Select Gene Expression Across Variable PrPSc Permissive Ovine Microglia

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Abstract

Abstract **Background:** Transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that affect multiple species, including sheep, cattle, and humans. A misfolded, pathogenic isoform (PrP^D) of the normal, host-encoded, cellular prion protein (PrP^C) is the causative agent for TSEs. While there have been advances in understanding TSEs, antemortem diagnostic tests are limited in many species, and there are no effective treatment protocols. Filling these reagent gaps will require knowledge of the molecular pathophysiology of PrP^D accumulation. Previous work has suggested that the extracellular matrix (i.e., fibronectin 1) and physiological functions (i.e., cell division) maybe key factors for cellular prion permissibility, at least in specific cell culture models. Using a natural scrapie isolate, six immortalized, ovine microglial clones, of varying permissiveness to classical scrapie were evaluated for differential gene expression in seven genes based on previous RNASeq studies (fibronectin 1 [FN1], follistatin-like 1 [FSTL1], osteonectin [SPARC], survivin [BIRC5], syndecan 4 [SDC4], AXL receptor tyrosine kinase [AXL], and prion protein [PRNP]), and to determine correlations with prion permissibility. **Results:** Significant differential gene expression was frequently observed for survivin, follistatin-like 1 and osteonectin between clones, and when evaluated relative to PRNP expression. However, only fibronectin 1 and survivin were significantly correlated with prion permissibility, and only when evaluated relative to PRNP expression. Inoculation had a significant effect on follistatin-like 1, syndecan 4, and osteonectin. **Conclusions:** Similar to previous studies in other systems, fibronectin and mitotic rate show promise as potential determinants of prion permissibility in ovine microglia. As determinants of prion permissibility, the expression of fibronectin 1 and survivin coupled with PRNP could be utilized as biomarkers for detection of prion permissibility phenotype in ovine microglia, and perhaps other cell culture models of prion disease.

Background

Transmissible spongiform encephalopathies (TSEs) are a group of progressive, fatal neurodegenerative diseases that affect cattle (bovine spongiform encephalopathy), small ruminants (scrapie), cervids (chronic wasting disease), felids (feline spongiform encephalopathy), humans (Creutzfeldt-Jakob disease and Kuru) [1, 2], and now camelids [3]. Classical scrapie is the oldest described prion disease, dating back to over 250 years ago [4]. Due to the public concern for all prion diseases, and the economic and financial devastation associated with this disease, eradication efforts have been implemented in various countries for the control and elimination of classical scrapie.

TSEs are caused by a misfolded, pathogenic isoform (PrP^D; D for disease-associated) of the normal, host-encoded, cellular prion protein (PrP^C; C for cellular) [5-10]. In this disease, PrP^D can promote autocatalytic conversion of normal PrP^C to PrP^D in a perpetuating, positive feedback cycle [5-9]. While most TSEs are initiated after exposure to PrP^D from an infected animal, PrP^C has also been reported to spontaneously convert to PrP^D [10-12]. Although there are variations in several of the key components contributing to prion infection in hosts, which include heterogeneity of PrP^D strains [13], neuroanatomical distribution of

lesions and PrP^D deposits [2, 13], and post-translational PrP^C modifications [9], the common denominator is the conversion of PrP^C to its pathological isoform, PrP^D.

PrP^C is a protein encoded by the host gene PRNP which is evolutionarily conserved amongst mammals [1, 14, 15] and is constitutively expressed in several cell types, but notably in adult neurons [16], glial cells [17], lymphocytes, and monocytes [18]. In sheep, PrP^C is 256 amino acids long [15, 19]. Polymorphisms in PrP^C have been reported in codons 136 (A/alanine or V/valine), 154 (R/arginine or H/histidine) and/or 171 (Q/glutamine, R/arginine, or H/histidine) that increase susceptibility (i.e. VRQ/VRQ, ARQ/VRQ, ARQ/ARQ) or confer resistance (ARR/ARR) to classical scrapie in sheep [15, 20, 21].

Although there are variations in several of the key components contributing to prion infection in hosts, which include heterogeneity of PrP^D strains [13], neuroanatomical distribution of lesions and PrP^D deposition [2, 13], and post-translational PrP^C modifications [9], the common denominator is the conversion of PrP^C to its pathological isoform, PrP^D. While certain cofactors such as glycosaminoglycans [22], and lipids [23] have been suggested to facilitate this conversion, the intricate mechanisms contributing to this conformational change are not completely elucidated. Hence, this generalized paucity of knowledge in prion disease pathogenesis hinders the development of diagnostic aids, and of most importance, treatments for prion disease.

Various experimental model systems have been used to study and further characterize classical scrapie. In vivo models provide excellent systems to study the clinical aspects of the disease, and serve as the final test for potential therapies [24-26]. However, with in vivo models, assessment of cellular and extracellular factors that may influence disease onset or progression could be challenging to identify. Cell culture models serve as practical and informative ex-vivo systems, providing an avenue to study and dissect the role of cellular and extracellular constituents in prion disease pathogenesis, that cannot be dissected at the tissue or animal level. Furthermore, these models allow for the development and testing of treatments prior to animal testing. While transgenic cell culture models, such as ovinized rabbit kidney epithelial cells (Rov) [27], and ovinized mouse cerebellar astrocytes [1] allow for the expression of PrP^C from a normal prion host (e.g., sheep), and have provided useful information in prion research, these models cannot account for species-to-species variation in all non-PrP^C proteins. To increase the chances that PrP-non-PrP protein interactions are species matched, non-transgenic cell culture lines can be used. Immortalized, ovine microglia cells [28, 29] are an example of a non-transgenic cell culture line, and were hence utilized in this study. Microglia are innate, myeloid immune cells of the brain that have similar functions to macrophages [30, 31]. These resident phagocytic cells have also been localized to the neuroanatomical vicinity of PrP^D deposits and contain intracellular PrP^D, which contributes to prion protein degradation but also potential dissemination [31]. As a possible mode of prion propagation in the nervous system, the use of microglia could provide further insight on prion disease pathogenesis.

Using N2a cells inoculated with a mouse-adapted scrapie isolate (ScN2a), Marbahi *et al.* demonstrated that the downregulation of 9 extracellular matrix genes through silencing increased susceptibility to prion

infection, and when fibronectin 1 was inhibited from binding to integrin $\alpha 8$, prion propagation was enhanced [32]. Similar investigations into gene expression were also performed using a subline of ovine microglia established within our laboratory. Munoz-Gutierrez *et al.* evaluated the transcriptomic profile in immortalized clones of this subline, between one permissive and one relatively resistant to scrapie prions, and twenty-two genes were differentially expressed [33]. Additionally, several other genes were potentially differentially expressed or regulated (i.e., AXL, BIRC5, SPARC) [33]. Due to the limited nature of a single pairwise analysis, the relevance of these findings is unknown. For this study, we expanded the analysis of AXL, BIRC5, FSTL1, FN1, SPARC, SDC4 and PRNP into three additional immortalized, ovine microglia clones of varying prion permissibility, and one clone that reverted from highly to intermediately permissive from a previous study [28] to determine if their expression correlated with prion permissibility.

Results

3.1 Assessment of gene expression normality for Mock and Utah-inoculated clones

Seven genes with a potential role in prion permissibility were assessed with RT-qPCR,

and normalized to 18s rRNA and hPRT1b. Gene expression data from Mock-inoculated clones showed a normal distribution (Goodness-of-Fit; $p > 0.05$). Utah-inoculated clones were not normally distributed for SDC4 (Goodness-of-Fit; $p < 0.001$) and SPARC (Goodness-of-Fit; $p < 0.01$) but were normally distributed for the remaining genes. For SDC4 and SPARC, raw expression values from Utah-inoculated clones were transformed with Box-Cox Y transformation test, and these values were deemed as normally distributed after assessment with histograms and Goodness of Fit test ($p > 0.05$) and subsequently utilized in parametric statistical analyses as such.

3.2 Transcript levels in Mock-inoculated clones

To determine if the RNA levels of the targeted genes significantly differed between clones, fold changes in normalized gene expression were statistically compared amongst all of the clones from at least 3 independent replicates. Statistically significant differences in expression were observed for BIRC5 ($p < 0.001$), SPARC ($p < 0.001$) and FSTL1 ($p < 0.01$) (Figure 1). For BIRC5, differential gene expression was significantly decreased for both 439Late

and 439Early compared to all other clones ($p < 0.05$), and this decreased expression was a four-to-eight-fold change compared to clone 438. Expression of FSTL1 in clones 439Late ($p < 0.05$) and 439Early ($p < 0.05$) was significantly decreased compared to clone 434, and clone 439Late was also significantly decreased compared to clone 438 ($p < 0.05$), but these changes were less than two-fold compared to clone 438. Additionally, clone 440 had significantly decreased expression of FSTL1 compared to clones 438 ($p < 0.05$) and 434 ($p < 0.05$), but these changes were also less than two-fold compared to clone 438. With SPARC, clones 439Late and 439Early had significantly diminished expression compared to clones 438 ($p < 0.0001$ and $p < 0.01$) and 434 ($p < 0.001$ and $p < 0.05$), and these changes were two-to-three-fold compared to clone 438. Additionally, 439Late SPARC expression was significantly decreased compared

to clone 441 ($p = 0.0378$). Furthermore, clone 438 had significantly increased SPARC expression compared to clones 441 ($p < 0.05$) and 440 ($p < 0.01$), and clone 434 was also significantly increased compared to 440 ($p < 0.05$), but these changes were less than two-fold compared to clone 438. Significant differential gene expression was lacking for AXL, FN1, PRNP and SDC4 in all pairwise comparisons.

Since clone 439 exhibited variation in permissibility over time, differences in gene expression were also assessed for 439Late relative to the most permissive clone, 439Early in all evaluated genes. Only in BIRC5 was there a two-fold change expression in 439Late relative to 439Early, but this change was not statistically significant ($p > 0.05$).

There were no statistically significant correlations between mean normalized gene expression of Mock-inoculated clones and prion permissibility ($p > 0.05$).

3.3 Transcript levels in Utah-inoculated clones

As described in section 3.2, analysis of fold changes in gene expression and correlations with prion permissibility were also performed for Utah-inoculated clones for at least 3 independent replicates, with the exception of clone 439Early for BIRC5 in which data from only 2 out of 3 independent replicates was available to assess (one replicate had undetectable transcript levels, consistent with low expression levels detected in the other replicates). Individual, significant variations in gene expression were present for PRNP ($p < 0.05$) and SPARC ($p < 0.01$) (Figure 2). A single, significant increase in PRNP expression was observed for clone 439Late as compared to clone 438 ($p < 0.05$); however, this change was less than two-fold compared to clone 438. With SPARC, expression levels were significantly increased for clone 439Late as opposed to clone 438 ($p < 0.01$), and clone 434 ($p < 0.01$), but this variation was also less than two-fold compared to clone 438. Although a two-fold change in BIRC5 expression was present for 439Late and 439Early relative to clone 438, these differences were not statistically significant ($p > 0.05$) due to the low expression levels in these clones. BIRC5 tended to amplify late in RT-qPCR with Utah-inoculated clones. Significant differential gene expression was also lacking for AXL, FN1, FSTL1, and SDC4 in all pairwise comparisons. No significant differential expression existed for clones 439Late versus 439Early in any of the examined genes.

As also described for the Mock-inoculated clones, significant correlations were not observed between mean normalized gene expression and prion permissibility ($p > 0.05$).

3.4 Transcript levels of Mock and Utah-inoculated clones combined

The data for the Mock and Utah-inoculated clones were combined to increase the sample sizes. For FSTL1, SPARC, and SDC4, the effect of inoculation was significant ($p < 0.05$), and the interaction of inoculation status and clone were also significant for SDC4 and SPARC ($p < 0.05$), so these genes were not included in this aspect of the study. However, data could be combined for AXL, FN1, BIRC5, and PRNP. For BIRC5 ($p < 0.001$) and PRNP ($p < 0.01$), the effect of clone remained statistically significant with two-

way ANOVA, frequently involving the same original clones as described in sections 3.2 and 3.3, but with some modifications to be addressed. When data was combined for BIRC5, the significant variation in gene expression of clones 439Early and 439Late, compared to clone 440 were no longer significant ($p > 0.05$). In PRNP, although clone 439Early lacked a two-fold change in expression to other clones, collective data indicated that 439Early is weakly but significantly ($p < 0.05$) increased relative to 438. No significant correlations were identified between genes ($p > 0.05$) or with prion permissibility ($p > 0.05$).

Since inoculation status was influential to gene expression for FSTL1, SPARC, and SDC4 with two-way ANOVA, the ratio of normalized mean expression derived from both treatment groups (i.e., Mock-inoculated/Utah-inoculated) was constructed to evaluate if inoculation with scrapie prions influenced transcript levels of these three genes (Figure 3).

For SPARC, transcript levels were approximately three-fold higher for clones 438 and 434 ($p < 0.05$) as compared to clone 439Late subsequent to inoculation with scrapie prions.

3.5 Gene expression values relative to PRNP in Mock-inoculated clones

Although normalized PRNP expression was determined to lack two-fold changes in expression and displayed absent to minimal, significant variation amongst the clones in this, as well as another previous study [28], the influence of target genes relative to PRNP expression of other genes was evaluated to determine if target gene/PRNP ratios were significant relative to prion permissibility. Raw target gene/PRNP ratios were calculated using the relative quantity of expression (i.e., non-normalized) for target genes and PRNP, per clone. Fold changes in expression and correlations with prion permissibility were statistically evaluated as previously described in section 3.2. Statistically significant differences in expression were observed for BIRC5/PRNP ($p < 0.001$), SPARC/PRNP ($p < 0.001$), FSTL1/PRNP ($p < 0.01$) and SDC4/PRNP ($p < 0.01$) (Figure 4). In BIRC5/PRNP, differential gene expression of 439Late and 439Early were significantly decreased compared to clones 438 ($p < 0.001$ and $p < 0.01$), and 441 ($p < 0.01$ and $p < 0.05$), and this decreased expression was a five-to-eight-fold change for 439Late and 439Early compared to clone 438. Earlier in the study, significant fold changes with BIRC5 in 439Early and 439Late were also described for both treatment groups, independent of PRNP expression. Moreover, for BIRC5/PRNP, clone 439Late had significantly diminished expression compared to clones 434 ($p < 0.01$) and 440 ($p < 0.05$). SPARC/PRNP had significant decreased expression for clones 439Late and 439Early compared to clones 438 ($p < 0.001$ and $p < 0.01$) and 434 ($p < 0.001$ and $p < 0.05$), and a two-to-three-fold change in expression existed in clones 439Late and 439Early compared to clone 438. Also, for SPARC/PRNP, clones 440 and 441 had significantly diminished expression compared to 438 ($p < 0.01$ and $p < 0.05$), and clone 440 was significantly decreased from 434 ($p < 0.05$), but these changes were slightly less than two-fold relative to clone 438. In FSTL1, clone 439Late had significantly diminished expression from clones 438 ($p < 0.05$) and 434 ($p < 0.05$), but these differences were less than two-fold. For SDC4/PRNP expression, 439Late was significantly decreased compared to clones 438 ($p < 0.01$), 440 ($p < 0.05$), and 434 ($p < 0.05$), but these differences were also less than two-fold relative to clone 438. Relative to PRNP expression, significant differential gene expression remained absent for AXL and FN1.

Although an approximately three-fold change in BIRC5/PRNP expression was present in clone 439Late compared to 439Early, this difference was not significant ($p > 0.05$). No significant differences in expression of clones 439Early versus 439Late were present for any of the remaining gene ratios.

No significant correlations to prion permissibility were identified amongst all gene ratios.

3.6 Gene expression values relative to PRNP in Utah-inoculated clones

Fold change data and correlations to prion permissibility were constructed and statistically assessed as previously described in section 3.5. Statistically significant differences in expression were observed for AXL/PRNP ($p < 0.05$) (Figure 5). For AXL/PRNP, clone 441 had significantly diminished expression compared to clone 438 ($p < 0.05$), but this change was less than two-fold. Clones of BIRC5/PRNP lacked significant differential expression, but an

approximately three-to-six-fold change in expression was noted in clones 439Late and 439Early relative to clone 438. In FN1/PRNP, there was also an absence of significant differential expression amongst the clones, but two-fold changes in expression were present in clones 439Late, 439Early, 434, and 441, relative to clone 438, and mean expression was highest in the least permissive clone (i.e., 438). In FSTL/PRNP, no significant differential expression was observed amongst the clones, but a two-fold change was present in clone 439Late relative to clone 438. Significant differential gene expression was lacking for SDC4/PRNP and SPARC/PRNP.

For BIRC5/PRNP, clone 439Late had a two-fold higher transcript level relative to 439Early, but this change lacked statistical significance. No additional significant differences in expression were present in clone 439Early versus 439Late for any of the remaining gene ratios.

No significant correlations with prion permissibility were detected amongst the gene ratios.

3.7 Gene expression values relative to PRNP in Mock and Utah-inoculated clones

Target gene/PRNP expression ratios were pooled from Mock and Utah-inoculated clones for AXL/PRNP, BIRC5/PRNP and FN1/PRNP, and the impact of clone and inoculation status with expression, as well as correlations to prion permissibility were evaluated using similar procedures as described in section 3.4 (Figure 6). Neither inoculation status, nor the

interaction of clone with inoculation status were influential to expression of genes selected for this portion of the study, so treatment groups were pooled to increase sample sizes. For AXL/PRNP, the effect of clone was significant ($p < 0.01$) for clones 438 and 441. In BIRC5/PRNP, although the effect of clone remained significant to gene expression ($p < 0.001$) with many of the same clone comparisons depicted in section 3.5, further significant differential comparisons included clones 439Early and 434 ($p < 0.01$), clones 441 and 439Late ($p < 0.01$), and clones 440 and 438 ($p < 0.05$). Furthermore, BIRC5/PRNP differential expression became insignificant between clones 440 and 439Late ($p > 0.05$). Of interest, when FN1/PRNP was assessed jointly for both treatment groups, the effect of clone became significant ($p <$

0.01), and expression of clone 438 was significantly increased compared to 439Late ($p < 0.05$), 441 ($p < 0.01$) and 439Early ($p < 0.05$), with clones 441 and 439Early previously characterized as highly permissive to scrapie prion infection.

Additionally, BIRC5/PRNP ($r = -0.7187$, adjusted p -value < 0.05), and FN1/PRNP ($r = -0.7841$, adjusted p -value < 0.01) were strongly and negatively correlated with prion permissibility (Table 1).

Table 1. Significant correlations between target genes/PRNP and prion permissibility.

Gene-Gene interaction ^a	Pearson's Correlation	Adjusted p-value ^b
FN1/PRNP	-0.7841	0.0076
BIRC5/PRNP	-0.7187	0.0127

^a Data was derived from at least 3 independent replicates. A p -value < 0.05 was considered significant. Ratio expression was scaled to 438.

^b Adjusted p -value were derived from the formula (Pearson's p -value x (total number of p -values/ p -value rank)). The value obtained from this calculation was compared to the previous adjusted p -value, and the smaller of the two was recorded. This value is also referred to as the Benjamini-Hochburg p -value (www.biostathandbook.com/multiplecomparisons.html).

Discussion

Identifying definitive determinants of prion permissibility is key in unraveling prion disease pathogenesis, and it also acts as a catalyst in the development of preventative measures and effective treatment protocols. Multiple studies of classical scrapie have implemented the use of cell culture models, and from these studies, potential determinants of prion permissibility have been identified [28, 32, 33]. In this study, 6 genes (AXL, BIRC5, FN1, FSTL1, SPARC, SDC4) that previously demonstrated either differential gene expression or the potential to affect prion permissibility between two clones (i.e., 438 and 439) of contrasting prion permissibility [33], were further characterized. The previous results were expanded into a set of six sheep microglia clones of varying prion permissibility, all derived from the same subline as formerly stated. Raw gene expression levels were evaluated, as were gene expression values relative to PRNP (i.e., target gene/PRNP).

Overall, there were no significant differences in gene expression observed between clones 439Late and 439Early. Significant correlations with prion permissibility were identified in expression ratios only (i.e., target gene/PRNP), and no significant correlations were detected between genes. Clone was the most common, influential factor to gene expression.

As previously stated, clone 439 was originally determined to have high permissibility to infection with naturally-derived classical scrapie isolates [29], but over time and subsequent subpassage, a shift to a more intermediate permissibility phenotype manifested [28]. Thus, this provided an opportunity to

temporally compare a clone to itself. Clone 439Late was consistently decreased by a two-to-three-fold change from 439Early with BIRC5 raw expression and BIRC5/PRNP ratios, but these changes were not statistically significant. Unlike clone 439Early, clone 439Late was significantly decreased in FSTL1 raw expression, FSTL1/PRNP and SDC4/PRNP expression ratios, but these variations were also less than two-fold. Similar to a previous study including 439Late and 439Early [28], these limited transcriptional analysis studies failed to find significant difference. A transcriptome-wide approach could be used in future studies to determine the differences between the 439Late and 439Early that result in the change of permissibility phenotype, as well as the rest of the clones, which have not been evaluated on a transcriptomic level (i.e., 434, 440, 441).

FN1/PRNP expression was strongly and negatively correlated with prion permissibility in ovine microglia. Furthermore, with pooled, Mock and Utah-inoculated FN1/PRNP expression, the effect of clone became significant with decreased expression in clones 439Late, 439Early, and 441 compared to clone 438. These findings suggest that the expression profile of FN1, relative to PRNP may contribute to prion permissibility. Marbiah *et al.* observed that FN1 silencing enhanced the levels of PrP^D accumulation in prion-susceptible murine neuroblastoma cells (i.e., R7 cells) [32]. The influence of clone parallels findings reported by Munoz-Gutierrez *et al.* in which FN1 expression was downregulated in highly-permissive clone 439 compared to poorly-permissive clone 438, both infected with scrapie prion [33]. Collectively, these findings suggest that transcript levels of FN1 influence prion permissibility and susceptibility, and that this applies across different prion isolates and types of cell culture model systems. While the mechanism of fibronectin's influence on permissibility is unknown, possible causes include prion protein binding or extracellular matrix remodeling. Growth factors have been reported to bind to FN1, becoming concealed and able to evade proteolytic degradation, with subsequent accumulation in the FN1 fibrillar matrix [34]. Potentially, prion protein could have a similar interaction with FN1. Additional studies of FN1 are warranted to determine if similar correlations with prion permissibility exist in other cell culture systems (i.e., Rov or cpRK13[35]) and prion strains (i.e., natural scrapie isolates X124 or SSBP-1, Nor98, human prion isolates). Furthermore, future studies to silence FN1 or deletion of FN1 from culture media in order to create cell culture models for human prions may be warranted. With these subsequent studies, further knowledge may be provided to strengthen the classification of FN1 as a biomarker of prion permissibility.

BIRC5 had consistently low expression in clones 439Late and 439Early. This is consistent with previous results [33]. However, BIRC5 expression was very low (nearly undetectable). While BIRC5/PRNP expression was strongly and negatively correlated with prion permissibility, this correlation was likely skewed by the results of the related 439Early and 439Late clones. Thus, the significance of these comparisons, involving such a poorly expressed gene is questionable. Since BIRC5 has a role in apoptosis (i.e., it directly inhibits activation of caspase-9, and formation of the apoptosome, suppressing the intrinsic signaling pathway of apoptosis) [36], future studies may be best targeted toward apoptosis, rather than BIRC5 specifically.

SPARC and SPARC/PRNP expression were significantly different in multiple pairwise comparisons, but lacked a correlation with prion permissibility. While likely not important to prion permissibility amongst

the clones, it is possible that the permissibility of different clones have different genetic determinants. With specialized functions in cellular adhesion, proliferation, migration [37, 38], and matrix metalloproteinase activity [38], it is possible that decreased expression of SPARC may result in pathological abnormalities in these functions that enhance prion infectivity. Booth *et al.* identified differential expression of SPARC between non-infected and scrapie-infected brain tissue from C57BL/6 mice experimentally inoculated (i.e., intracerebral) with one of two mouse-adapted prion strains (i.e., ME7, 79a) [39]. Interestingly, SPARC was also significantly affected by the inoculation status of the microglia clones. These findings indicate that for some genes, inoculation influences transcript levels, which may potentially contribute to prion permissibility, in contrast to other genes studied in ovine microglia [28, 33]. It is also possible that the pre-inoculation state of the cell is critical for initial prion replication. To the authors' knowledge, this report depicts the first characterization of SPARC in prion permissive ovine microglia clones.

PRNP expression was significantly increased in inoculated clone 439Late compared to 438, and collectively in Mock and Utah-inoculated clones 439Late and 439Early compared to clone 438, but these variations in expression were less than two-fold. Previous studies using these clones also identified some variation in expression that was of a similar magnitude in one study [28], but in the remaining study, the results were not statistically significant [33]. Additionally, in this study, many significant differential comparisons were present in target gene/PRNP expression ratios (i.e., FN1/PRNP, BIRC5/PRNP, FSTL1/PRNP). These findings suggest that transcript level ratios of certain target genes and PRNP may also influence prion permissibility. Future gene expression studies of prion diseases are suggested to evaluate gene transcript levels relative to PRNP, to account for the potential of PRNP levels interacting with the target gene levels.

FSTL1, AXL and SDC4 results demonstrated sporadic pairwise comparisons that were significant, but these genes lacked patterns or correlations. Based on these results, it is unlikely that FSTL1, AXL, or SDC4 affect prion permissibility.

Some limitations were present in this study. First, only 2 out of 3 replicates of Utah-inoculated 439Early could be assessed for BIRC5, which hindered full assessment of BIRC5 for this clone; however, this was due to the very low transcript levels for BIRC5, with one replicate being so low as to be undetectable. Second, only a single scrapie isolate was used (i.e., Utah), and assessed for differential gene expression amongst the clones. Currently, only clone 439Early from the subline has been assessed for permissiveness to other natural scrapie isolates (i.e., Pullman 1, Pullman 3, X124, 13-7) [29]. If the remaining clones are also discovered to be permissive to these isolates, evaluation and comparison of the transcriptomic profiles between clones and scrapie isolates would be beneficial. This would allow the identification of similar or disparate genes that could be differentially expressed, providing more insight on prion disease pathogenesis in ovine microglia. Thirdly, assessment of gene expression and correlations with prion permissibility were only performed for ovine microglia of VRQ/VRQ genotype, which is characterized by a rapid incubation period and fast accumulation of PrP^{Sc}. Extrapolation of the results from this study towards the development of an ARQ/ARQ sheep prion culture system could aid in

the development of the first cell culture system for slowly accumulating PrP^{Sc}, which is more characteristic of most human prion diseases.

Conclusions

Currently, definitive determinants of prion permissibility are limited, but one extracellular matrix gene identified in this study (FN1), shows considerable promise in becoming an acknowledged determinant of prion permissibility. The studies previously performed by Marbiah *et al.*, Munoz-Gutierrez *et al.*, and this current study suggest that alterations in FN1 expression levels consistently correlate with prion permissibility and susceptibility across two distinct cell culture model systems. For this study, FN1/PRNP expression was strongly and negatively correlated with prion permissibility. These findings suggest prion permissibility is not dependent on individualized gene expression in ovine microglia cells, but other factors may be involved to include cell of origin, type of scrapie isolate or strain, post-transcriptional or post-translational factors, and possibly additive effects of gene expression (e.g., target gene levels relative to PRNP levels).

Future studies are needed to fully characterize the functionality of these genes and their role in prion permissibility in different cell culture model systems, with various prion isolates or strains, and transcriptomic analysis.

Methods

2.1 Cell culture line

In previous studies, ovine microglia were isolated from the fetal brain of a pregnant, near-term, Suffolk-cross ewe [40], and immortalized (hTERT) with construction of sublines, and cloning procedures implemented for a single subline (subline H) in our lab [29], which was approved by the Institutional Animal Care and Use Committee of Washington State University, Pullman (ASAF04575). Six ovine microglia clones from subline H were further evaluated in the experimental study (438, 440, 434, 439Late, 441, and 439Early), arranged in order of diminishing permissibility to scrapie prion infection [28]. Clone 438 was least permissive; clones 440, 434 and 439Late of intermediate permissiveness, and clones 441 and 439Early as highly permissive [28, 29, 33]. 439Late and 439Early refer to a single clone whose permissibility changed after continual passage [41], and are treated as two separate clones in the study. These clones were previously inoculated with either a natural, sheep-derived scrapie isolate (Utah) (derived from Animal Disease Research Unit; USDA Agricultural Research Service, Pullman, WA from a natural infected sheep in Utah, USA), or uninfected ovine brain homogenate (Mock) [28, 29]. Cells were cultured under standard conditions of Opti-MEM media (Gibco), supplemented with 10% complement-inactivated fetal bovine serum (Atlanta Biologicals), 10,000 units/mL penicillin (Hyclone), 10,000 µg/mL streptomycin (Hyclone), and 200 mM L-Glutamine (Hyclone). Cells were passaged every 3 to 4 days at a 1/5 dilution.

2.2 Extraction, collection and analysis of RNA

Cell suspensions for use with five experiments were previously collected at passage 5 (P-5) for Mock-inoculated clones, and for Utah-inoculated clones at passages: P-2 (experiment 2.3Redo), P-4 (experiment 2.2), P-5 (experiment 1), P-6 (experiment 2.3), and P-7 (experiment 2.7) [28]. Over the course of all experiments, a total of at least 3 independent replicates of each clone, per inoculation status, were tallied. Cell suspensions were homogenized with QIAshredders (Qiagen) according to manufacturer's instructions. RNA was extracted from lysates using RNeasy Mini Kit (Qiagen), per manufacturer's directions. Eluted RNA was collected into DNA LoBind microcentrifuge tubes (Eppendorf) and stored at -80 °C until further use. When ready to use, RNA purity and concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.3 DNase-treatment of RNA

RNA was standardized at 10 µg/50 µL, and residual DNA was removed using Invitrogen DNA-free DNase Treatment and Removal kit (Fisher Scientific). A total volume of 50 µL was prepared per sample using 5 µL of 10x DNase I buffer, 1 µL of rDNase I, eluted RNA, and nuclease-free water. Each sample was incubated at 37 °C for 30 minutes before adding 5 µL of DNase Inactivation reagent. After addition of the inactivation reagent, the sample was mixed frequently and incubated at room temperature (~ 25 °C) for 2 minutes. Subsequent to centrifugation of the sample at 10,000 x g for 1.5 minutes, RNA was collected and transferred to a new, 0.5 mL DNA LoBind microcentrifuge tube (Eppendorf). RNA was either immediately stored at -80 °C, or purity and concentration were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific), before storage for later use.

2.4 First strand cDNA synthesis

DNase-treated RNA was standardized to 1 µg/20 µL, and first strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis Supermix for qRT-PCR (ThermoFisher Scientific). Each sample consisted of 10 µL of 2x RT reaction mix, 2 µL of RT enzyme mix, 1 µg of DNase- treated RNA, and DEPC-treated water for a total volume of 20 µL. Samples were gently mixed, then incubated at 25 °C for 10 minutes, 50 °C for 30 minutes, and 85 °C for 5 minutes. After incubation, samples were chilled on ice for 5 minutes, and then 1 µL of *E. coli* RNase H was added before an additional incubation at 37° C for 20 minutes. After the last incubation, samples were chilled on ice for 5 minutes, and immediately stored at -80 °C.

2.5 Primers

Genes identified from previous RNAseq data in two clones of varying prion permissibility [33] were further assessed (Table 2).

Table 2. Primers for qRT-PCR and RT-PCR.

Genes	Gene name	Accession number for sequence	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	Amplicon size (bp)
AXL ^a	AXL receptor tyrosine kinase	XM_004015278.4	AAGGACAGCCAATCCACCAG	ACACCTCTCCATAACGGGTCT	165
BIRC5 ^a	Baculoviral IAP repeat-containing protein 5/Survivin	XM_004013098.3	CCCGACTTGGCTCAGTGTTT	AGCACAAACCAGATGAATGCTT	102
hPRT1 ^a	Hypoxanthine phosphoribosyltransferase	XM_015105023.1	GGCTCCGTTATGGCGGC	TTCGGTCCTGTCCATAATTAGTCC	150
18srRNA ^a	18s ribosomal RNA	XR_003587981.1	GAGGCCCTGTAATTGGAATGA	GCAGCAACTTTAATACGCTATTGG	120
FN1 ^c	Fibronectin 1	XM_004004911.4	TCTCGTGCCAGTGCTTAG	CTGACGATCCCACTTCTCTCC	110
FSTL1 ^a	Follistatin-like 1	XM_027980274.1	TCATTCCAGACGGCTGGTTC	CTGGAGTCCAAGCGAGAGTC	100
PRNP ^b	Prion protein	NM_001009481.1	CCGTTACCCCAACCAAGTGT	CGCTCCATTATCTTGATGTCAGT	159
SPARC ^a	Osteonectin	XM_012177565.3	CTGTGACCTGGACAACGACA	GGAGGCGTGGCTTTAGATCA	109
SDC4 ^a	Syndecan 4	XM_027977234.1	CTGATGACGAGGACATCGGG	ATAGGGACCAAGGGGTGCAT	127

^a Primers were designed using either presumed or curated mRNA from selected genes in the database program of NIH, US National Library of Medicine, National Center for Biotechnology Information (NCBI) [42].

^b PRNP primer sequences were derived from a previous publication [43].

^c FN1 primer sequences were designed using PriFi [44].

These annotated genes include fibronectin 1 (*FN1*), follistatin-like 1 (*FSTL1*), osteonectin (*SPARC*), survivin (*BIRC5*), syndecan 4 (*SDC4*), AXL receptor tyrosine kinase (*AXL*), and prion protein (*PRNP*). 18s ribosomal RNA (*18s rRNA*) and hypoxanthine phosphoribosyltransferase 1 (*hPRT1*) are both reference genes that have shown stable expression as internal controls in quantitative PCR analyses [45-47], and were hence utilized as reference genes in this study. With the exception of PRNP [43] and FN1 [44], which were previously published, primers were designed for each gene according to a reference sequence listed for the gene using the database program from the National Center of Biotechnology Information [42]. Standard curves for each primer set were performed to determine efficiency, confirm predictability across dilutions ($r^2 > 0.85$), and establish a single, repeatable product melting temperature. The resulting products were also evaluated by standard agarose gel electrophoresis to confirm the product size.

2.6 Sequencing of PCR products

All PCR products were cloned and sequenced to confirm amplification of the correct gene target. Superscript IV first strand cDNA synthesis system kit for RT-PCR (ThermoFisher Scientific) was used, according to manufacturer's instructions. Each reaction contained 50 ng of RNA, derived from naïve ovine microglia clone 439. First strand cDNA samples were then immediately used or stored at -20 °C. Conventional PCR was implemented using Advantage II PCR Enzyme System (Clontech) according to manufacturer's directions. Each reaction was composed of 40 µL of nuclease-free water, 5 µL of 10X PCR buffer, 1 µL of 50x dNTPs, 1 µL forward primer, 1 µL reverse primer, either 1 µL of Taq polymerase or nuclease free water, and 1 µL of cDNA. The PCR protocol was as follows: 95 °C for 5 minutes; 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds; and 72 °C for 7 minutes. Gel

electrophoresis was used to assess the size and solitary nature of the products. Bands not exposed to ultraviolet imaging were cut from the gel, and the DNA was purified using QIAEX II Agarose Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. Eluted, purified DNA was ligated into a pGEM-T easy vector system (Promega) per manufacturer's directions. Two microliters of each ligation reaction were transformed into 50 μ L of JM109 high efficiency competent cells and plated, according to manufacturer's instructions with few modifications. Cells were heat shocked at 43°- 45 °C, transformation cultures were incubated for 2 hours at 37 °C with a shaking speed of 420 rpm, and approximately 200 μ L of transformation cultures were plated onto LB/Ampicillin plates. After an overnight incubation at 37 °C, up to 9 clones were selected per plate, and evaluated for the presence of the correct gene product using conventional PCR and gel electrophoresis. Selected clones were isolated on an additional LB/Ampicillin agar plate, and placed in a 37 °C incubator for 8 – 12 hours. For each gene, a single clone of the correct size was further isolated in 3 μ L of ampicillin, 3 mL of sterile LB medium, and a pipette tip containing the colony, in an overnight 37 °C incubation with a shaking speed of 420rpm. After incubation, DNA was eluted (QIAprep Spin Miniprep Kit, QIAGEN), per manufacturer's directions, and measured for concentration and purity using NanoDrop 2000 spectrophotometer (Thermo Scientific). Eluted DNA was submitted for commercial Sanger sequencing using GENEWIZ (www.genewiz.com) or MCLAB (www.mclab.com). Forward and reverse sequences were aligned using software programs Mega 6 (Molecular Evolutionary Genetics Analysis; www.megasoftware.net) and Geneious Prime (www.geneious.com), and the resulting consensus sequences were aligned to the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov) nt database, using megablast.

2.7 qPCR

Each reaction contained 10 μ L SsoAdvanced Universal SYBR Green Supermix, 0.4 μ L forward primer, 0.4 μ L reverse primer, and 7.2 μ L nuclease-free water. Either 2.5 μ L of appropriately diluted cDNA or nuclease-free water for negative control was added to each reaction, and a single non-reverse transcriptase (NRT) control was run for each clone and gene. Reactions were then run with CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following protocol for all primers: 95 °C for 30 minutes; 35 times of 95 °C for 15 seconds then 60 °C for 30 seconds; followed by 95 °C for 10 minutes, 60 °C for 5 minutes, and an increase in temperature in 0.5 °C increments from 60 °C to 95 °C.

2.8 Data Analysis and Statistical computation

Gene expression data normalized to reference genes 18s rRNA and hPRT1 was retrieved and evaluated from at least 3 independent replicates using the software program CFX Manager 3.1 (www.bio-rad.com: Bio-Rad, Hercules, CA). Statistical analyses were performed using JMP version 14 (www.jmp.com; SAS Institute, Cary, NC.). However, for Utah-inoculated clone 439Early, only 2 of the 3 independent replicates had the acquisition of data for BIRC5 (i.e., undetectable levels of transcript in one replicate). Relative prion permissibility was previously determined [28]. Normality of the data distribution was assessed per inoculation status, using histograms and Goodness-of-fit test. For data that was not normally distributed, Box-Cox Y transformation was used for approximation to normality, and this newly configured data was

assessed for normality using histograms and Goodness of Fit test. Fold changes were computed manually using raw normalized expression (Mode in CFX Manager: Normalized expression ($\Delta\Delta Cq$)) collectively, and then relative to the previously identified least permissive clone, 438 [28], for each gene by treatment group. Statistical significance of fold change (i.e., clone) was assessed using One-way ANOVA and *post hoc* Tukey-Kramer HSD for multiple pairwise comparisons ($p < 0.05$). Correlations of mean normalized gene expression between genes, and with prion permissibility per treatment group, were calculated using multivariate analysis with Pearson's correlation coefficient (Pearson's r), and Benjamini-Hochberg multiple comparison correction. The effect of inoculation status, and the interaction of clone and inoculation status on gene expression were analyzed using pooled, raw expression data by gene in Two-way ANOVA and *post-hoc* Tukey HSD for multiple pairwise comparisons ($p < 0.05$). For genes in which inoculation status was not influential to expression, mean normalized gene expression data were pooled by gene and clone, and correlations with prion permissibility were calculated using Pearson's r and Benjamini-Hochberg multiple comparison correction. qRT-PCR analyses and statistical calculations as previously described were also performed for relative density expression (Mode in CFX Manager: Relative quantity (ΔCq)) of target genes relative to PRNP (target gene/PRNP ratios) using at least 3 independent replicates.

Abbreviations

Scrapie prion protein (PrP^{Sc}), transmissible spongiform encephalopathies (TSEs), cellular prion protein (PrP^C), disease-associated prion protein (PrP^D), prion protein (PrP), fibronectin 1 (FN1), follistatin-like 1 (FSTL1), osteonectin (SPARC), survivin (BIRC5), syndecan 4 (SDC4), AXL receptor tyrosine kinase (AXL), prion protein gene (PRNP), valine-arginine-glutamine (VRQ), alanine-arginine-glutamine (ARQ), alanine-arginine-arginine (ARR), ovinized rabbit kidney epithelial cells (Rov), murine neuroblastoma cells inoculated with mouse-adapted scrapie isolate (ScN2a), 18s rRNA (18s ribosomal RNA), hypoxanthine phosphoribosyltransferase 1 (hPRT1), E (Early), L (Late), human telomerase (hTERT), caprinized rabbit kidney epithelial cells (cpRK13), reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Declarations

a) Ethics approval and consent to participate

A previously established immortalized (hTERT) ovine microglia cell subline (Subline H) was utilized [29, 40], which was approved by the Institutional Animal Care and Use Committee of Washington State University (ASAF04575).

b) Consent for publication

Not applicable

c) Availability of data and material

All data generated or analysed during this study are included in this published article.

d) Competing interests

The authors declare that they have no competing interests.

e) Funding

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f) Author's contributions

JBS provided guidance on experimental design, collection and analysis of data. KD inoculated and maintained cell culture clones used in this study. ZN performed validation and efficiency testing of some primers. VRM performed the experiments, collected and analyzed the data. All authors have read and approved this manuscript.

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Figures

Figure 1

Fold change in gene expression for Mock-inoculated clones. Fold changes were assessed using normalized gene expression relative to the least permissive clone (438) for Mock-inoculated clones. Expression data was normalized to 18s rRNA and hPRT1, scaled to 438, and then the geometric mean and geometric standard deviation factor were calculated for graphical representation. Solid, black horizontal lines (i.e., 2¹ or 2⁻¹) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axes are partitioned into panels by gene (AXL, BIRC5, FN1, FSTL1, PRNP, SDC4, SPARC). Error bars indicate 1 geometric standard deviation factor from the geometric mean. In differentially expressed genes, clones that do not share the same letter had significant differential mean expression (p < 0.05). E = Early, L = Late

Figure 2

Fold change in gene expression for Utah-inoculated clones. Fold changes were assessed using normalized gene expression, relative to the least permissive clone (438) for Utah-inoculated clones. Expression data was normalized to 18s rRNA and hPRT1, scaled to 438, and then the geometric mean and geometric standard deviation factor were calculated for graphical representation. Solid, black, horizontal lines (i.e., 2¹ or 2⁻¹) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axes are partitioned into panels by gene (AXL, BIRC5, FN1, FSTL1, PRNP, SDC4, SPARC). Error bars indicate 1 geometric standard deviation factor from the geometric mean. In differentially expressed genes, clones that do not share the same letter had significant differential mean expression ($p < 0.05$). E = Early, L = Late

Figure 3

Effect of inoculation on transcript levels of FSTL1, SDC4, SPARC. The effect of inoculation on transcript levels was calculated by dividing the Mock-inoculated value by the Utah-inoculated value. Geometric mean and geometric standard deviation were calculated for graphical representation. Solid, black horizontal lines (i.e., 21 or 2 -1) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axis is partitioned into panels by gene (FSTL1, SDC4, SPARC). Error bars indicate 1 geometric standard deviation factor from the geometric mean. In differentially-expressed genes, clones that do not share the same letter had significant differential mean expression ($p < 0.05$). E = Early, L = Late

Figure 4

Fold change in target gene/PRNP expression in Mock-inoculated clones. Fold changes were assessed using relative expression data, scaled to the least permissive clone (438) for Mock-inoculated clones. Relative expression ratios were constructed (i.e., target gene/PRNP expression), and then the geometric mean and geometric standard deviation were calculated for graphical representation. Solid, black horizontal lines (i.e., 2¹ or 2⁻¹) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axes are partitioned into panels by gene (AXL, BIRC5, FN1, FSTL1, SDC4, SPARC). Error bars indicate 1 geometric standard deviation factor from the geometric mean. In differentially-expressed genes, clones that do not share the same letter had significant differential mean expression (p < 0.05). E = Early, L = Late

Figure 5

Fold change in expression of target gene/PRNP expression in Utah-inoculated clones. Fold changes were assessed using relative expression data, scaled to the least permissive clone (438) for Utah-inoculated clones. Relative expression ratios were constructed (i.e., target gene/PRNP expression), and then the geometric mean and geometric standard deviation factor were calculated for graphical representation. Solid, black, horizontal lines (i.e., 2¹ or 2⁻¹) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axes are partitioned into panels by gene (AXL, BIRC5, FN1, FSTL1, SDC4, SPARC). Error bars depict 1 geometric standard deviation factor from the geometric mean. In differentially-expressed genes, clones that do not share the same letter had significant differential mean expression (p < 0.05). E = Early, L = Late.

Figure 6

Comparison of Target gene/PRNP expression for Mock and Utah-inoculated clones. Expression ratios were pooled from Mock and Utah-inoculated clones, relative to PRNP expression, for genes in which inoculation was insignificant. Geometric mean and geometric standard deviation factor were calculated per ratio for graphical representation. Solid, black, horizontal lines (i.e., 21 or 2 -1) demarcate the two-fold change threshold. The x-axis is ordered by increasing prion permissibility phenotype (left to right). The horizontal axis is partitioned into panels by gene (AXL, BIRC5, FN1). Error bars depict 1 geometric standard deviation factor from the geometric mean. In differentially-expressed genes, clones that do not share the same letter had significant differential mean expression ($p < 0.05$). E = Early, L = Late.