity One Software, Bio-Rad) and band densities were analyzed using Fiji (version 1.50a; <http://fiji.sc/#>) (Schindelin et al., 2012; Schindelin et al., 2015; Schneider et al., 2012). MMP2 levels were expressed as the average log₁₀-transformed density from at least 3 independent replicate assays.

2.7 Data handling and statistical analysis

All statistical analyses were conducted using procedures implemented in JMP, version 12 software (SAS Institute Inc., Cary, NC, USA). To evaluate permissiveness between clones, SSCA spot numbers were log₁₀-transformed and statistically compared using a two-way ANOVA for the main and interaction effects of clone and passage. *Post hoc* analysis was conducted using Tukey's test for all-pairwise multiple comparisons ($P < 0.05$). Least squares means were calculated as estimates of clone permissiveness over all passages (P-3 through P-8).

Rates of cell proliferation, levels of selected gene transcripts, and levels of MMP2 between naïve clones were statistically compared using one-way ANOVA for the main effect of clone and Tukey's test for all-pairwise comparisons ($P < 0.05$). Correlation of these measures with clone permissiveness was calculated by multivariate analysis as the Pearson Product-Moment Correlation (Pearson's r).

In the Utah- and mock-inoculated clones, two-way ANOVAs were used to calculate the main and interaction effects of clone and inoculation status on cell proliferation rates, MMP2 levels, or levels of selected gene transcripts. A separate one-way ANOVA was used to calculate the main effect of clone for Utah-inoculated cells. All *post hoc* comparisons were made using Tukey's test for all-pairwise comparisons ($P < 0.05$). Correlations for each of these measures with overall estimates of clone permissiveness were calculated by multivariate analysis as either the Pearson Product-Moment or Kendall's Tau correlation.

3. Results

3.1 Evaluation of clonal prion permissiveness

The relative prion permissiveness of five hTERT-ovine microglia clones was determined by the standard scrapie cell assay (SSCA) following inoculation with a sheep-derived isolate of scrapie (Utah) (Fig. 1). PrP^{res}-positive spot numbers were compared between clones at three passages (P-3, P-5, and P-8) post-inoculation. Preliminary experiments suggested that the permissiveness of clone 439 had declined after being maintained in culture for approximately 4 months prior to inoculation (data not shown). Loss of permissiveness has been observed following subsequent passages and subcloning of other prion-infected cultures, including murine neuroblastoma cells (Klohn et al., 2003). Therefore, to provide a

within-clone comparison, 439 was tested using cultures with fewer (i.e., 439 Early) or more (i.e., 439 Late) passages.

A two-way ANOVA calculated for the main and interaction effects of clone (6 levels) and passage (3 levels) on SSCA spot number was significant ($F = 7.4443$, $P < 0.0001$). While the main effect of clone was significant ($P < 0.001$), significant effects were not detected for passage number ($P = 0.3393$) or the interaction term ($P = 0.4984$). This suggests that PrP^{res} accumulation levels differed between clones and that these levels were maintained at steady state in each clone from P-3 to P-8. The collective passage results and overall statistical groupings of clone permissiveness are shown in Fig. 1. Consistent with our previous findings (Munoz-Gutierrez et al., 2016; Munoz-Gutierrez et al., 2015), 438 was the least permissive clone (statistical group A; all comparisons $P < 0.01$). A significant difference was not detected between intermediately permissive clones 440, 434, and 439 Late (B; $P > 0.4$), nor between 439 Late and 441 (C; $P = 0.24$). The most permissive clone, 439 Early, was significantly higher than all other clones (D; all $P < 0.05$) except 441 ($P = 0.85$).

The accumulating PrP^{res} was characterized by immunoblotting at P-6 post-inoculation (Fig. 2). PrP^{res} bands were detected at molecular weights expected for the di-, mono-, and unglycosylated glycoforms. Further examination is required to elucidate the relevance of the 50 kDa band that was inconsistently present in most clones. Consistent with the SSCA data, PrP^{res} levels were so low in 438 that only one replicate assay had visible bands (Fig. 2b).

3.2 Comparison of hTERT-ovine microglia proliferation

To determine the influence of cell division on the permissiveness of hTERT-ovine microglia clones, we compared the 24-hour incorporation of BrdU into dividing cells prior to (i.e., naïve clones) and after inoculation (i.e., Utah and mock clones). Statistical analysis failed to detect significant differences in proliferation rates amongst the naïve clones (Fig. 3a, $F = 2.0019$, $P = 0.1300$), and BrdU incorporation lacked significant correlation with SSCA spot numbers ($r = -0.5763$, $P = 0.23$).

To determine if scrapie infection altered cell division rates, BrdU incorporation into Utah-inoculated clones was compared to that in mock-inoculated clones at P-8 post-inoculation (Fig. 3b). A two-way ANOVA calculated for the main and interaction effects of clone (6 levels) and type of inoculation (2 levels) on normalized BrdU incorporation was not significant ($F = 1.0824$, $P = 0.4115$). Thus, the experiment failed to detect differences in cell proliferation between clones ($P = 0.41$), between inoculation status ($P = 0.42$), and in the interaction of these model effects ($P = 0.78$). Comparison of cell proliferation rates with SSCA spot numbers in Utah-inoculated clones revealed a weak-to-moderate, negative correlation ($r = -0.4448$, $P = 0.049$). Taken together, the data suggest that prion permissiveness is not affected by baseline rates of cell division and that cell division is not altered following scrapie infection in these clones of hTERT-ovine microglia.

3.3 Quantitative analysis of transcript levels in naïve clones

To gain further insight into factors influencing prion permissiveness, eleven genes (Table 1) with roles in heparin binding, cell proliferation, protein degradation, and ECM maintenance were evaluated by RT-qPCR in naïve hTERT-ovine microglia clones. Fold-changes in

transcript levels were normalized to *GAPDH* transcript levels and expressed relative to 438 (i.e., the least permissive clone). The results are graphically presented in Fig. 4 and numerically tabulated in Table S1. Relative transcript levels were further compared to permissibility and rates of cell division.

PRNP transcript levels are shown in Fig. 4a. Though significant increases relative to 438 were observed for two of the five clones (i.e., 434 and 441), these differences represent less than 2-fold changes. In addition, levels of *PRNP* transcript in the highly permissive clone, 439 Early, were indistinguishable from 438 and there was no detectable change between the two 439 clones of varying permissibility. Further, a significant correlation was not detected between *PRNP* levels and permissibility, suggesting that while *PRNP* transcript levels may contribute to the permissibility of clones 434 and 441, it fails to account for the permissibility of clones 440 and 439.

TGFBI and *RARRES1* transcript levels were significantly increased in multiple naïve clones (Fig. 4b and Fig. 4c). When compared to 438, the highly permissive clone 441 had significantly increased levels of *TGFBI* transcripts (Fig. 4b); however, statistically significant correlations were not detected for cell permissiveness. *RARRES1* transcript levels were also increased in several clones; however, this activity was not limited to clones of higher permissiveness, as significantly higher *RARRES1* transcript levels were observed in the intermediately permissive clone 440 (Fig. 4c). Unlike *TGFBI*, a statistically significant direct correlation was detected between *RARRES1* transcript levels and permissiveness (Table 2). When analyzing only the change in permissiveness of 439 (i.e., 439 Early vs. 439 Late), significant differences were not detected between transcript levels of either *TGFBI* (Fig. 4b, $P = 0.28$) or *RARRES1* (Fig. 4c, $P = 0.2$). Thus, while *TGFBI* transcript levels may not affect permissiveness in all clones, the correlation detected between *RARRES1* transcript levels and permissiveness suggests a possible role for *RARRES1* in clonal permissiveness to PrP^{res}.

Statistically significant decreases in transcript level were observed for *CTSB*, *SEPP1*, *MMP14*, *PTN*, and *DPT* in most clones when compared to 438 (Fig. 4d–h); however, all five genes lacked any significant correlation with permissiveness. When comparing only the 439 clones of varying permissibility, no significant differences in transcript levels of *CTSB* (Fig. 4d, $P = 0.2$), *SEPP1* (Fig. 4e, $P = 0.79$), or *MMP14* (Fig. 4f, $P = 0.71$) were detected, suggesting that drift in permissibility is not due to the altered transcription of these genes. The relative fold-change for *PTN* (Fig. 4g, $P = 0.01$) and *DPT* (Fig. 4h, $P < 0.01$) was significantly lower in 439 Late versus 439 Early; however, neither gene had significant correlations with permissibility and the transcriptional changes from 439 Early to 439 Late were in the opposite direction of the 438 transcript levels. This data suggest a potential role for *CTSB*, *SEPP1*, *PTN*, *MMP14*, and *DPT*; however, the lack of a correlation with permissibility suggests at most an inconsistent role in permissibility.

No statistically significant differences were found in the transcript levels for *SQSTM1*, *DCN*, and *NCKAP1L* when compared to 438 (Fig. 4i–k) or when compared between 439 Late and 439 Early. Thus, none of the three genes had evidence of contributing to permissibility in the naïve hTERT-ovine microglia clones. The transcript levels for all eleven

genes failed to correlate with cell proliferation (Fig. 3a), suggesting that none contributed to variation in baseline mitotic activity. Multiple statistical comparisons identified significant direct correlations between transcript levels in several genes and can be found in Table S2.

3.4 Quantitative analysis of transcript levels in inoculated clones

To investigate gene alteration due to PrP^{res} accumulation, transcript levels were compared between Utah- and mock-inoculated clones at P-5 post-inoculation. Transcripts were normalized to *GAPDH* transcript levels and fold-changes in Utah-inoculated clones were expressed relative to mock-inoculated clones. The two-way ANOVA calculated for the main and interaction effects of clone (6 levels) and inoculation status (2 levels) on transcript level was significant for all genes (*F* range: 2.51 to 47.48, $P < 0.05$). The main effect of clone was significant ($P < 0.05$), but significant effects were not detected for inoculation status ($P > 0.1$) or the interaction term ($P > 0.05$). This suggests that while gene transcript levels differed between clones, transcript levels were not altered following scrapie inoculation. Quantitative summaries for relative transcript levels can be found in Tables S3 and S4.

Quantitative summaries of individual transcript levels in the Utah-inoculated clones alone are listed in Table 3 and graphically presented in Fig. S1. In the Utah-inoculated clones, significant indirect correlations were detected between permissiveness and transcript levels of *SEPP1* and *PTN* (Table 2). When comparing between 439 Early and 439 Late, no detectable differences were observed for any of the tested transcripts, further suggesting that these transcripts are not contributing to drift in permissibility of this clonal line (Table 3). When compared to rates of cell proliferation (Fig. 3b), significant direct correlations were detected with transcript levels of *PTN* and *SEPP1*, and an indirect correlation was detected with *RARRES1* (Table 2). Significant correlations were also detected between the transcript levels of multiple genes and can be found in Table S5. Overall, these data support the contribution of *SEPP1* and *PTN* to prion permissiveness in the hTERT-ovine microglia clones following scrapie infection.

3.5 Evaluation of gene transcript levels relative to PRNP

Given the general importance of *PRNP* expression during prion infection (Bueler et al., 1993; Vilette et al., 2001), it is possible that the effect of non-PrP^C factors may be strongly affected by their level relative to the level of PrP^C. For example, if the key non-PrP^C factor is leading to degradation of PrP^C, then in cells with low PrP^C expression levels, relatively low levels of that non-PrP^C factor may be sufficient to maintain low permissibility. In contrast, in cells highly expressing PrP^C, then the non-PrP^C factor may need to be more strongly expressed to maintain low permissibility. Therefore, we evaluated target gene transcript levels in relation to *PRNP* transcript levels (i.e., target:*PRNP* ratios).

Ratios in the naïve clones expressed relative to clone 438 are graphically presented in Fig. 5 and numerically tabulated in Table S6. In general, the relative scale of fold-changes in target:*PRNP* ratios for the naïve clones were similar when compared to transcript levels only normalized to *GAPDH* (Fig. 4, Table S1). This is consistent with the less than 2-fold changes in *PRNP* transcript observed as shown in Fig 4a. In addition, significant correlations were not detected between permissibility and the target:*PRNP* ratio for *TGFBI*, *MMP14*,

PTN, *DPT*, *DCN*, and *NCKAP1L* (Fig. 5a–f), which is consistent with the analysis described in subsection 3.3 (Fig. 4, Table 2). In contrast, no significant correlation was detected for *RARRES1:PRNP* transcript levels (Fig. 5g), and strong, indirect correlations were detected between permissiveness and the target:*PRNP* ratios for *CTSB*, *SEPP1*, and *SQSTM1* (Fig. 5h–j, Table 4). Therefore, the data suggest that *CTSB*, *SEPP1*, and *SQSTM1* may influence permissiveness, but that the level of *PRNP* expression affects that influence.

To investigate target:*PRNP* ratios following PrP^{res} accumulation, transcript levels were compared between Utah- and mock-inoculated clones at P-5 post-inoculation as described in subsection 3.4. Fold changes in ratios can be found in Tables S7 and S8. Quantitative summaries of individual ratios in the Utah-inoculated clones alone are listed in Table 5 and graphically presented Fig. S2. Consistent with the analysis described in subsection 3.4 (Table 2, Fig. S1), significant indirect correlations in the Utah-inoculated clones were detected between permissiveness and *SEPP1:PRNP* and *PTN:PRNP* ratios (Table 4, Fig. S2). This further supports the potential roles of *SEPP1* and *PTN* in the permissiveness of the hTERT-ovine microglia clones to prions. Additional significant correlations with permissiveness and target to *PRNP* ratios included *CTSB* and *DCN* (Table 4).

3.6 Comparison of MMP2 levels by gelatin zymography

Since MMP2 levels were associated with varying permissibility in murine neuroblastoma cells (Marbiah et al., 2014) and a similar pathway was noted in initial assays of microglial cells (Munoz-Gutierrez et al., 2016), MMP2 levels were directly measured and compared. Significant differences in MMP2 levels were detected between naïve clones (Fig. 6a; $F = 10.2239$, $P < 0.001$). When compared to the least permissive clone 438, statistically significant reductions in MMP2 levels were detected for intermediately permissive clones 434 ($P = 0.01$) and 439 Late ($P < 0.01$) (Fig. 6a). However, the highly permissive clone (439 Early) lacked any difference to 438 ($P = 0.45$) and the MMP2 level in 439 Late was significantly lower than 439 Early ($P < 0.01$). Further, a significant correlation was not detected between MMP2 level and permissiveness ($r = -0.3766$, $P = 0.46$), suggesting that baseline levels of MMP2 do not dictate prion permissiveness in the hTERT-ovine microglia clones. In naïve cells, a positive correlation of MMP2 level was detected with transcript levels of *DPT* (Table 2).

To evaluate the effect of PrP^{res} accumulation on MMP2, levels were compared after Utah- and mock-inoculation at P-3 (Fig. 6b). A two-way ANOVA calculated for the main and interaction effects of clone (6 levels) and inoculation status (2 levels) on MMP2 level was significant ($F = 2.1887$, $P = 0.0406$); however, significant effects of clone ($P = 0.0573$), inoculation status ($P = 0.0504$), and the interaction term ($P = 0.9824$) were not detected. Furthermore, a significant difference in MMP2 level between clones after Utah-inoculation was not detected ($F = 1.4009$, $P = 0.2832$) and MMP2 levels were not significantly correlated with permissiveness ($r = -0.4004$, $P = 0.08$), suggesting that MMP2 levels are not affected by scrapie inoculation. In Utah-inoculated cells, MMP2 levels were significantly correlated with transcript levels of *NCKAP1L* (Table 2).

4. Discussion

While it is well known that PrP^C is a critical requirement for prion propagation (Bueler et al., 1993; Vilette et al., 2001), it insufficiently predicts cellular permissiveness to prions (Belt et al., 1995; Lloyd et al., 2001; Neale et al., 2010; Polymenidou et al., 2008). Previous studies have suggested that numerous PrP^C-related and non-related factors may influence prion replication by altering PrP^D conversion, localization, or degradation (Ghaemmaghami et al., 2007; Grassmann et al., 2013; Klohn et al., 2003). In this study, these factors were evaluated using five clonal populations derived from an immortalized subline of ovine microglia.

The differences between poor, intermediate, and highly permissive clones were stable from the 3rd through 8th passages post-inoculation. Consistent with previous studies (Munoz-Gutierrez et al., 2016; Munoz-Gutierrez et al., 2015), 438 was poorly permissive and 439 was highly permissive. However, as described for other model cell culture systems (Klohn et al., 2003; Neale et al., 2010), the permissiveness of 439 decreased with extended time (4 months) in maintenance culture prior to inoculation. Thus, cloning does not necessarily select for stable factors affecting cellular permissiveness to prions.

The negative influence of rapid cell division on levels of prion accumulation has been previously described in murine neuroblastoma cells (Ghaemmaghami et al., 2007). Results of this study suggest that when the rate of prion conversion exceeds that of cell division, PrP^D accumulates over time. Conversely, if the rate of cell division exceeds that of prion conversion, accumulated PrP^D is diluted out over time. A direct measure of mitotic activity through BrdU incorporation showed no detectable differences between cell division rates in all five microglial clones, whether tested before or after scrapie infection. While a negative correlation of permissiveness with mitotic rate was observed, this correlation was weak-to-moderate and only observed after incubation with the Utah-isolate of scrapie. Taken together, the rate of cell proliferation did not widely vary between these clones and is thus unlikely to have played a primary role in determining permissiveness. It is possible that mitotic rates may not be a causation of permissiveness, but is rather a by-product of other changes, such as that to the extracellular matrix (Marbiah et al., 2014).

To further identify factors related to prion conversion and degradation, the transcript levels of genes with multiple cellular functions were compared between clones. Previous work comparing the transcriptional profiles of PrP^D-inoculated clones 438 and 439 revealed the differential regulation of numerous genes, including those with roles specific to ECM homeostasis, mitosis, and protein degradation (Munoz-Gutierrez et al., 2016). In the present study, a subset of these genes (*MMP14*, *DCN*, *PTN*, *SEPP1*, *RARRES1*, *SQSTM1*, *CTSB*, *TGFBI*, *DPT*), and one previously untested gene (*NCKAP1L*), were selected to further characterize the impact of transcript level on the variable permissiveness of the entire clonal population.

The differences in transcript levels between naïve clones 438 and 439 Early were consistent with their previous findings in PrP^D-inoculated clones (Munoz-Gutierrez et al., 2016). Furthermore, inoculation failed to demonstrate a significant effect on transcript levels. This

is also consistent with our previous study (Munoz-Gutierrez et al., 2016), as well as additional studies which found a limited transcriptional response following PrP^D inoculation in primary ovine microglia (Stanton et al., 2009) and murine neuronal (Julius et al., 2008) cell lines. Also consistent with previous findings (Munoz-Gutierrez et al., 2016; Munoz-Gutierrez et al., 2015), cell permissiveness was at least incompletely affected by *PRNP* expression, as no significant correlation was detected. Thus, when developing cell culture models, high levels of *PRNP* expression are unlikely to be the sole predictor of permissibility.

RARRES1 transcript levels, upregulated in highly permissive clones 441 and 439 Early, were directly correlated with PrP^{res} permissiveness. However, *RARRES1* to *PRNP* ratios were not correlated with permissiveness, suggesting that the effect of *RARRES1* expression on permissiveness is likely independent of *PRNP* expression. *RARRES1* encodes for retinoic acid receptor responder protein 1 (i.e., Tazarotene-induced gene 1 [TIG1]), a protein shown to regulate cellular differentiation. Although a direct effect of TIG1 on prion accumulation has not been reported, prion permissibility was increased in murine neuroblastoma cells following treatment with retinoic acid (Marbiah et al., 2014). Previous studies have also reported the down regulation of TIG1 expression in cancer cell lines, indicating a role in the suppression of cell growth (Wu et al., 2011). *RARRES1* transcript levels failed to statistically correlate with cell proliferation in the naïve clones ($P = 0.0573$), but correlated indirectly with cell proliferation in the Utah-inoculated clones ($P = 0.0164$). Thus, the data suggests that a retinoic acid-inducible pathway may be key to permissibility in microglia, and that the effect may be due to its alteration of cell division.

MMP14, *PTN*, *SEPP1*, *CTSB*, and *DPT* were all downregulated in the intermediately and highly permissive clones. In the naïve clones, *CTSB*, *PTN*, and *DPT* transcript levels were significantly lower in 439 Late than 439 Early. However, based on the overall trends of permissiveness observed, the changes in transcript levels of these genes were opposite of what would be expected, suggesting a role for additional factors in drift in permissiveness over time in maintenance culture. A significant correlation was detected between permissiveness and *CTSB:PRNP* ratios, suggesting a potential role for *CTSB* in PrP expression and prion permissiveness. *CTSB* encodes for cathepsin B, an enzyme involved in the degradation of lysosomal proteins. Evidence has suggested the involvement of lysosomal compartments in prion conversion as it is a site of complete (PrP^C) or partial (PrP^{res}) degradation (Campana et al., 2005) and cathepsin B has been shown to degrade PrP^D in mouse neuronal cells (Luhr et al., 2004). Thus it is possible that PrP^{res} permissiveness is altered by *CTSB* expression through the degradation of PrP^{res} and/or PrP^C by cathepsin B. Thus, when screening for clones of high permissibility, the levels of cathepsin B may warrant consideration. Both *SEPP1* and *SEPP1:PRNP* transcript levels were negatively correlated with prion permissibility in the naïve and Utah-inoculated clones, suggesting a role for *SEPP1* in prion permissiveness. *SEPP1* encodes for selenoprotein P, a protein which binds heparin and defends against oxidative stress and DNA damage (Burk and Hill, 2005). Heparin is a sulfated glycan previously shown to bind PrP^D (Hijazi et al., 2005) and enhance prion conversion (Imamura et al., 2016; Yokoyama et al., 2011); thus, low levels of *SEPP1* transcripts may be associated with altered levels of heparin and prion misfolding. Further investigations into the role of selenoprotein P in prion permissiveness are pending.

Permissiveness also negatively correlated with both *PTN* and *PTN:PRNP* transcript levels. *PTN* encodes for pleiotrophin which, like selenoprotein P, has a high affinity for heparin. Pleiotrophin is a cytokine shown to promote cell growth and differentiation, and is upregulated following injury to brain tissue (Deuel et al., 2002; Maeda et al., 1999; Maeda et al., 1996). Transcript levels for both *SEPP1* and *PTN* were directly correlated with cell proliferation in the Utah-inoculated clones, suggesting an additional contribution of these genes to differential permissiveness through rates of cell division. *DPT*, *TGFBI*, and *MMP14* failed to correlate with permissibility; however, given the significant differences in transcript level between clones with differing levels of permissibility, we cannot exclude the possibility of indirect or multifactorial effects from these pathways.

In the naïve clones, the differences observed in *SQSTM1* transcription levels were below 2-fold and not detected between 439 Early and 439 Late; however, a strong, indirect correlation was observed between permissiveness and *SQSTM1:PRNP* transcript levels. *SQSTM1* encodes for sequestosome 1 (i.e., p62), a protein that plays a role in the degradation of misfolded proteins and has been previously shown to contribute to the clearance of PrP^{res} in cell cultures (Homma et al., 2014). Similar to that of *CTSB*, it is possible that *SQSTM1* expression affects permissiveness through effects on PrP expression and the degradation of PrP^{res}.

DCN:PRNP transcript levels in the Utah-inoculated clones indirectly correlated with prion permissiveness. *DCN* encodes for decorin, an ECM sulfated glycoprotein with roles in collagen fibril assembly and cell proliferation (Jarvinen and Prince, 2015). An indirect relationship between *DCN* expression and cancer cell growth has been previously reported (Jarvinen and Prince, 2015); however, *DCN* transcript levels were not correlated with cell proliferation in the ovine microglia clones. In addition, studies have reported the overexpression (i.e., direct relationship) of *DCN* in the mesenteric lymph nodes of scrapie infected sheep (Filali et al., 2014), a relationship that is inconsistent with that found herein. Alternatively, it has been reported that the undersulfation of glycan chains (e.g., heparan sulfate) is associated with prion permissibility in murine neuroblastoma cells (Marbiah et al., 2014). The relationship between reduced *DCN* transcript levels and permissibility in Utah-inoculated clones corroborate these findings and suggest that sulfated glycans, possibly that of decorin, may be a factor for prion propagation in ovine microglia. Further investigation is required to determine this role, including the expression of additional proteoglycans.

As previously described, *MMP14* encodes MMP14, a protein of which is a critical activator of MMP2 (Brinckerhoff and Matrisian, 2002). MMP2 downregulation leads to an increase in permissiveness of cultured neuroblastoma cells to prion propagation (Marbiah et al., 2014). In the present study, *MMP14* transcript levels were neither significantly correlated with permissiveness nor statistically different between cultures of clone 439. While significant differences in MMP2 levels were detected between the naïve clones, correlations between MMP2 levels and permissiveness lacked significance. Unlike the previous study using murine neuroblastoma cells (Marbiah et al., 2014), we failed to find a consistent correlation in the hTERT-immortalized ovine microglia clones. This suggests that the contribution of MMP2 to prion permissiveness may be cell type, cell line, and/or species specific.

5. Conclusions

The present study has evaluated the roles of cell proliferation, selected gene transcript levels, and MMP2 levels in the differential permissiveness of hTERT-ovine microglia clones. Within the 5 clones tested, a spectrum of permissiveness has been demonstrated. However, as shown in clone 439, this permissiveness can be lost after prolonged maintenance in culture. Prion permissiveness was not related to levels of MMP2, suggesting that MMP2 is a poor predictor for permissibility in ovine microglial cells. In addition, significant differences were not detected between rates of cell division; however, a significant correlation was observed between permissiveness and genes associated with cell proliferation (i.e., *RARRES1* and *PTN*). Thus, it remains possible that small changes in rates of cell division may impact PrP^{res} accumulation. Prion permissiveness was also significantly correlated with transcript levels of genes with important roles in protein degradation (i.e., *CTSB* and *SQSTM1*) and heparin binding (i.e., *SEPP1* and *PTN*). Taken together, these associations implicate a single or multifactorial contribution of genetic factors that may alter rates of prion expression, prion degradation, or cell division, and thus impact differential permissiveness. To determine a causative relationship with prion permissiveness, a more complete characterization of these factors is required. Studies are currently being performed to determine the significance and mechanism for the role of these genes, particularly *SEPP1*, in PrP^{res} accumulation. These experiments include an examination of targeted gene protein levels and the significance of these genes on permissiveness in additional cell types. Determining the influence of these genes on cell permissibility will allow for the optimization of PrP^{res} permissiveness in current cell lines, provide a rational method to engineer human cell lines permissive to prions, and can provide potential targets for inhibitors of prion replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Clonal differences in scrapie permissiveness were detected.
- The rate of cell proliferation did not differ between clones.
- Cell growth and protein degradation genes correlated with scrapie permissiveness.
- Scrapie permissiveness negatively correlated with heparin-binding genes.
- Variable MMP2 activity was observed, but did not correlate with permissiveness.

Fig. 1.

Quantification of PrP^{res} permissiveness in hTERT-ovine microglia clones. Following inoculation with a natural sheep scrapie isolate (Utah) or treatment with OMEM (mock), permissiveness to PrP^{res} was quantified by the SSCA at multiple passages post-inoculation. Box plots represent the statistical summary for the average number of PrP^{res}-positive spots from each independent replicate assay at P-3 (closed circles), P-5 (open circles), and P-8 (triangles). The horizontal line within the box represents the median spot number for each clone. The y-axis reference line is a positive/negative cutoff based on the upper 95% confidence level of the average spot number from 10 assays of mock-inoculated microglia.

The x-axis is ordered by permissibility ranking (i.e., from least to most permissive). Log-transformed spot numbers were statistically compared between clones using a two-way ANOVA and Tukey's test for all-pairwise *post-hoc* comparisons. Clones that do not share a letter are significantly different ($P < 0.05$).

Fig. 2.

Detection of PrP^{res}-specific bands in hTERT-ovine microglia clones. Utah-inoculated clones were cultured as described above and evaluated for PrP^{res} accumulation by immunoblot. (A) A representative immunoblot from cells at P-6 demonstrating PrP^{res}-specific bands. Vertical lanes are ordered by permissibility ranking (i.e., from least to most permissive), as determined in Fig. 1. (B) An immunoblot from a separate replicate assay to depict banding patterns from clone 438 at P-6. The arrow indicates an artifact of the immunoblot.

Fig. 3.

Comparison of cell proliferation in hTERT-ovine microglia clones. The cell proliferation rates of (A) naïve and (B) P-8 inoculated clones were quantified by BrdU assay. BrdU incorporation into dividing cells (i.e., cell proliferation) was quantified by measurement of OD at 450 and 550 nm. Absorbance values were normalized to an interassay standard control and cell proliferation was expressed as the average normalized absorbance value. Box plots represent the summary of data points representing the average normalized value of BrdU incorporation from the technical replicates of each independent replicate assay. The horizontal line within the box represents the median value from at least 3 independent experiments. The x-axis is ordered by permissibility ranking. BrdU incorporation was statistically compared between clones using a (A) one-way or (B) two-way ANOVA.

Fig. 4.

Comparison of gene transcript levels in naïve hTERT-ovine microglia clones. The impact of transcript levels on PrP^{res} permissiveness prior to inoculation was evaluated. RNA was collected from naïve clones and tested by RT-qPCR to quantify the transcript levels of multiple genes. Transcripts were normalized to *GAPDH* and expressed relative to the least permissive clone, 438 (i.e., 2⁰ = no change). Columns represent the fold-change in (A) *PRNP*, (B) *TGFBI*, (C) *RARRES1*, (D) *CTSB*, (E) *SEPP1*, (F) *MMP14*, (G) *PTN*, (H) *DPT*, (I) *SQSTM1*, (J) *DCN*, and (K) *NCKAP1L* transcripts. The y-axis reference lines represent the 2-fold cutoff levels suggestive of biological significance. Error bars represent the standard error of the geometric mean fold-change from 3 independent experiments. The x-axis is ordered by permissibility ranking. Fold-change values were statistically compared to clone 438 using a One-Way ANOVA and Tukey's test for all-pairwise *post-hoc* comparisons. *: *P* < 0.05, †: *P* < 0.01.

Fig. 5.

Comparison of target gene transcript levels relative to *PRNP* in naïve hTERT-ovine microglia clones. The impact of target:*PRNP* transcript levels on PrP^{res} permissiveness prior to inoculation was evaluated as described in Fig. 4, except that transcripts were expressed relative to *PRNP* and compared to the least permissive clone, 438. Columns represent the fold-change in (A) *TGFBI*, (B) *MMP14*, (C) *PTN*, (D) *DPT*, (E) *DCN*, (F) *NCKAP1L*, (G) *RARRES1*, (H) *CTSB*, (I) *SEPP1*, and (J) *SQSTM1* transcripts. Fold-change values from 3 independent experiments were statistically compared to clone 438 using a One-Way ANOVA and Tukey's test for all-pairwise *post-hoc* comparisons. *: $P < 0.05$, †: $P < 0.01$.

Fig. 6.

Comparison of MMP2 levels in hTERT-ovine microglia clones. MMP2 levels were quantified by gelatin zymography and densitometry in (A) naïve and (B) Utah- and mock-inoculated clones at P-3 post inoculation. Data points within box plots represent the density from each independent replicate assay. The horizontal line within the box represents the median value from at least 3 independent experiments. The x-axis is ordered by permissibility ranking. MMP2 levels were statistically compared between clones using a (A) one-way or (B) two-way ANOVA and Tukey's test for all-pairwise *post-hoc* comparisons. *, MMP2 levels are significantly different from clone 438 ($P < 0.05$).

TABLE 1

RT-PCR primer information.

Target	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Ref.	Gene description
<i>GAPDH</i>	GGCGTGAAACACACGAGAAAGTATAA	CCCTCCACGATGCCAAAGT	120	Budhia et al., 2006	Glyceraldehyde 3-Phosphate Dehydrogenase
<i>PRNP</i>	CCGTTACCCCAACCAAGTGT	CGTCCATTATCTTGATGTCAGT	156	Stanton et al., 2012	Prion Protein
<i>MMP14</i>	CGCTATGCCCATCCAGGGACT	CTCCCACACCTCGGGAATGCCT	126	Muñoz-Gutiérrez et al., 2016	Matrix Metalloproteinase-14
<i>DCN</i>	GCTGGCCGACCAATAAGTACA	TGGGTTGCTTGAAAGGGCTCA	139	Muñoz-Gutiérrez et al., 2016	Decorin
<i>PTN</i>	GCAGACTCCACAGTACCCTGC	ACACACACTCCACTGCCATT	163	Muñoz-Gutiérrez et al., 2016	Pleiotrophin
<i>TGFBI</i>	TGGGCGGCAAGAACTGAGA	GGATTGTCCCCCTTCAGGA	170	Muñoz-Gutiérrez et al., 2016	Transforming Growth Factor Beta Induced
<i>SEPP1</i>	ACCGTGGTTGCTCTTCTTCAA	TCTCCAGTTTCTTACTCGCAGGTC	85	Muñoz-Gutiérrez et al., 2016	Selenoprotein P
<i>CTSB</i>	TTGGAAGGCTGGACAACT	TCCCTGGTCTCGGATCTCTT	190	Muñoz-Gutiérrez et al., 2016	Cathepsin B
<i>RARRES1</i>	GGCAGCTCTTACGTGATGTG	CCAGACCAAGTGAAATACGGCA	177	Muñoz-Gutiérrez et al., 2016	Retinoic Acid Receptor Responder 1
<i>SQSTM1</i>	TTGTACCCACATCTGCCACC	AGCGCCTTCATCAGAGAAC	91	Muñoz-Gutiérrez et al., 2016	Sequestosome-1
<i>NCKAP1L</i>	CCGAACACAGCACGCAACATT	TGGCAGGCATCAATGGTGTT	147		Nck-Associated Protein 1-Like
<i>DPT</i>	GCTGGTGGGAGGAGATCAAC	GACTCGAAGTAGCGGCTCTG	97	Muñoz-Gutiérrez et al., 2016	Dermatopontin

TABLE 2

Significant correlations with transcript levels in hTERT-ovine microglia clones.

Cell status	By activity	Gene ^b	^a Multivariate correlation		
			Pearson's <i>r</i>	Kendall's τ	<i>P</i> -Value
Naïve	Prp ^{res} Permissiveness	<i>RARRES1</i>	0.8605		0.0278
	MMP2 Level	<i>DPT</i>	0.8296		0.0411
Utah-inoculated	Prp ^{res} Permissiveness	<i>SEPP1</i>	-0.4833		0.0309
		<i>PTN</i>	-0.609		0.0044
	Cell Proliferation	<i>PTN</i>		0.3579	0.0274
		<i>SEPP1</i>	0.4605		0.041
		<i>RARRES1</i>		-0.3895	0.0164
	MMP2 Levels	<i>NCKAP1L</i>		-0.3368	0.0379

^aCorrelations based on values from at least 3 independent experiments.

^bTranscripts were normalized to *GAPDH*.

TABLE 3

uantitative RT-PCR analysis of specific transcript levels in Utah-inoculated hTERT-ovine microglia clones

<i>a</i> Fold-change relative to least permissive clone (438)																													
438					440					434					439 Late					441					439 Early				
Gene ^b	Mean	SEM	+	SEM	Mean	SEM	+	SEM	Mean	SEM	+	SEM	Mean	SEM	+	SEM	Mean	SEM	+	SEM	Mean	SEM	+	SEM					
<i>PRNP</i> ^c	1.00	0.95	1.05	0.94	0.98	0.94	1.02	1.02	1.60	1.52	1.68	1.68	0.75	0.73	0.77	1.45	1.40	1.34	1.45	1.00	0.95	1.00	0.95	1.05					
<i>MMP14</i>	1.00	0.81	1.24	0.62	0.88	0.62	1.26	1.73	1.27	0.94	1.73	1.73	0.20 [*]	0.16	0.25	1.93	1.40	1.02	1.93	0.30	0.25	0.30	0.25	0.35					
<i>DCN</i>	1.00	0.84	1.18	0.31	0.39 ^{**}	0.31	0.47	2.47	2.15 [*]	1.87	2.47	2.47	0.18 ^{***}	0.16	0.21	1.23	1.10	0.98	1.23	0.16 ^{***}	0.15	0.16 ^{***}	0.15	0.16					
<i>PTN</i>	1.00	0.73	1.38	0.24	0.28 [*]	0.24	0.33	0.87	0.80	0.74	0.87	0.87	0.02 ^{***}	0.02	0.03	0.15	0.12 ^{***}	0.10	0.15	0.06 ^{***}	0.05	0.06 ^{***}	0.05	0.07					
<i>TGFBI</i>	1.00	0.89	1.13	0.18	0.23 ^{**}	0.18	0.29	0.82	0.63	0.48	0.82	0.82	0.59	0.54	0.64	0.60	0.46	0.34	0.60	0.46	0.41	0.41	0.51						
<i>SEPP1</i>	1.00	0.78	1.28	0.22	0.36	0.22	0.58	0.91	0.73	0.58	0.91	0.91	0.01 ^{***}	0.01	0.01	0.48	0.40	0.34	0.48	0.01 ^{***}	0.01	0.01 ^{***}	0.01	0.02					
<i>CTSB</i>	1.00	0.87	1.15	0.5	0.63	0.5	0.8	1.18	0.95	0.77	1.18	1.18	0.29 ^{**}	0.27	0.31	1.04	0.80	0.61	1.04	0.42	0.36	0.42	0.36	0.48					
<i>RARRES1</i>	1.00	0.95	1.06	0.15	0.19 ^{***}	0.15	0.24	0.44	0.33 [*]	0.26	0.44	0.44	1.30	1.17	1.45	0.41	0.37 [*]	0.33	0.41	1.37	1.03	1.37	1.03	1.80					
<i>SQSTM1</i>	1.00	0.9	1.11	0.92	1.01	0.92	1.09	1.47	1.27	1.10	1.47	1.47	0.58 [*]	0.55	0.60	1.73	1.46	1.23	1.73	0.67	0.63	0.67	0.63	0.71					
<i>NCKAP1L</i>	1.00	0.84	1.19	1.23	1.23	1.03	1.48	1.67	1.57	1.47	1.67	1.67	0.80	0.71	0.90	1.62	1.32	1.07	1.62	1.29	1.22	1.29	1.36						
<i>DPT</i>	<i>d</i> _{1,00}	0.84	1.18	0.01	0.01 ^{***}	0.01	0.01	0.12	0.08 ^{***}	0.06	0.12	0.12	0.17 ^{**}	0.15	0.20	0.04	0.03 ^{***}	0.02	0.04	0.29 [*]	0.26	0.29 [*]	0.26	0.33					

^aTranscripts were normalized to *GAPDH*. Fold-changes in transcript levels were normalized to clone 438.

^bMean and ± SEM for all genes except *PRNP* based on 3 independent experiments.

^cMean and ± SEM for *PRNP* based on 30 independent experiments.

^{*}Transcript level statistically different from clone 438;

^{*}P-value 0.05,

^{**}P-value 0.01,

^{***}P-value 0.001.

Bold Fold-change in transcript level is both statistically different and greater than 2.00 when compared to clone 438.

^dAt least one sample was not detected due to low transcript level and was thus estimated to be a value of at least the lowest Cq detected on the plate.

TABLE 4
Significant correlations between target: *PRNP* transcript levels and *Pp^{res}* permissiveness in hTERT-ovine microglia clones

Cell status	Gene ^b	^a Multivariate correlation	
		Pearson's <i>r</i>	<i>P</i> -Value
Naïve	<i>CTSB</i>	−0.8209	0.0452
	<i>SEPP1</i>	−0.838	0.0372
	<i>SQSTM1</i>	−0.9121	0.0112
Utah-inoculated	<i>CTSB</i>	−0.6499	0.0019
	<i>DCN</i>	−0.4979	0.0255
	<i>SEPP1</i>	−0.558	0.0106
	<i>PTN</i>	−0.6898	0.0008

^aCorrelations based on values from at least 3 independent experiments.

^bTarget gene transcripts were expressed relative to *PRNP* transcripts.

TABLE 5
Quantitative RT-PCR analysis of specific target: *PRNP* transcript levels in Utah-inoculated hTERT-ovine microglia clones

	<i>a</i> Fold-change relative to least permissive clone (438)														
	438			440			434			439 Late			441		
Gene ^b	Mean	SEM	+	Mean	SEM	+	Mean	SEM	+	Mean	SEM	+	Mean	SEM	+
<i>MMP14</i>	1.00	0.82	1.21	0.83	0.63	1.09	0.69	0.59	0.81	0.25 ^{**}	0.20	0.30	0.92	0.71	1.18
<i>DCN</i>	1.00	0.71	1.40	0.37	0.28	0.50	1.22	1.01	1.47	0.24 ^{**}	0.19	0.30	0.76	0.70	0.83
<i>PTN</i>	1.00	0.88	1.14	0.34 [*]	0.32	0.36	0.62	0.49	0.78	0.03 ^{***}	0.03	0.04	0.11 ^{***}	0.10	0.12
<i>TGFB1</i>	1.00	0.85	1.17	0.23 ^{***}	0.17	0.31	0.35 [*]	0.29	0.41	0.68	0.59	0.79	0.32 ^{**}	0.28	0.36
<i>SEPP1</i>	1.00	0.88	1.14	0.35 [*]	0.23	0.52	0.47	0.44	0.51	0.01 ^{***}	0.01	0.02	0.28 ^{***}	0.24	0.32
<i>CTSB</i>	1.00	0.84	1.19	0.64	0.59	0.69	0.55	0.51	0.59	0.40 ^{**}	0.34	0.46	0.57	0.48	0.69
<i>RARRES1</i>	1.00	0.83	1.21	0.23 ^{***}	0.19	0.28	0.23 ^{***}	0.20	0.26	2.01	1.77	2.28	0.29 ^{***}	0.27	0.33
<i>SQSTM1</i>	1.00	0.87	1.14	1.04	0.99	1.09	0.89	0.84	0.93	0.78	0.72	0.85	1.08	0.99	1.17
<i>NCKAP1L</i>	1.00	0.97	1.03	1.20	1.15	1.25	0.99	0.81	1.20	1.01	0.83	1.24	0.91	0.81	1.02
<i>DPT</i>	1.00	0.69	1.45	0.01 ^{***}	0.01	0.01	0.05 ^{***}	0.04	0.06	0.21 ^{**}	0.18	0.25	0.02 ^{***}	0.01	0.02

^aTarget gene transcripts were expressed relative to *PRNP*. Fold-changes in transcript levels were normalized to clone 438.

^bMean and ± SEM for all genes based on 3 independent experiments.

^{*}Transcript level statistically different from clone 438;

^{*}P-value 0.05,

^{**}P-value 0.01,

^{***}P-value 0.001.

Fold Fold-change in transcript level is both statistically different and greater than 2.00 when compared to clone 438.

^cAt least one sample was not detected due to low transcript level and was thus estimated to be a value of at least the lowest Cq detected on the plate.