# IDENTIFYING FACTORS THAT ENHANCE PRION ACCUMULATION IN CULTURED SHEEP MICROGLIAL CELLS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JAMES BRANTLY STANTON find it satisfactory and recommended that it be accepted.

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Chair

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Abstract

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Transmissible spongiform encephalopathies (TSE, prion diseases) are invariably fatal, neurodegenerative diseases. Unlike other neurodegenerative diseases, such as Alzheimer's disease, many TSE are transmissible. Based on the protein-only hypothesis, this transmissibility is thought to be conferred by the ability of the abnormal isoform ($PrP^{Sc}$) to catalyze the conversion of the normal cellular form of the host encoded prion protein ($PrP^{C}$) to additional $PrP^{Sc}$ molecules. The conformational difference between $PrP^{C}$ and $PrP^{Sc}$ is the abundance of beta-sheets within the $PrP^{Sc}$ molecules, which renders $PrP^{Sc}$ detergent insoluble and partially resistant to protease digestion. The stability of $PrP^{Sc}$ results in the aggregation of $PrP^{Sc}$ in affected tissues and serves as the marker for prion diseases. TSE can be found in many species and include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease (CJD) and kuru in humans. The similarities between scrapie and CJD have long been recognized and the use of scrapie as an experimental model allows for the investigation of a natural prion disease in a natural host. This work utilized a sheep-model of prion diseases to investigate the role of possible co-factors involved in the accumulation of $PrP^{Sc}$. In the first study, it was determined that coinfection of microglial cell cultures with both $PrP^{Sc}$ and a noncytopathic small ruminant

lentivirus (SRLV) resulted in a relative increase in the amount of $PrP^{Sc}$ accumulated within and released by the cultured microglial. Additional studies are required to determine the biological significance and mechanism of this pathogen synergism. The second study sought to describe the cellular response of sheep microglia to accumulation of $PrP^{Sc}$ in an attempt to identify proteins that might directly interact with $PrP^{C}$ or $PrP^{Sc}$. Forty-nine genes were determined to be differentially regulated in this second study. Several of these genes have previously been determined to be differentially expressed in mouse and human models of prion disease. Determining if any of these identified genes play a direct role in the pathogenesis of prion diseases, requires additional studies.

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## Dedication

This dissertation is dedicated to

Dr. Corrie C. Brown; mentoring the next generation is one of the greatest gifts that a person can give. I hope that I can pass the legacy on.

My wife, Julie, for leading the way into an academic career and for her constant support during the turbulent times that are always associated with research.

My son, Spencer; who doesn't know it, but he helped me to remember what is important in life.

# CHAPTER 1

# SMALL RUMINANT LENTIVIRUS ENHANCES $PrP^{Sc}$ ACCUMULATION IN CULTURED SHEEP MICROGLIAL CELLS

## ABSTRACT

Sheep scrapie (Sc) is the prototypical transmissible spongiform encephalopathy (prion disease), which has a fundamental pathogenesis involving conversion of normal cellular prion protein ($PrP^{C}$ [C superscript stands for cellular]) to disease-associated prion protein ($PrP^{Sc}$ [Sc superscript stands for scrapie]). Sheep microglial cell cultures, derived from a PRNP 136VV/171QQ near-term fetal brain, were developed to study sheep scrapie in the natural host and to investigate potential cofactors in the prion conversion process. Two culture systems, a primary cell culture and a cell line transformed with the large T antigen of SV40, were developed and both were identified as microglial in origin as indicated by expression of several microglial phenotype markers. Following exposure to $PrP^{Sc}$, sheep microglial cells demonstrated relatively low (transformed cell line) to high (primary cell line) levels of $PrP^{Sc}$ accumulation over time. The accumulated $PrP^{Sc}$ demonstrated protease resistance, an inferred beta-sheet conformation (as determined by commercial ELISA), specific inhibition by anti-PrP antibodies, and was transmissible in a dose-dependent manner. Primary microglia coinfected with a small ruminant lentivirus (caprine arthritis encephalitis virus-Cork strain) and $PrP^{Sc}$ demonstrated an approximate twofold relative increase in $PrP^{Sc}$ accumulation as compared to primary microglia infected with $PrP^{Sc}$ alone. The results demonstrate the in vitro utility of $PrP^{Sc}$-permissive sheep microglial cells in investigating the biology of natural prion diseases, and show that small ruminant lentiviruses enhance prion conversion in cultured sheep microglia.

## INTRODUCTION

Prion diseases (transmissible spongiform encephalopathies, TSE) are a group of invariably fatal, transmissible, neurodegenerative diseases, which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease (CJD) and kuru in humans (38). The similarities between scrapie and CJD have long been recognized (36) and the use of scrapie as an experimental model allows for the investigation of a natural prion disease in a natural host. The central feature of prion pathogenesis is the conversion of the normal cellular form of the host encoded prion protein ($PrP^{C}$) to an abnormal isoform, designated $PrP^{Sc}$ (6, 10, 13). The conversion occurs post-translationally and involves a conformational change resulting in the generation of a detergent insoluble, partially protease resistant molecule that aggregates in affected tissues and serves as the marker for prion diseases. The principal component of the transmissible agent is thought to be the abnormal prion protein and provides the basis for the protein-only hypothesis of prion diseases (50).

There are at least twenty-one cell lines that have been used to study prion diseases in vitro (59). However, only four of these are susceptible to $PrP^{Sc}$ derived from a natural TSE host, while the remaining seventeen cell lines are only susceptible to rodent-adapted strains of $PrP^{Sc}$. Further, only one of the cell lines is derived from a natural TSE host, mule deer (*Odocoileus hemionus*) (51). While rodent-derived cells have many benefits, including the availability of reagents and highly inbred genetics, much of the work accomplished in these cells has to be verified in a natural host-TSE system. The lack of a sheep-derived, scrapie-permissive cell line also prevents full investigation into such species specific phenomena as allelic usage variation,

allelic-predicted susceptibility, and species-specific cofactors. Therefore, the development of a sheep cell culture system would provide an excellent model for such studies.

In addition to creating cell lines that accumulate $PrP^{Sc}$, it is also desirable to use cells that contribute to the pathophysiology of the clinical disease. Microglia (resident brain macrophages) are such cells, which not only accumulate infectivity in vivo (5), but are also thought to play a role in the neuropathology by their activation and release of immune mediators such as IL-6 (8, 52, 63). Additionally, peripheral macrophages have demonstrated both accumulation (9, 23, 28, 41, 49) and proteolysis of $PrP^{Sc}$ (9, 28). Only one of the current cell culture systems demonstrates microglial or macrophage characteristics, and this cell line is mouse-derived and overexpresses the murine prion gene (33). While overexpression of $PrP^{C}$ often increases the permissiveness of cells to $PrP^{Sc}$ accumulation, it can also introduce spontaneous cell pathology (62) and is a confounding factor when trying to study the effects of $PrP^{Sc}$ accumulation at the cellular level.

Another area that would benefit from a natural host-TSE cell culture system is the investigation into possible cofactors for the prion conversion process (55). Identification of these accessory molecules is still unresolved; however, several studies suggest that nucleic acids are a possible family of cofactors (2, 14, 15, 18, 19, 21, 64). Interestingly, recombinant prion protein has demonstrated the ability to bind and chaperone retroviral RNA, which is similar to retroviral nucleocapsid's function (18, 19, 42). Other interactions between prion protein and retroviruses have been identified including an increase in murine leukemia viral (MLV) titers and replication, and a shortened scrapie incubation period in the brains of coinfected mice (12, 40). In vitro coinfection studies have also demonstrated increased scrapie infectivity release into the cell culture supernatant in murine cell cultures coinfected with MLV (39).

Sheep, the natural host of scrapie, are also clinically affected by retroviruses, most notably the small ruminant lentiviruses (SRLV) visna/maedi virus (VISNA) and caprine arthritis-encephalitis virus (CAEV) (47). These viruses have a worldwide distribution and are the target of eradication programs (47). However, there has been little work published regarding any possible correlation between scrapie and infection with SRLV, with only one report to the authors' knowledge demonstrating a correlation between VISNA induced lymphofollicular mastitis and resulting $PrP^{Sc}$ accumulation within macrophages and follicular dendritic cells (41). Other studies have demonstrated that chronic inflammation of various organs results in $PrP^{Sc}$ accumulation within those organs that normally lack $PrP^{Sc}$ in prion-affected animals (26, 37), suggesting that the effect of VISNA on $PrP^{Sc}$ in mastitis was indirect. To directly determine if coinfection with a small ruminant lentivirus increases accumulation of $PrP^{Sc}$, a sheep microglial cell culture system was developed and utilized in coinfection studies with $PrP^{Sc}$ and SRLV.

## MATERIALS AND METHODS

**Primary ovine brain cell cultures.** Primary mixed glial cell cultures were obtained from an ovine fetal brain using a mechanical dissociation technique for small ruminants previously described in our laboratory (7). The ovine fetus was obtained from a near term pregnant Suffolk-cross ewe that was housed and cared for in accordance with Institutional Animal Care and Use Committee at Washington State University, Pullman, WA. At approximately day 102 of gestation, the ewe was euthanized by intravenous overdose of barbiturate and the fetal brain was removed in toto. Approximately 250 mg of brain tissue was removed from the cerebral cortex and used for genotyping of the fetal prion gene, as previously described (3). Periventricular white matter tissue from the remainder of the cerebral cortices and

midbrain was collected, cut into approximately 5-mm cubes and disassociated by mechanical triturating in a 25-ml pipette. The resulting brain tissue explants were plated into 75-$cm^2$ tissue culture flasks with Dulbecco's Modification of Eagle's Medium (Cellgro) supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 IU/ml of penicillin, 10 mg/ml streptomycin, and 2.5 μg/ml Amphotericin B and left undisturbed for approximately one week. Upon reaching confluency, aliquots of cells were frozen in 90% heat-inactivated FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Aliquots of cells were thawed as needed and serially passaged, using standard techniques, in OPTI-MEM® I Reduced Serum Medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 IU/ml of penicillin, and 10 mg/ml of streptomycin (OMEM).

**Transformation of brain cells.** Cells were transfected, as per manufacturer's instructions using the LF2000 reagent (Invitrogen), with a plasmid containing the SV40 virus large T antigen, as described previously (25, 27). Primary cells were split in triplicate into 24-well plates and allowed to grow to approximately 60% confluency. Three wells of cells were incubated with plasmid DNA in LF2000 reagent (Invitrogen) and OPTI-MEM® for eight hours at 37°C in 5% $CO_2$, while the three remaining wells were sham-transfected by incubating in LF2000 reagent and OPTI-MEM® without plasmid DNA. Following eight hours, the cell culture medium was changed to OMEM. Cells were fed every four days and allowed to grow for 12 days. Cells were then lifted from the plate and passed without dilution into 25-$cm^2$ plastic tissue culture flasks. Cells were fed every 3–5 days as needed and serially passed 1/10 after reaching confluency. Transfected cells demonstrated an increase in mitotic activity and a loss of contact inhibition. Immunocytochemistry, with the SV40 large T specific mAb DP02A (Oncogene Res. Prod., Cambridge, MA), was used to confirm transfection, as previously described (27).

**Characterization of microglial cell cultures.** For characterization the brain cell cultures were phenotyped using various cell markers and functional activity assays. The microglial and endothelial marker biotinylated *Ricinus communis* agglutinin-1 (RCA-1) (Dako Cytomation) was used in immunocytochemistry and flow cytometry as previously described (7). Non-specific esterase activity (Sigma) was performed as per manufacturer's directions (7). Two additional markers, CD14 (MM61A, IgG1, VMRD, Inc.) and CD68 (EBM11, IgG1, Dako), that are predominately found on cells of the monocytic lineage were tested by immunocytochemistry and flow cytometry. In cells of the monocyte lineage, CD14 is a membrane-bound receptor for lipopolysaccharide (34). CD68 is a lysosomal associated glycoprotein used to identify macrophages (30), and while the mAb EBM11 was raised against human CD68, it has previously demonstrated immunoreactivity against bovine macrophages (1), thus suggesting its utility in this study of sheep cells. For flow cytometric detection of CD14 expression, cells were trypsinized and incubated with the primary antibody. Following three washes, cells were incubated with a secondary fluorescein isothyocyanate-conjugated goat anti-mouse immunoglobulin antibody. Cells were then washed twice and fixed in 2% formaldehyde. CD68 is a predominately intracellular antigen; therefore, cells were fixed for 2 days in 10% neutral buffered formalin, permeabilized in 0.1% Triton X-100 in PBS for 5 minutes, washed and then labeled as described above. Five thousand events were analyzed on a FACSort flow cytometer (Becton Dickinson) and counts determined with Macintosh CellQuest software (BD Biosciences). Results were graphically analyzed using FCS Express (De Novo Software). The Kolmogorov Smirnov test (CellQuest), with a cut-off *P*-value of 0.05, was used to determine significance. Negative controls included the use of isotype matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies.

For immunocytochemistry, cells were grown in chambered glass slides (Nunc) and allowed to grow to approximately 70% confluency. Cells were rinsed in PBS and fixed in 100% ethanol for ten minutes. Following quenching of endogenous peroxidase with hydrogen peroxide for 10 minutes, cells were assayed for expression of the antigens using the Signet™ kit (Covance), as per manufacturer's instructions, and the antibodies listed above. The immunolabeling was visualized with 3-Amino-9-ethylcarbazole (Dako) with nuclear counterstaining by Mayer's hematoxylin. Negative controls included the use of isotype matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies.

**Inoculation of primary microglia with $PrP^{Sc}$.** Rov9 cells, which are rabbit renal epithelial cells that are stably transfected with the sheep VRQ (Val-136, Arg-154, and Gln-171) allele of the prion gene under the control of a tetracycline inducible promoter and are susceptible to sheep $PrP^{Sc}$ (60), were used as the inoculum. Rov9 cells with detectable amounts of $PrP^{Sc}$ ($Rov9^{Sc}$) and Rov9 cells that were never exposed to $PrP^{Sc}$ ($Rov9^{C}$) were obtained (B. Caughey with permission from D. Vilette) and maintained in OMEM supplemented with 1 µg/ml Doxycycline (OMEM-Dox), as previously described (60). $PrP^{Sc}$ within $Rov9^{Sc}$ cells was verified by $PrP^{Sc}$ specific immunoblotting and ELISA (see below). For use as an inoculum, mechanical lysates of the $Rov9^{Sc}$ and $Rov9^{C}$ cells were prepared as previously described (60). Briefly, the Rov9 cells were grown to confluency in two 75-$cm^2$ plastic tissue culture flasks. $Rov9^{Sc}$ and $Rov9^{C}$ cells were rinsed three times with sterile 1× Dulbecco's phosphate buffered saline (D-PBS) and scraped into 10 ml of PBS. The cell pellets were collected by centrifugation at 170 × *g* at room temperature for 10 minutes and resuspended in 0.5 ml of filter-sterilized 5% glucose. The cell suspensions were frozen and thawed four times, and then subjected to 1–2 minutes of

sonication in a cup horn sonicator. The inoculum was stored at -20°C. For inoculation, primary microglia were passed into 6-well plates and allowed to grow to approximately 60% confluency. Microglia were rinsed once with PBS and then overlaid with 200 µl of a 1/20 dilution of either the $Rov9^{Sc}$ lysate ($microglia^{Sc}$) or the $Rov9^{C}$ lysate ($microglia^{C}$) in OPTI-MEM®. $Microglia^{Sc}$ and $microglia^{C}$ were incubated for six hours and then 200 µl of OMEM was added to each well. Following an additional two days of incubation an additional 0.5 ml of OMEM was added to each well and microglia were incubated for four days at which time they were expanded into 25-$cm^2$ tissue culture flasks. Microglia were fed every three to four days with OMEM as necessary and serially passed 1/5 after reaching confluence.

**Detection of $PrP^{Sc}$ by ELISA.** At selected passages following trypsinization, 4/5 of the microglial cell suspension from a 25-$cm^2$ tissue culture flask was rinsed in D-PBS and then lysed in 120 µl of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0, 5mM EDTA, and 150 mM NaCl) for 3 minutes at room temperature, followed by centrifugation at approximately 2300 × *g* at room temperature for 5 min. One hundred microliters of the supernatant was then used for $PrP^{Sc}$ detection by the HerdChek™ Scrapie Antigen Test Kit ELISA (IDEXX) following the manufacturer's instructions. The proprietary ELISA positive and negative controls were used as per manufacturer's instructions. A standard curve prepared from diluted $Rov9^{Sc}$ inoculum (1/20, 1/100, and 1/400) was used to normalize corrected optical density results. $Microglia^{Sc}$ results from P-2 were set at 1 and all other results were normalized to this value. The $PrP^{Sc}$ signal from P-5 was compared to P-2 using an independent t-test with a cut-off *P*-value of 0.05 (SigmaPlot). Cells were considered positive for $PrP^{Sc}$ accumulation if the corrected optical density was greater than 0.18 + the negative control value (as per manufacturer's methods). To assay for accumulation of $PrP^{Sc}$ over time and to

compare the amount of $PrP^{Sc}$ within $microglia^{Sc}$ to the amount in the original inoculum, the $PrP^{Sc}$ signal within late passage $microglia^{Sc}$ lysates was compared to early passage $microglia^{Sc}$ lysates and to dilutions of the inoculum, respectively, using an independent t-test with a cut-off *P*-value of 0.05 (SigmaPlot). $Microglia^{C}$ lysates served as the experimental negative controls.

**Demonstration of $PrP^{Sc}$ infectivity from $microglial^{Sc}$-derived lysates.** Inocula were derived from mechanical lysates of $microglia^{Sc}$ and $microglia^{C}$ as described above for the preparation of inocula from Rov9 cells. $Rov9^{C}$ cells (the target cells) were then inoculated as described above with three dilutions (1/20, 1/200, and 1/2000) of the $microglia^{Sc}$ and $microglia^{C}$ inocula. The Rov9 cells were then expanded once and split 1/5 three times. Lysates from the Rov9 cells were analyzed for $PrP^{Sc}$ accumulation in a single HerdChek™ Scrapie Antigen Test Kit ELISA (IDEXX) plate and reported as the corrected optical density. Cells were considered positive for $PrP^{Sc}$ accumulation if the corrected optical density was greater than 0.18 + the negative control value (as per manufacturer's methods). The $PrP^{Sc}$ signal of each of the $microglia^{Sc}$-exposed Rov9 cells was compared to the signal of the respective $microglia^{C}$-exposed Rov9 cells using an independent t-test with a cut-off *P*-value of 0.05 (SigmaPlot).

**Inoculation of immortalized microglia with $PrP^{Sc}$ and antibody-based inhibition.** Immortalized microglial cultures were cloned by limiting dilution in 96-well plastic, flat-bottom tissue culture plates. Twenty wells contained small colonies that were visualized and split 1/2 into replicate 96-well formats. One replicate was inoculated with 1 µl of the $Rov9^{Sc}$ lysate, and the other replicate was inoculated with 1 µl of the $Rov9^{C}$ lysate (negative control) in 100 µl of OMEM. Following confluency, cells and medium (with residual inoculum) were expanded in toto into 24-well plates and cultured in approximately 1 ml total volume of OMEM. One week later, an additional 5 µl of $Rov9^{Sc}$ lysate ($Rov9^{C}$ lysate for negative control cells) was added to

the appropriate wells, and four days later, 0.5 ml of OMEM was added to each well. At confluency, cells and medium were first expanded into 6-well tissue culture plates (approximately 3 ml total of OMEM) and then finally into 75-$cm^2$ tissue culture flasks (25 ml total of OMEM), each time without dilution. Cells were then serially passed 1/5 at confluency in OMEM using standard techniques. At select passage points, an aliquot of cells was collected, lysed, and evaluated for $PrP^{Sc}$ accumulation by ELISA, as described above. Cells were considered positive for $PrP^{Sc}$ accumulation if the corrected optical density was greater than 0.18 + the negative control value (as per manufacturer's methods) after five 1/5 splits or later. $PrP^{Sc}$ was verified by immunoblotting (see below).

$PrP^{Sc}$ accumulation within the $PrP^{Sc}$-positive SV40 large T transformed cell line (B6) was then inhibited by a prion specific antibody. Similar to the previously described method, B6 cells were treated for thirteen days with the recombinant anti-prion protein Fab D18 (InPro Biotechnology), which has demonstrated the ability to bind membrane bound $PrP^{C}$ and inhibit $PrP^{Sc}$ accumulation in murine neuroblastoma cells (ScN2a cells) (46). Treated B6 cells were then split 1/5, without antibody, every four days for four weeks and then collected for immunoblot analysis as described below. The recombinant anti-prion protein Fab R72 (InPro Biotechnology), which does not bind to cell surface $PrP^{C}$ and thus does not inhibit $PrP^{Sc}$ accumulation (46), was used as the negative control. All antibody inhibition experiments were performed in triplicate.

**Immunoblot detection of $PrP^{Sc}$ in cell cultures.** Since the ELISA does not utilize the standard method of protease digestion to discriminate between $PrP^{Sc}$ and $PrP^{C}$, cells were also collected for immunoblot confirmation of protease resistance. Primary cells were immunoblotted similar to previously described (32). Approximately 2–3 million $microglia^{Sc}$ and

microglia[C] were trypsinized into solution and washed with 1x D-PBS. The resulting pellet was lysed for 1–2 hours in 1 ml of lysis buffer containing 0.5% (v/v) Nonidet P-40 (Roche), 0.5% (w/v) sodium deoxycholate (Sigma) in 10 mM Tris buffer pH 7.4. Aliquots (100–250 µl) of lysate were brought up to a total volume of 500 µl by using additional lysis buffer. An equal volume of 4% (w/v) N-lauroylsarcosine sodium salt (Sigma) solution in PBS was added to the lysate and incubated for 15 minutes at 37°C, followed by DNase I (100 µg/ml) (Roche) treatment for 30 minutes at 37°C. Samples were centrifuged 1100 × *g* for 5 minutes at room temperature, and 1 ml of the supernatant was treated with Proteinase K (50 µg/ml) (Roche) for 1 hour at 37°C. Replicate samples were incubated for 1 hour at 37°C without Proteinase K. Eighty microliters of 4% (w/v) phosphotungstic acid in 170 mM $MgCl_2$ was added, the samples were incubated for one hour at 37°C, and following centrifugation at 16,500 × *g* at room temperature, the pellet was resuspended in 16 µl of water. Each sample (16 µl) was mixed with 7 µl of 4× NuPAGE sample buffer and 2.5 µl of 10× reducing agent (Invitrogen) and then boiled for 10 minutes. The samples were electrophoresed for 50 minutes at 200 V using the NuPAGE precast 12% Bis-Tris buffered sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel system (Invitrogen) with MOPS running buffer. NuPAGE antioxidant (Invitrogen) was added to the inner chamber running buffer. Gels were electroblotted for 50 minutes at 300 mA onto a 0.45 µm polyvinylidene fluoride (PVDF) (Millipore) membrane, using the semi-dry Biometra Fastblot B33 apparatus and transfer buffer containing 25 mM Tris pH 8.3, 150 mM glycine, and 10% (v/v) methanol. Following the transfer, the PVDF membrane was fixed for 20 seconds in 100% methanol, air dried, and then immunoblotted or stored dry overnight. PVDF membranes were blocked in casein blocker (Pierce) with 0.05% (v/v) Tween-20 for one hour at room temperature. The prion protein was detected by a one hour, room temperature incubation with the monoclonal

antibody F99/97.6.1 (produced in our laboratory, also available from VMRD, Inc.) at a concentration of 3.5 μg/ml in blocking buffer. The membrane was then washed three times in TBST (150 mM NaCl, 0.05% Tween-20 in 10 mM Tris pH 8.0) and incubated for thirty minutes at room temperature with a goat anti-mouse IgG1 antibody, conjugated with horseradish peroxidase (Southern Biotechnology Associates, 1070-05) diluted 1:5000 in blocking buffer. Following five additional washes in TBST, the membrane was incubated with chemiluminescent substrate (Western Lightning, Perkin Elmer) for 3 minutes. Signals were visualized by exposing the membrane to radiographic film (ISC BioExpress) and evaluated for the proper banding pattern associated with PK-resistant $PrP^{Sc}$.

For detection of $PrP^{Sc}$ within the SV40 large T transformed cell line (B6), the TeSeE™ western blot kit (Bio-Rad, France) was used as per manufacturer's instructions. Fifteen to twenty microliters of the final sample suspension was electrophoresed 60-90 minutes at 250 V using precast 12% Tris-HCl Ready gels (Bio-Rad) in 25 mM Tris pH 8.3, 192 mM glycine, and 0.1% SDS running buffer. Gels were electroblotted for 60 minutes at 115 V onto a 0.22 μm nitrocellulose membrane (Bio-Rad), using the a Mini-PROTEAN® 3 cell (Bio-Rad) and transfer buffer containing 1× Tris/Caps (Bio-Rad) and 15% ethanol. Following the transfer, the nitrocellulose membrane was fixed for 10 seconds in 100% ethanol and briefly transferred to distilled water. The immunoblotting procedure for the detection of the prion protein was similar to the method described above for the primary microglia, except for the use of reagents, including antibodies, provided by the TeSeE™ kit.

**Inoculation of $PrP^{Sc}$-accumulating primary microglia with CAEV and evaluation of effect of CAEV on extracellular $PrP^{Sc}$ release.** At passage ten post $PrP^{Sc}$ inoculation, primary $microglia^{Sc}$ were plated $1 \times 10^5$/well in 24-well plastic tissue culture plates. $Microglia^{C}$ were

treated similarly and served as negative controls for this experiment. The Cork strain of CAEV, in 0.5 ml of total OMEM, was added to the cells (microglia$^{Sc\text{-}CAEV}$ and microglia$^{C\text{-}CAEV}$) at a concentration sufficient to infect the majority of the cells within 7 days (based on a previous viral infection experiment, data not shown) as assayed by immunocytochemistry, which is described below. Aliquots of both cell types were also sham inoculated with medium only (microglia$^{Sc\text{-}sham}$ and microglia$^{C\text{-}sham}$) to serve as negative controls for the viral inoculation. All inoculations were performed with six replicates. At days 4, 8, 12, 16, and 19 post virus inoculation, culture supernatants were collected and saved at -80°C for analysis by HerdChek™ Scrapie Antigen Test Kit ELISA (see above) and for transmission studies (see below). A standard curve prepared from diluted Rov9$^{Sc}$ inoculum (1/50, 1/100, 1/400, 1/800, and 1/1600) was used to normalize corrected optical density results between ELISA plates. The PrP$^{Sc}$ signal of microglia$^{Sc\text{-}CAEV}$ supernatants was compared to the PrP$^{Sc}$ signal of microglia$^{Sc\text{-}sham}$ supernatants using an independent t-test with a cut-off *P*-value of 0.05 (SigmaPlot).

**Demonstration of PrP$^{Sc}$ infectivity from microglial$^{Sc\text{-}CAEV}$-derived supernatants and evaluation of effect of CAEV on intracellular PrP$^{Sc}$ accumulation.** Primary microglia$^{C}$, at passage twenty-one, were plated in 24-well plates at $2 \times 10^5$ cells per well. One day later, 400 µl of the 4 day post-viral inoculation cell culture supernatant (see previous section) was applied to the cells, in triplicate for each of the four treatment groups (microglia$^{Sc\text{-}CAEV}$, microglia$^{Sc\text{-}sham}$, microglia$^{C\text{-}CAEV}$, and microglia$^{C\text{-}sham}$). At confluency, cells were expanded sequentially into 12-well plates and into 25-cm$^2$ flasks. At day 26 post inoculation, an aliquot of microglial cells were plated into chambered slides for confirmation of CAEV infection (see below) and 4/5 of the total cells were collected and lysed (as described above) for analysis by HerdChek™ Scrapie Antigen Test Kit ELISA. A standard curve prepared from diluted Rov9$^{Sc}$ inoculum (1/100,

1/400, 1/800, and 1/1600) was used to normalize corrected optical density results. The $PrP^{Sc}$ signal of $microglia^{Sc\text{-}CAEV}$ lysates was compared to the $PrP^{Sc}$ signal of $microglia^{Sc\text{-}Sham}$ lysates using an independent t-test with a cut-off p-value of 0.05 (SigmaPlot).

**Immunocytochemical detection of CAEV in primary microglia.** Cells were fixed with 4% buffered zinc formaldehyde (Z-fix; Anatech Ltd) for 2 minutes and then rinsed in 70% ethanol. CAEV antigen was detected using the monoclonal antibody 10A1 (VMRD, Inc.) directed against 28 kDa capsid protein. Negative controls included the use of isotype matched antibodies raised against an irrelevant antigen, omission of the primary antibody, and omission of both the primary and secondary antibodies. CAEV infected goat synovial membrane cells, derived from neonatal goats (29), served as positive controls for the immunocytochemistry.

## RESULTS

**Establishment and characterization of primary and transformed fetal sheep brain cells.** To study the cellular and molecular changes associated with prion conversion in a natural host-TSE system, primary brain cell cultures were derived from a Suffolk-cross domestic sheep fetus that was homozygous for the PRNP VRQ allele (Val-136, Arg-154, and Gln-171). Primary cell cultures were adherent and viable for approximately 20 passages. Additionally, a cell line derived from the primary brain cells was created by transformation with a plasmid containing the SV40 virus large T antigen. Increased rate of growth, lack of contact inhibition, and immunoreactivity for the large T antigen confirmed transformation of the cell culture (data not shown). The transformed cell line has demonstrated viability up sixty passages, as of this writing, and have shown no evidence of cell pathology. Both the primary brain cultures and the transformed cell line demonstrated expression of several microglial markers by both

immunocytochemistry and flow cytometry (Table 1), although the primary microglia cultures lacked CD68 expression. By immunocytochemistry approximately 90% of cells demonstrated immunoreactivity for the given markers (data not shown). The approximately 10% of cells lacking immunoreactivity were morphologically indistinguishable from those cells that were demonstrating immunoreactivity. Based on the phenotypic features we classified both the primary cell cultures and the transformed cell line as sheep microglia.

**$PrP^{Sc}$ infection of primary microglia.** To determine the permissiveness of cultured sheep microglia to $PrP^{Sc}$ infectivity, primary microglia were exposed to the PRNP genotype-matched $Rov9^{Sc}$-derived $PrP^{Sc}$. Lysates of the inoculated microglia ($microglia^{Sc}$) were collected at several time points and commercially available ELISAs were used to screen the lysates for $PrP^{Sc}$. Primary $microglia^{Sc}$ demonstrated accumulation of $PrP^{Sc}$ over time, as illustrated by an increasing $PrP^{Sc}$ signal in cell lysates, even after dilution of the cells and residual inoculum due to several 1/5 splits (Fig. 1A). $Microglia^{Sc}$ and $microglia^{C}$ showed no differences between each other in cell growth rate or morphology, which is consistent with previously reported findings (11).

To determine the relative level of $PrP^{Sc}$ accumulation as compared to the original inoculum and to further verify the detection of newly converted $PrP^{Sc}$ versus detection of residual inoculum, the amount of $PrP^{Sc}$ in a lysate of microglial cells at passage 9 (representing two expansions and seven 1/5 splits) was compared to the amount of $PrP^{Sc}$ in the inoculum. The inoculum was tested at its original concentration and at a $5^7$ (1/78,125) dilution, representing the seven 1/5 splits (and two expansions) of the $microglia^{Sc}$ cells at passage 9. The level of $PrP^{Sc}$ in the $1/5^7$ diluted inoculum was below the detection limit of the ELISA, as evidenced by results similar to the uninoculated microglia cells. The $microglial^{Sc}$ lysates from passage 9

demonstrated a significantly higher $PrP^{Sc}$ signal as compared to the $1/5^7$ diluted inoculum (representing the expected $PrP^{Sc}$ signal after seven splits if no accumulation was occurring in the $microglia^{Sc}$) (Fig. 1B). If the cells lacked the ability to form nascent $PrP^{Sc}$, then the expected result would be that the passage 9 sample would be equal to the $1/5^7$ diluted inoculum. As this was not seen, it was concluded that de novo conversion and accumulation of $PrP^{Sc}$ was occurring within the primary sheep microglia in order to compensate for the dilution of $PrP^{Sc}$ that occurs during splitting of cells during routine cell culture.

The ELISA that was utilized in this study uses a proprietary chemical, which preferentially binds beta sheets, to distinguish $PrP^{Sc}$ from $PrP^{C}$. Therefore, immunoblotting of microglial lysates was used to confirm the proteinase K resistance, which is the gold-standard for $PrP^{Sc}$ detection, of the microglial-derived $PrP^{Sc}$ and to compare the glycoform profile as compared to the $Rov9^{Sc}$-based inoculum. $Microglia^{Sc}$ accumulated proteinase K resistant $PrP^{Sc}$ that migrated with a profile different from scrapie positive brain material (Fig. 2A), but with a profile similar to the $Rov9^{Sc}$-based inoculum (Fig. 2B).

The infectivity of the intracellular $PrP^{Sc}$ produced by the microglia was analyzed by exposing $Rov9^{C}$ cells to three dilutions (1/20, 1/200, and 1/2000) of microglial cell lysates. The $Rov9^{C}$ cells inoculated with $microglia^{Sc}$ lysates demonstrated accumulation of $PrP^{Sc}$ over time as evidenced by an increasing ELISA optical density at each subsequent time point (Fig. 3). Additionally, $Rov9^{C}$ cells exposed to higher concentrations of $microglia^{Sc}$ inoculum demonstrated higher levels of $PrP^{Sc}$ accumulation (Fig. 3). The dose-responsiveness of the microglia-derived $PrP^{Sc}$ is consistent with infectivity.

**$PrP^{Sc}$ infection of transformed microglia and inhibition of $PrP^{Sc}$ accumulation.** Transformed microglial cells were cloned and inoculated to test whether sheep microglial cell

cultures with a longer culture lifespan, as compared to the primary sheep microglial cell culture, support $PrP^{Sc}$ accumulation. Clones were inoculated with Rov9-derived $PrP^{Sc}$ and then screened by ELISA for evidence of $PrP^{Sc}$ accumulation. Twenty clones were inoculated, and fifteen of those clones were stable and were able to be expanded and tested for $PrP^{Sc}$ accumulation. One of the fifteen clones tested positive for $PrP^{Sc}$ by ELISA at passage five and later (data not shown). The positive clone, B6, was the clone that was the slowest to grow to confluency during the initial inoculation period, 16 days as compared to an average of 6 days for the fourteen negative clones. The $PrP^{Sc}$ signal in the transformed cell line remained consistently low, as compared to the primary sheep microglia, and spontaneous loss of $PrP^{Sc}$ was seen after approximately 15 passages.

To further characterize the $PrP^{Sc}$ being accumulated within the transformed microglia, the positive clone was treated with the recombinant anti-prion Fab D18. Following a thirteen day exposure to this Fab, and four weeks of culture without the Fab, the transformed microglial cell line demonstrated loss of ELISA detectable (data not shown) and immunoblot detectable $PrP^{Sc}$ as compared to the cultures treated with the negative control recombinant anti-prion Fab R72, which maintained detectable levels of $PrP^{Sc}$ (Fig. 4). This specific antibody-based inhibition of $PrP^{Sc}$ accumulation and the multiple assays used to detect $PrP^{Sc}$ increase over time indicate that the $PrP^{Sc}$ detected in the microglial cells represents $PrP^{Sc}$ molecules that are newly formed in microglia.

**Enhancement of $PrP^{Sc}$ levels by CAEV coinfection.** Previous studies with ScN2a cells and MLV, a gammaretrovirus, have demonstrated enhancement of prion infectivity release into the culture supernatant (39). While no gammaretroviruses of small ruminants have been identified (17), sheep and goats are susceptible to the small ruminant lentiviruses (SRLV)

visna/maedi virus and caprine arthritis encephalitis virus, which are closely related to one another and are considered members of the same lentivirus group (45). While gammaretroviruses and lentiviruses have unique properties, much of the basic biology between these two groups of retroviruses is shared. To determine if SRLV coinfection enhances the release of PrPSc into the culture supernatant, similar to the results of MLV in ScN2a cells, late passage primary microglia$^{Sc}$ and microglia$^{C}$ were inoculated with the Cork strain of CAEV. CAEV-Cork induced less cytotoxicity as compared to the other tested SRLV strains (VISNA 84-28, VISNA LMH11, and VISNA WLC-1) that each resulted in nearly 100% cell lysis within one week (data not shown). The culture supernatants were collected regularly and assayed for relative levels of PrP$^{Sc}$ concentration by normalized ELISA optical densities. There was no detectable difference between cultures infected and uninfected with SRLV in cell growth, as determined by rate to confluence, or phenotype. The results demonstrate that following subsequent infection by CAEV, microglia cells that were already PrP$^{Sc}$ infected have significantly enhanced, up to 1.5-fold, PrP$^{Sc}$ release into the culture supernatant as compared to microglia$^{Sc\text{-}Sham}$ (Fig. 5A). The supernatants from cultures of microglia$^{C\text{-}CAEV}$ and microglia$^{C\text{-}Sham}$ remained free from detectable levels of PrP$^{Sc}$ indicating that CAEV does not result in detectable levels of spontaneous conversion of PrP$^{C}$ into PrP$^{Sc}$.

It is known that the mechanism of PrP$^{Sc}$ transmission within one cell culture system may vary as compared to another cell culture system. For instance, in ScN2a cells, the spread of PrP$^{Sc}$ is mainly vertical (i.e., from mother to daughter cells) (22), whereas spread within Rov9 cells is horizontal to nearby cells, with rare spreading to spatially distant cells (44). To determine if supernatant-derived PrP$^{Sc}$, which was enhanced by SRLV coinfection in primary sheep microglia, is associated with infectivity towards primary microglia, microglia$^{C}$ were

inoculated with supernatants from the previous primary microglia experiment that contained both $PrP^{Sc}$ and CAEV. Additionally, this experiment also served three additional purposes: to repeat the observed enhancement of $PrP^{Sc}$ accumulation following CAEV coinfection, to determine if the increase in $PrP^{Sc}$ also applied to intracellular $PrP^{Sc}$, and to determine if simultaneous $PrP^{Sc}$/CAEV coinfection (versus CAEV infection of established $PrP^{Sc}$ infected microglia as shown above) yielded a similar enhancement of relative $PrP^{Sc}$ levels. Lysates were analyzed by ELISA to determine relative amount of $PrP^{Sc}$. Both $microglia^{Sc\text{-}CAEV}$ and $microglia^{Sc\text{-}Sham}$ contain detectable levels of $PrP^{Sc}$, thus confirming that primary $microglia^{Sc}$-derived $PrP^{Sc}$ is infectious to microglia and suggesting that horizontal spread within microglial cultures is likely (Fig. 5b). Additionally, $microglia^{Sc\text{-}CAEV}$ lysates demonstrated an approximate twofold higher $PrP^{Sc}$ signal as compared to $microglia^{Sc\text{-}Sham}$ (Fig. 5b). Thus, these results confirm the repeatability of CAEV-induced enhancement of $PrP^{Sc}$ accumulation in microglia, show that the intracellular $PrP^{Sc}$ levels are also elevated, and demonstrate that the $PrP^{Sc}$ enhancement is evident with simultaneous $PrP^{Sc}$ and CAEV coinfections. These two coinfection experiments (Fig 5) indicate an overall increase, up to twofold, in $PrP^{Sc}$ within primary sheep microglia that are coinfected with CAEV. As expected, primary microglia not infected with $PrP^{Sc}$ ($microglia^{C\text{-}CAEV}$ and $microglia^{C\text{-}Sham}$ treatment groups) did not demonstrate detectable levels of $PrP^{Sc}$. CAEV infection was verified by immunocytochemistry for the CAEV 28 kDa capsid protein (data not shown).

## DISCUSSION

Scrapie is an important disease in the United States and has been targeted for eradication (47). While scrapie has been recognized since the early $18^{th}$ century (48), many questions still remain including the details of transmission between animals and the identification of factors

that promote transmission and infection. Recent studies in mice and mouse-derived cell cultures have shown that gammaretroviral infections enhance the amount of prion infectivity produced by prion infected cells (39). Our results have extended this connection into sheep-derived microglia cultures coinfected with a small ruminant lentivirus. While the VISNA strains that were most readily available were too cytopathic for these studies, we have shown that coinfection with the very closely related small ruminant lentivirus CAEV-Cork strain results in increased $PrP^{Sc}$ accumulation in sheep brain macrophages (microglia), as evidenced by the increase in $PrP^{Sc}$ signal both within the supernatant and within the microglial cells. Additionally, we have demonstrated that the enhancement of $PrP^{Sc}$ accumulation occurs following either sequential $PrP^{Sc}$/CAEV infection or simultaneous coinfection and that both the intracellular and extracellular microglia-derived $PrP^{Sc}$ is infectious.

Studies analyzing prion and retroviral coinfections in natural hosts are limited. A previous report has demonstrated $PrP^{Sc}$ in macrophages within lesions of SRLV-induced lymphofollicular mastitis in sheep (41). However, the mechanism of colocalization was not determined. One possible mechanism is recruitment of macrophages and follicular dendritic cells, unrelated to prion pathogenesis, to tissues with SRLV-induced inflammation, which then increased the number of $PrP^{Sc}$-permissive cells in the mammary gland. A second possible mechanism is that the SRLV infection specifically enhances $PrP^{Sc}$ accumulation in the macrophages and follicular dendritic cells. While these two mechanisms are not mutually exclusive, our results demonstrating SRLV-induced enhancement of $PrP^{Sc}$ in microglia (brain-derived macrophages) suggest a specific interaction may be at least partially responsible for the presence of $PrP^{Sc}$ in SLRV-induced mastitis.

The SLRV-induced enhancement of $PrP^{Sc}$ in these microglia cultures is modest; thus, the in vivo relevance of any direct interaction between SRLV and $PrP^{Sc}$ is not known. However, prion diseases are extremely chronic diseases that take months to years to fully develop (61); thus, small changes in the rate of $PrP^{Sc}$ accumulation can potentially be significant following amplification over that extended time period. Furthermore both $PrP^{Sc}$ and SRLV are independently present in the same cell types, e.g., macrophages (20, 23, 24, 43, 49), dendritic cells (24, 31, 53), and microglia (5, 7, 16, 33). Additionally, nearly one in four domestic sheep within the United States are infected with VISNA (56). Thus, given the increased $PrP^{Sc}$ accumulation associated with SRLV coinfection that is reported herein, the overlapping cellular tropism of $PrP^{Sc}$ and SRLVs, and the prevalence of SRLV, it is important to determine the extent of interaction between these two agents in vivo and the effects, if any, on any scrapie eradication measures.

This study does not investigate the mechanism of interaction between SRLVs and $PrP^{Sc}$. Possible mechanisms for the enhanced $PrP^{Sc}$ accumulation in the sheep microglia include, but are not limited to, the coinfecting SRLV increasing $PrP^{C}$ protein expression, enhancing access to conversion accessory molecules, increasing colocalization of $PrP^{C}$ and $PrP^{Sc}$ during viral assembly, and increasing transmission of $PrP^{Sc}$ by co-transmission with the virus. The study using MLV and ScN2a cells, demonstrated that $PrP^{C}$ and $PrP^{Sc}$ are recruited to virions (39), suggesting that increased $PrP^{Sc}$ transmission is likely to play at least some role in the enhancement of $PrP^{Sc}$ accumulation. Another recent study investigated the interaction of $PrP^{Sc}$ and minute virus of mice, a parvovirus, and demonstrated that the parvoviral infected cells internalize exogenous $PrP^{Sc}$ more efficiently than the virus-free cells, most likely due to virus-induced changes in the cellular membranes (4). However, it remains to be determined if similar

mechanisms are induced by infections with retroviruses and other viruses. Another possible mechanism for the enhanced prion accumulation is that there are virus-induced changes in the abundance and/or character of RNA, which has been suggested as a possible cofactor for the conversion of $PrP^{C}$ to $PrP^{Sc}$ (2, 15, 19, 21). Determining the mechanism(s) of the virus-induced enhancement of $PrP^{Sc}$ accumulation warrants further study, which may also help to elucidate some of the basic cellular mechanisms of $PrP^{Sc}$ conversion.

During the course of scrapie infection numerous cell types, including glia, macrophages, and follicular dendritic cells, demonstrate accumulation of $PrP^{Sc}$ (58), but the cell specific mechanisms that allow for prion conversion are not known. The ability to investigate these mechanisms requires the tools to study $PrP^{Sc}$ infection in these various cell types, mainly having permissive cell culture systems from a variety of cell types. Herein we describe the second cell line derived from a natural TSE host, and the first microglial cell culture derived from a natural TSE host (59).

These microglial cells are of the highly susceptible genotype, (Val-136, Arg-154, and Gln-171). Acquiring $PrP^{Sc}$-containing VRQ/VRQ sheep brain for research material can be difficult in the United States due to the rarity (approximately 0.5%) of this genotype in the United States (57). While, it is recognized that this study used an inoculum derived from lagomorph cells, it should be noted that the inoculum's $PrP^{Sc}$ has an ovine, and not lagomorph, amino acid sequence (PRNP in Rov cells is the ovine insert). Thus, the disadvantage of utilizing material derived from a leporine source is outweighed in this particular case by the availability of Rov9-derived $PrP^{Sc}$ and its matched ovine amino acid sequence. Furthermore, now that the concept of $PrP^{Sc}$ susceptible sheep microglia cultures has been demonstrated, these cells can be

utilized in further studies to determine the relevance of prion allelic usage, prion allelic susceptibility, and other sheep-specific prion questions.

Cell culture models of $PrP^{Sc}$ accumulation have been used for numerous studies to screen for inhibitors of $PrP^{Sc}$ accumulation (59). However, recent work has indicated one of the pitfalls of using cell culture models, that the amount of $PrP^{Sc}$ accumulating in cells is inversely related to the mitotic rate of the cell cultures (22). Thus, the ability to cure cell culture systems of $PrP^{Sc}$ differs significantly as compared to mitotically inactive neurons, which are infected with $PrP^{Sc}$ in clinical patients. Our results support this conclusion, as the only positive cell line derived from the transformed microglia was the clone that was slow growing during the initial inoculation phase and even this clone lost the ability to maintain detectable levels of $PrP^{Sc}$ after the cultures began achieving confluency at a faster rate. While the mitotic activity of cell cultures systems may alter the biological relevance of using these systems to screen for antiprion compounds, cell culture systems do have the ability to screen for factors that promote $PrP^{Sc}$ accumulation. Multiple lines of evidence suggest a role for an accessory molecule(s) to enable $PrP^{Sc}$ accumulation (35, 54, 55). The coinfection model of $PrP^{Sc}$ and SRLV in primary sheep microglia is one example of using cell cultures to identify factors that enhance $PrP^{Sc}$ accumulation. Further studies, including screening for additional factors that promote $PrP^{Sc}$ accumulation in microglia and studying those cofactors in a variety of $PrP^{Sc}$ permissive cell lines, are warranted.

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## TABLES

TABLE 1. Phenotype of primary sheep brain cultures and transformed glial cell line

| Cells | Method | Markers | | | |
|---|---|---|---|---|---|
| | | RCA-I[a] | CD14[b] | CD68[c] | Nonspecific esterase |
| Primary | Immunocytochemistry | + | + | - | + |
| | Flow cytometry | + | + | - | NA[d] |
| Transformed | Immunocytochemistry | + | + | + | + |
| | Flow cytometry | + | + | + | NA |

[a] *Ricinus communis* agglutinin-1

[b] CD14 (MM61A, VMRD, Inc.),

[c] CD68 (EBM11, Dako),

[d] Not applicable

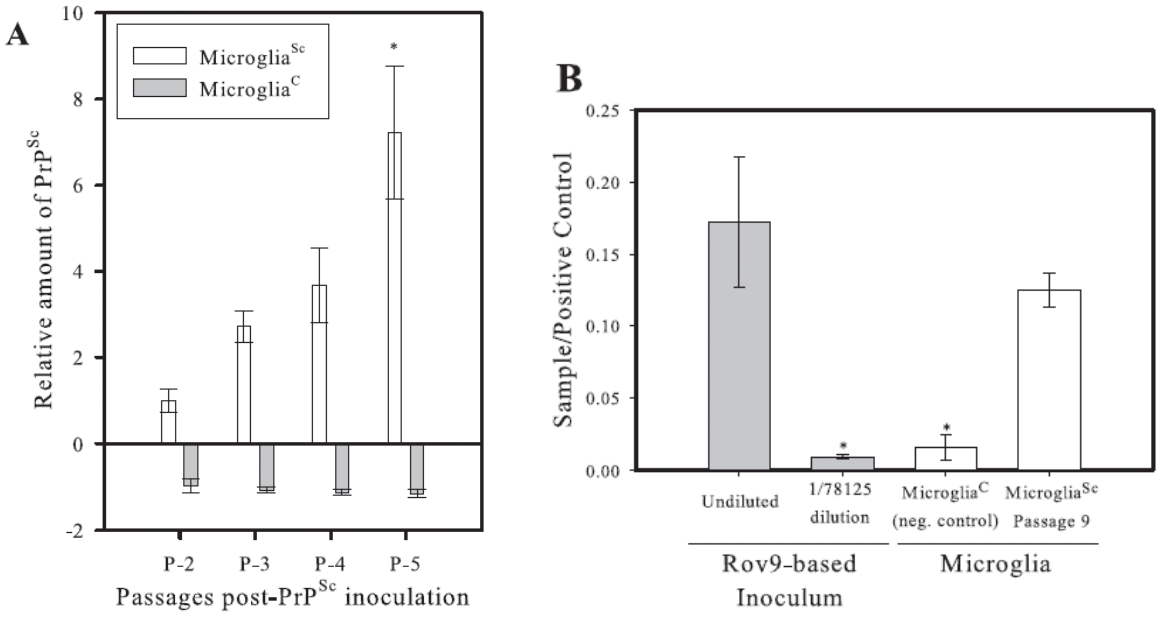


**Figure 1.** $PrP^{Sc}$ accumulation in primary microglia. Primary sheep microglia were inoculated in triplicate with either $PrP^{Sc}$-containing ($microglia^{Sc}$) or $PrP^{Sc}$-lacking ($microglia^{C}$) cell lysates derived from the $PrP^{Sc}$ positive or negative, respectively, Rov9 cell line (60) and then serially passaged. Lysates of $microglia^{Sc}$ were assayed for $PrP^{Sc}$ by commercial ELISA and the corrected optical density was determined. The equation for the corrected optical density is $OD_{450}$-$OD_{620}$, as per manufacturer's instructions. (A) ELISA results comparing the level of $PrP^{Sc}$ in late passage (P-5) $microglia^{Sc}$ versus early passage (P-2) $microglia^{Sc}$. Data were normalized using a standard curve. $Microglia^{Sc}$ results were set at one and all other results were normalized to this value (y-axis). Bars represent the means ± one standard deviation. Results of $microglia^{Sc}$ were statistically compared between P-5 and P-2; * = $p < 0.005$. (B) ELISA results comparing the relative level of $PrP^{Sc}$ in late passage (P-9) primary $microglia^{Sc}$ to the original inoculum. Data were normalized to the ELISA plate positive control (y-axis) to account for plate to plate variation between testing repeats performed at different times. Results for $microglia^{Sc}$ P-9 were statistically compared to $microglia^{C}$, and both $Rov9^{Sc}$-based inoculum data points; * = $p < 0.05$ as compared to P-9 $microglia^{Sc}$.

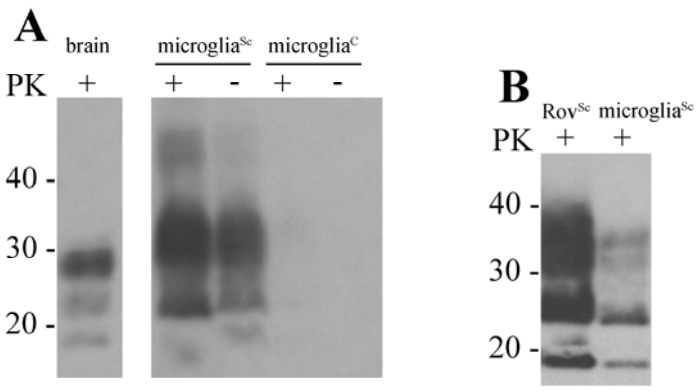


**Figure 2.** Immunoblot analysis of primary sheep microglia following exposure to $PrP^{Sc}$ ($microglia^{Sc}$) and unexposed microglia ($microglia^{C}$). Microglia were lysed, treated with proteinase K (+) or without proteinase K (-), precipitated with phosphotungstic acid, and immunoblotted using the monoclonal anti-PrP antibody F99.97.6.1. (A) Comparison of prion immunoreactivity from sheep brain (brain), $microglia^{Sc}$, and $microglia^{C}$. (B) Comparison of proteinase K resistant glycoform patterns between $Rov9^{Sc}$ and $microglia^{Sc}$. The position of molecular mass standards, in kilodaltons, is indicated on the left.

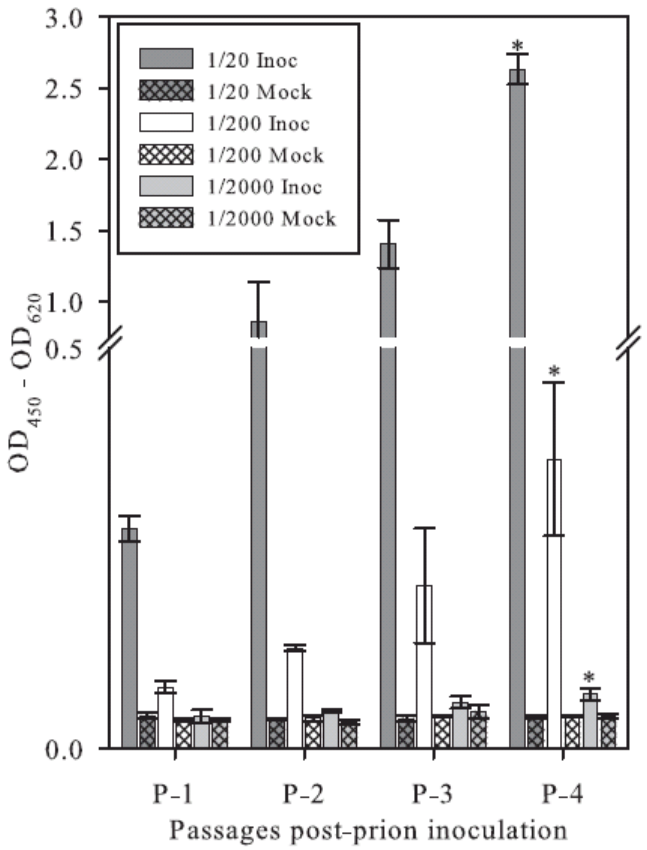


**Figure 3.** Transmission of microglial-derived $PrP^{Sc}$ to $Rov9^{C}$ cells. $Rov9^{C}$ cells were inoculated, in triplicate, with three dilutions of $microglia^{Sc}$ lysates. The inoculated Rov9 cells were serially passaged, lysed, and analyzed for $PrP^{Sc}$ levels by ELISA. Results at passage 4 for each group of inoculated cells were statistically compared to the corresponding mock inoculated cells; * = $p < 0.05$. All samples were run on the same ELISA plate; thus, results are reported as the corrected optical density (y-axis). Data represent the means ± one standard deviation of the sample corrected optical density. The equation for the corrected optical density is $OD_{450}$-$OD_{620}$, as per manufacturer's instructions.

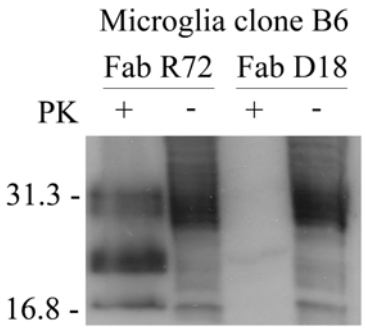


**Figure 4.** Immunoblot analysis of $PrP^{Sc}$-infected sheep microglial cell line after incubation with the recombinant anti-prion Fab D18. Primary sheep cultures were transformed with the SV40 virus large T antigen, limiting dilution cloned, and then inoculated with $PrP^{Sc}$. The clone that was positive for $PrP^{Sc}$ was incubated with Fab D18 for thirteen days and then cultured in the absence of the Fab for four weeks. Cells were lysed, treated with proteinase K (+) or without proteinase K (-) and immunoblotted using the TeSeE™ western blot kit (Bio-Rad, France). Lysates of replicate $PrP^{Sc}$-infected B6 cells that were treated with the control anti-prion Fab R72, which does not inhibit $PrP^{Sc}$ accumulation, are included as a control for spontaneous loss of detectable $PrP^{Sc}$. The position of molecular mass standards, in kilodaltons, is indicated on the left.

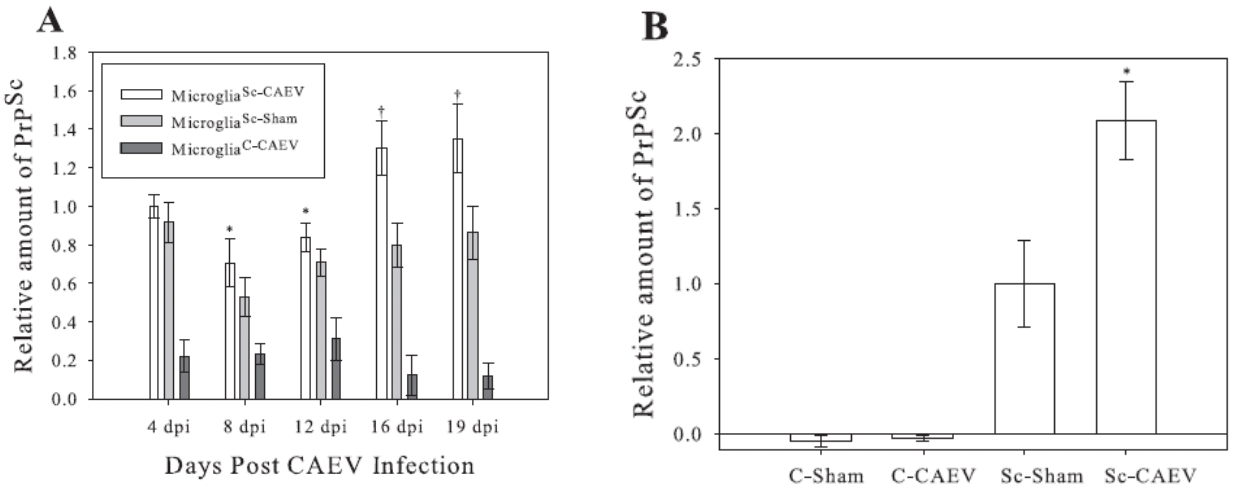


**Figure 5.** Effect of CAEV coinfection on $PrP^{Sc}$ accumulation in primary sheep microglia and transmission of microglial-derived $PrP^{Sc}$ to primary microglial cells. (A) $PrP^{Sc}$ accumulation in the culture supernatant of primary microglia infected with CAEV after establishment of $PrP^{Sc}$ accumulation. $Microglia^{Sc}$ and $microglia^{C}$ were inoculated with CAEV, and cell culture supernatants were collected at 4, 8, 12, 16, and 19 days post CAEV inoculation (dpi). Aliquots of supernatant were analyzed by ELISA for $PrP^{Sc}$ levels, which were normalized via a standard curve. Normalized 4 dpi $microglia^{Sc\text{-}CAEV}$ levels were set at one, and all other results were normalized based on this value (y-axis). Six replicates for each treatment group were used. At each time point, results for $microglia^{Sc\text{-}CAEV}$ were statistically compared to $microglia^{Sc\text{-}Sham}$; $* = p < 0.05$ and $† = p < 0.0005$. (B) $PrP^{Sc}$ levels in the lysates of primary microglia simultaneously coinfected with $PrP^{Sc}$ and CAEV. $Microglia^{C}$ were inoculated with the 4 dpi culture supernatants from the previous experiment: Sc-CAEV, Sc-Sham, C-CAEV, and C-Sham. At 26 dpi, cell lysates were analyzed by ELISA for $PrP^{Sc}$ levels, which were normalized via a standard curve. Normalized $microglia^{Sc\text{-}Sham}$ levels were set at one and all other results were normalized to this value (y-axis). Three replicates for each treatment group were used. Results for $microglia^{Sc\text{-}CAEV}$ were statistically compared to $microglia^{Sc\text{-}Sham}$; $* = p<0.01$. Error bars represent one standard deviation.

# CHAPTER 2

# TRANSCRIPTOMIC PROFILING OF PRION ACCUMULATING OVINE MICROGLIA

## ABSTRACT

Sheep scrapie (Sc) is the classical transmissible spongiform encephalopathy (prion disease). The conversion of normal cellular prion protein ($PrP^{C}$) to disease-associated prion protein ($PrP^{Sc}$) is a fundamental component prion disease pathogenesis. The molecular mechanisms contributing to prion diseases and the impact of $PrP^{Sc}$ accumulation on cellular biology are not fully understood. To define the molecular changes associated with $PrP^{Sc}$ accumulation, primary sheep microglia were infected with $PrP^{Sc}$ and then the transcriptional profile of these $PrP^{Sc}$-accumulating microglial cells was compared to the profile of $PrP^{Sc}$-lacking microglial cells using the Affymetrix bovine genome array. The experimental design included three biological replicates, each with three technical replicates, and samples that were collected at the measured maximal level of $PrP^{Sc}$ accumulation. The array analysis revealed 19 upregulated genes and 30 downregulated genes in $PrP^{Sc}$-accumulating microglia. Three transcripts (CCL2, SGK1, and AASDHPPT) were differentially regulated in a direction similar to previous reports from mouse or human models, whereas three other transcripts (MT1E, NR4A1, PKP2) responded oppositely from previous reports. Overall, the results demonstrated a limited transcriptional response to $PrP^{Sc}$ accumulation, in contrast to when microglia and macrophages are infected with other infectious agents such as viruses and bacteria. This is the first microarray-based analysis of prion accumulation in primary cells derived from a natural TSE-host.

## INTRODUCTION

Transmissible spongiform encephalopathies (TSE, prion diseases) are fatal, transmissible, neurodegenerative diseases, including scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and variant Creutzfeldt-Jakob disease (vCJD) in humans (50). A component of prion diseases is the post-translational conversion of the normal cellular form of the host encoded prion protein ($PrP^{C}$) to an abnormal isoform, designated $PrP^{Sc}$ (7, 15, 20). This conformational change generates a detergent insoluble, partially protease resistant molecule that aggregates in affected tissues and serves as the marker for prion diseases. $PrP^{Sc}$ is considered by many to be the principal component of the transmissible agent and provides the basis for the protein-only hypothesis of prion diseases (63).

Many of the underlying molecular mechanisms associated with the post-translational conformational change and the resulting cellular response are either unknown or poorly understood (87). Previous studies into the differential transcriptional regulation associated with prion diseases have employed a variety of methods, including suppression subtractive hybridization (24, 27, 28, 65), differential display RT-PCR (25, 58), RNA arbitrarily primed PCR (4), and most commonly cDNA microarray (5, 13, 14, 16, 17, 32, 39, 47, 56, 64, 69, 75, 78, 91-93). These transcriptomic studies permit investigation of the cellular response to prion accumulation, and may lead to identification of co-factors involved in prion diseases, identification of biomarkers of prion infection, and determination of cell specific and tissue specific responses to accumulation of $PrP^{Sc}$.

Numerous rodent-adapted prion strains are available (22, 23, 57, 81) and most of the transcriptomic studies listed above have used mouse models of scrapie (5, 13, 14, 16, 17, 25-27,

32, 39, 47, 48, 64, 69, 75, 78, 91, 93). The genomic annotation and wide variety of molecular tools available for the mouse model make it useful for such large-scale transcriptomic studies. Despite these advantages, no natural prion diseases are known to occur in rodents. In contrast, the similarities between sheep scrapie, a natural prion disease, and CJD are well recognized (49); therefore, sheep scrapie is a logical experimental model for the investigation of a natural prion disease in a natural host.

Many prion transcriptomic studies used extracted whole tissue material for analysis (13, 14, 16, 17, 24-26, 28, 48, 58, 64, 65, 69, 75, 78, 91-93). This provides the benefit of analyzing the response on a whole tissue level, which is applicable to the pathogenesis of prion diseases at the organismal level. Given the multitude of cell types present in tissue, however, it becomes difficult to determine which cell types are responsible for differential regulation of any group of genes. Furthermore, many of the responses may represent an indirect response to prion accumulation within other cell types (e.g. microglia responding to $PrP^{Sc}$-accumulating neurons). While animal based, large-scale transcriptomics studies are feasible in a highly inbred species such as mice, these studies are less feasible when using the natural TSE hosts (e.g. sheep, cattle, humans, etc.) due to the inherent biological variation in these outbred species.

Alternatively, the sample to sample variation can be reduced by removing many variables associated with whole animal studies and narrowing the transcriptional analysis to a specific subset of prion-accumulating cells by using cell cultures. While this strategy has been used before, many of the prion cell culture transcriptomics studies have again focused on the mouse models of scrapie including the readily available mouse neuroblastoma cells (ScN2a) and mouse hypothalamic neuronal cell line (GT1) (27, 32, 39, 47). To the authors' knowledge, there is only one report of a large-scale transcriptomic study based on a cell line from a natural TSE host

(human SH-SY5Y neuroblastoma cell line) (56). Herein, we describe the results of the first microarray-based large-scale transcriptomic analysis of cultured cells from sheep, the host of the prototypical prion disease, in response to $PrP^{Sc}$ accumulation.

Specifically, sheep primary microglia cultures were used in the current study. Microglia (resident brain macrophages) are cells that accumulate infectivity in vivo (6), and likely contribute to prion-induced neuropathology by their activation and release of immune mediators (9, 67, 89). While the transcriptional response of murine microglia to $PrP^{Sc}$ has been evaluated previously (5, 6, 53), these studies isolated the microglia after $PrP^{Sc}$ inoculation of the whole animal. Thus, these studies include variation due to sampling of cells between two or more different animals. For the present study sheep microglia were isolated from a single homozygous Val-136, Arg-154, and Gln-171 animal and then inoculated with $PrP^{Sc}$ material. This study extends transcriptomic results of prion accumulation into the sheep model, but it also investigates the specific response by resident brain macrophages to $PrP^{Sc}$ accumulation.

## MATERIALS AND METHODS

**Primary ovine brain cell cultures.** Primary mixed glial cell cultures were obtained from an ovine fetal brain using a mechanical dissociation technique for small ruminants previously described (8, 79). The ovine fetus was collected from a near term pregnant Suffolk-cross ewe that was housed and cared for in accordance with Institutional Animal Care and Use Committee at Washington State University, Pullman, WA. The genotype of the fetus was previously determined to be VRQ (Val-136, Arg-154, and Gln-171) homozygous (2, 79). Aliquots of cells were thawed as needed and serially passaged, using standard techniques, in

OPTI-MEM I Reduced Serum Medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 IU/ml of penicillin, and 10 mg/ml of streptomycin (OMEM).

**Inoculation of primary microglia with $PrP^{Sc}$.** As previously discussed (79), the rarity (approximately 0.5%) of the VRQ (Val-136, Arg-154, and Gln-171) genotype in the United States makes acquisition of $PrP^{Sc}$-positive, VRQ brain material difficult. A readily available source of VRQ derived $PrP^{Sc}$ are Rov9 cells, which were used as the inoculum (obtained from B. Caughey with permission from D. Vilette), as previously described (79). Rov9 cells are rabbit renal epithelial cells that are stably transfected with the VRQ allele of the sheep prion gene under the control of a tetracycline inducible promoter, and they are susceptible to sheep $PrP^{Sc}$ (86). The presence and absence of $PrP^{Sc}$ within inoculated ($Rov9^{Sc}$) and uninoculated ($Rov9^{C}$) Rov9 cells, respectively, was previously verified (79). For use as an inoculum, mechanical lysates of the $Rov9^{Sc}$ and $Rov9^{C}$ cells were prepared as previously described (79, 86). Briefly, the Rov9 cells were grown to confluency in two 75-$cm^2$ plastic tissue culture flasks. $Rov9^{Sc}$ and $Rov9^{C}$ cells were rinsed three times with sterile 1× Dulbecco's phosphate buffered saline (D-PBS) and scraped into 10 ml of PBS. The cell pellets were collected by centrifugation at 170 × *g* at room temperature for 10 min and resuspended in 0.5 ml of filter-sterilized 5% glucose. The cell suspensions were frozen and thawed four times and then subjected to 1–2 min of sonication in a cup horn sonicator. The inoculum was stored at -20°C.

For inoculation, primary microglia were passed into 6-well plates and grown to approximately 60% confluency. Three wells were arbitrarily chosen as the three biological inoculation replicates (InocA, InocB, and InocC) and the other three wells served as three mock inoculations (MockA, MockB, and MockC). Microglia were rinsed once with PBS and then overlaid with 200 µl of a 1/20 dilution of either the $Rov9^{Sc}$ lysate (Inoc A, B, and C) or the

Rov9$^{C}$ lysate (Mock A, B, and C) in OPTI-MEM. All replicates (A, B, and C) of both treatment groups (Inoc and Mock) were incubated for six hours and then 200 µl of OMEM were added to each well. Following an additional two days of incubation, 0.5 ml of OMEM was added to each well, and microglia were incubated for four days at which time they were expanded into 25-cm$^2$ tissue culture flasks. Microglia were fed every three to four days with OMEM as necessary and serially passed 1/5 after reaching confluence.

**Detection of PrP$^{Sc}$.** As previously described (79), at selected passages following trypsinization, 4/5 of the microglial cell suspension from a 25-cm$^2$ tissue culture flask was rinsed in D-PBS and then lysed in 120 µl of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0, 5mM EDTA, and 150 mM NaCl) for 3 min at room temperature, followed by centrifugation at *ca*. 2300 × *g* at room temperature for 5 min. Supernatant (100 µl) was then used for PrP$^{Sc}$ detection by the HerdChek™ Scrapie Antigen Test Kit ELISA (IDEXX) following the manufacturer's instructions. The proprietary ELISA positive and negative controls were used as per manufacturer's instructions. To compare between ELISA plates, the corrected optical densities for the test samples were normalized to the corrected optical density of the manufacturer supplied positive control.

**Collection, preparation, and microarray hybridization of sample RNA.** To determine the transcriptional differences following PrP$^{Sc}$ accumulation, experiments were performed in biological triplicates, each with its own set of technical triplicates. To reduce the effect of the inoculum-based PrP$^{Sc}$ and to maximize the effect of the nascent PrP$^{Sc}$ on the transcriptional response of the microglial cells, RNA was collected at time points estimated to be near the point of maximal PrP$^{Sc}$ accumulation (as determined by ELISA). Near maximal PrP$^{Sc}$ accumulation, each biological replicate (Inoc A, B, and C or Mock A, B, and C) was passaged

into three 75-$cm^2$ flasks for technical triplicates of RNA collection (Inoc A1-A3 and so on, and Mock A1-A3 and so on). At approximately 90-100% confluency, each technical replicate was independently trypsinized and shredded in QIAshredder spin columns (Qiagen). Total RNA from each technical replicate was eluted into 50 µl total volume using RNeasy mini spin columns (Qiagen) per manufacturer's instructions and the quantity and quality of the total RNA were assayed by spectrophotometry and agarose gel electrophoresis. Samples for InocB and MockB were collected and analyzed by microarray at one time point post inoculation (passage 8 post-$PrP^{Sc}$ inoculation), and samples for InocA, InocC, MockA, and MockC were collected and analyzed at a second time point post inoculation (passage 6 post-inoculation)

Biotinylated cRNA targets were generated from total RNA using the One-Cycle Target Labeling kit (Affymetrix) per manufacturer's instructions. Briefly, single stranded cDNA was generated from total RNA (10 µg) using an oligo(dT) primer with a T7 promoter. The single-stranded cDNA was then converted to double-stranded cDNA via RNase H-mediated second-strand cDNA synthesis. The resultant double-stranded cDNA was then subjected to in vitro transcription to generate biotinylated, antisense cRNA. The biotinylated cRNA was fragmented and hybridized to the Affymetrix GeneChip Bovine Genome Array, representing approximately 23,000 transcripts, as per manufacturer's directions. An Affymetrix GeneChip Fluidics Station 400 was used for array processing and the arrays were scanned using a Gene/Array Scanner 2500A (Agilent). Reverse transcription, hybridization, and scanning steps were performed at the Laboratory for Biotechnology and Bioanalysis 1 at Washington State University (Pullman, WA).

**Data analysis.** Eighteen (two treatment groups, three biological replicates per treatment group, and three technical replicates per biological replicate) raw Affymetrix ".CEL" files were analyzed by the Bioconductor (37) package "affy" (36), which uses the statistical R

programming language (82). The affyQCReport package (C. Parman, C. Halling, and R. Gentleman. affyQCReport: QC Report Generation for affyBatch objects. R package version 1.14.0) was used to assess quality control parameters.

To compare the overall number of transcripts detected in this study with previous reports that used either ovine and bovine mRNA on the Affymetrix GeneChip Bovine array, our results were initially processed similar to those previous studies (35, 70, 74). Probe-level robust linear model fitting was accomplished via the affyPLM package (Ben Bolstad (2007). affyPLM: Methods for fitting probe-level models. R package version 1.12.0. http://bmbolstad.com).

To evaluate differential transcriptional regulation associated with $PrP^{Sc}$ accumulation, data were preprocessed using several different algorithms to determine the algorithm best suited for this data set (Micro Array Suite 5.0 (MAS5) (43), robust multiarray average (RMA) (44), multiplicative model-based expression index (MBEI) (52), probe logarithmic intensity error (PLIER) (1) and GC robust multiarray average (GCRMA) (90). The MAS5 algorithm was determined to be the most useful with this dataset since it maintained the highest percentage of absent and present probe sets in their respective categories (i.e. the highest percentage of probe sets determined to be absent in all samples by PMA calls remained absent following normalization, likewise for those present in all samples). Following preprocessing, probe sets that were consistently present in at least one treatment group, as determined by the cutoff established by the MAS5 algorithm, and demonstrated at least a 0.5 log2 change in the 75%–25% interquartile range were selected for further analysis. Finally, hierarchical clustering using the hclust function (Euclidean metrics, complete linkage) demonstrated a batch effect resulting from the biological replicate group B being hybridized at a different time point from the groups A and C. The resultant batch effect was corrected using the empirical Bayes-based ComBat

algorithm (http://statistics.byu.edu/johnson/ComBat/) (46). The values for the technical replicates were then averaged to attain a single value for each biological replicate, and differential transcriptional levels were determined by fitting the LIMMA linear model (77) to each probe set and correcting for multiple comparisons by controlling the false discover rate via the Benjamini and Hochberg method (10) with $\alpha < 0.05$.

The results of this study using the Bovine Genome Array were compared to previous studies that used mouse and human models. The mouse and human homologues of the differentially expressed transcripts were determined via the annotations provided by Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx, accessed July 23, 2008). Probe sets representing the differentially expressed transcripts were also annotated to gene ontology (GO) using the GO annotations available on NetAffx (accessed July 23, 2008).

**Quantitative RT-PCR.** Quantitative RT-PCR was performed on several genes to verify microarray results. Total RNA that was originally isolated for microarray analysis was aliquoted and saved a -80°C pending microarray analysis. Contaminating DNA was removed using the DNA-free kit (Ambion), which includes a 30 min incubation at 37°C with rDNase I and removal of DNase activity by 2 min incubation with Dnase Inactivation Reagent (Ambion) and centrifugation at 10,000 × g for 1.5 min. The DNase-treated RNA was pooled for each set of technical replicates resulting in six (three Inoc and three Mock) samples for testing. One microgram of RNA was then reverse transcribed into cDNA using the SuperScript™ III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen) as per manufacturer's instructions. GAPDH primers for sheep have been previously reported (18); all other gene specific primers (Table 1) were designed by using PrimerQuest (Integrated DNA Technologies, http://www.idtdna.com/Scitools/Applications/Primerquest/). Known ovine sequences were used

for primer design when available (Table 1). When ovine sequences for genes were unavailable, primer design was limited to those regions of the Affymetrix target sequence (available on NetAffx™ Analysis Center, http://www.affymetrix.com/analysis/index.affx) that had high PM/MM probe hybridization intensity ratios, as determined by GeneChip Operating Software 1.4 (Affymetrix). Quantitative real-time PCR was performed in an iCycler iQ (Bio-Rad). The 20 µl reaction mix contained containing 1x SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), 200 nM of each specific primer, 8 µl of 1/100 diluted cDNA, and water. Reaction conditions were 50°C for 2 min, 95°C for 8.5 min, 40 cycles of denaturation at 95°C for 15 sec and annealing at 59°C for 1 min. A melt curve analysis (denature at 95°C for 1 min, followed by annealing at 55°C for 1 min, and 80 cycles of 55°C + 0.5°C/cycle for 10 sec) was performed immediately following the PCR to verify the presence of single dsDNA products in each reaction. Biological replicates were assayed in triplicate. Negative controls for quantitative RT-PCR included RNA processed without reverse transcriptase, and no-template controls for qRT-PCR. Relative transcript levels were calculated using the ΔΔCT method with normalization to GAPDH (3).

## RESULTS

**Confirmation of $PrP^{Sc}$ accumulation.** Accumulation of $PrP^{Sc}$ was verified and the level of $PrP^{Sc}$ was monitored over time with a commercially available ELISA for $PrP^{Sc}$ that was shown previously to correlate with $PrP^{Sc}$ immunoblots (79). RNA for transcriptomic analysis was collected at time points of maximal $PrP^{Sc}$ accumulation, as determined by relative ELISA optical density.

**Ability of bovine-based microarray to detect ovine-derived transcripts.** Before identifying genes differentially regulated following $PrP^{Sc}$ exposure, it was first determined if the ovine mRNA used in this study yielded detectable transcripts when hybridized to the Affymetrix Bovine Genome Array. To be considered expressed in a single biological replicate, the transcript must have been detected in all three of the corresponding technical replicates. In the $PrP^{Sc}$ inoculated group, the total number of transcripts detected in a single biological replicated ranged 14,902–15,373 (61.8%–63.7%) with approximately 13,300 (55.2%) transcripts detected in all three of the $PrP^{Sc}$ inoculated biological replicates. In the mock-treated group, the total number of transcripts detected in a single biological replicate ranged 15,142–15,713 (62.8%–63.1%) with approximately 13,600 (56.6%) transcripts detected in all three of the mock-treated biological replicates. The number of detected transcripts was consistent both with previous reports of using ovine mRNA (35) or bovine mRNA (70, 74) on the Affymetrix Bovine Genome Array.

**Verification of microglial cell phenotype using transcript analysis.** While the cell isolation procedure has been previously described in sheep and shown to result in cultures of microglia (79), the transcript levels of five genes commonly used to identify microglia were analyzed (Table 2) to further characterize the cells. Toll-like receptor 4 (TLR4), CD68, and cathepsin K (CTSK) were detected in all technical replicates for each of the six biological groups (three inoculated groups and three uninoculated groups). CD14 and CD163L1 were detected in the majority of technical replicates for each of the biological groups.

To further exclude the possibility of significant contamination of the cultures with astrocytes and endothelial cells, the transcript levels of marker genes for these two cell types were examined (Table 2). Glial fibrillary acidic protein (GFAP), a commonly used marker for astrocytes, was not detected in any of the biologically replicates. Four probe sets on the Bovine

Genome Array represent von Willebrand factor (VWF), a commonly used marker for endothelial cells. VWF was considered present by only one probe set in only two of the six biological groups, and in each of those groups, it was only detected in two of the three technical replicates. Based on these transcript features, the primary cell cultures were classified as sheep microglia.

**Identification of genes differentially regulated in $PrP^{Sc}$-accumulating primary sheep microglia.** To determine which transcripts were significantly upregulated or downregulated in $PrP^{Sc}$-accumulating microglia, the array data were subjected to statistical analysis as described above in the *Material and Methods*, with cutoff values for significance defined as a Benjamini and Hochberg (BH) false discover rate < 0.05. Nineteen genes were significantly upregulated in $PrP^{Sc}$ accumulating microglia (Fig. 1A). Of these 19 genes, six had BH false discovery rate < 0.01. The maximum fold upregulation was observed for platelet factor 4 (PF4, CXCL4), which was upregulated 2.2-fold in the $PrP^{Sc}$ inoculated group. Only one other transcript, milk fat globule-EGF factor 8 protein (MFGE8, lactadherin), was detected with at least a twofold increase. Twelve of the remaining 17 genes had at least a 1.5-fold increase of transcript levels in the treated group. Thirty transcripts were considered significantly (BH false discovery rate < 0.05) downregulated in the $PrP^{Sc}$ treated group (Fig. 1B). Fourteen of these transcripts were downregulated at least 1.5-fold. None of the transcripts in the $PrP^{Sc}$ treated group were decreased more than twofold.

**Functional categorization of differentially regulated genes.** The differentially regulated transcripts were further analyzed in order to identify patterns in the cellular response to $PrP^{Sc}$ accumulation. The gene ontology (GO) terms for the differentially regulated probe sets were acquired via Affymetrix NetAffx™ Analysis Center. The terms were slimmed to select gene ontology parent terms (nodes) and then graphed as the percentage of probe sets within a

node (Fig. 2). A higher percentage of the upregulated transcripts annotated to the extracellular region; whereas, a higher percentage of the downregulated transcripts annotated to the nucleus (Fig. 2A). Examination of the molecular function ontologies demonstrated two upregulated probe sets, compared to zero downregulated probe sets, annotated to the cytokine activity node (Fig. 2B), suggesting a mild activation of the microglial cells' immune function. Three downregulated probe sets, compared to zero upregulated probe sets, annotated to the transcription factor node, although the genes controlled by these downregulated transcription factors are not determined. In the biological process ontologies, GO nodes consistent with activation of microglia (chemotaxis, proteolysis, cell communication, and response to stimulus) are represented at higher percentages in the upregulated probe set group as compared to the downregulated probe set group (Fig. 2C).

It should be noted that the ontological categorization of these transcripts is incomplete due to high percentage of bovine genes with unknown GO terms and the fact that even genes with one or more GO terms are likely associated with more, as yet undetermined, GO terms. Nevertheless, using the currently available knowledge of these genes there is a minimal common response to $PrP^{Sc}$ accumulation, which most likely represents limited activation of the immune function of microglial cells.

**Verification of microarray results by RT-PCR.** To verify the microarray results, quantitative RT-PCR was used to assay seven of the upregulated transcripts and three of the downregulated transcripts. Qualitative differences (up- or downregulation) were confirmed in all cases (Fig. 3), although for MFGE8 the qPCR results demonstrated a minimal increase in transcript levels.

## DISCUSSION

Prion diseases manifest as chronic neurodegenerative diseases with limited, localized inflammation characterized by activation of astrocytes and microglia (30). The complete pathogenesis leading to this neurodegeneration and limited inflammation is poorly understood. To further understand the microglial responses to $PrP^{Sc}$ accumulation and to extend the prion transcriptomic studies into the ovine model, we investigated the transcriptional response of primary ovine microglial cells to $PrP^{Sc}$ accumulation.

**Overall transcriptional response.** The transcriptional response by the primary microglia was limited with only a total of 49 transcripts being differentially regulated with relatively small magnitudes of change (largest absolute value fold change = 2.2). This is markedly lower than what is found when microglia or macrophages are activated in culture either by lipopolysaccharide (LPS) (54) or when infected by viruses (21, 41) or bacteria (38), in which 200–600 genes are upregulated with 10-fold to 100-fold changes in transcript levels. While the transcriptional response of primary microglia infected with $PrP^{Sc}$ in vitro has not been studied, previous studies that analyzed transcriptional response of neuronal cell cultures to $PrP^{Sc}$ yielded mixed results. In two of the earlier studies using SH-SY5Y neuroblastoma cells (56), ScN2a murine neuroblastoma cells (39), and murine hypothalamic neuronal cells (GT1) (39) approximately 100–200 differentially regulated genes are found for each cell type, with maximum absolute values of fold change up to 22-fold. In contrast, a subsequent study of ScN2a cells demonstrates only one significantly altered gene (approximately 1.5-fold decrease) (47), and a recent study comparing $PrP^{Sc}$-accumulating GT1 cells to quinacrine-cured GT1 cells demonstrates only thirteen differentially regulated genes (fold change absolute value 2.0–3.6). The reasoning offered for the fewer altered genes in the two latter studies is that in both cases the

experiments use more temporally related cells (i.e. minimal time in culture between splitting of samples for inoculation) and use more biological replicates as compared to the previous studies. The study described herein is similarly designed. Thus, the relatively few genes differentially regulated in these sheep microglial cultures and their relatively limited change are consistent with the more recent findings of minimal transcriptional alterations in cell culture models of $PrP^{Sc}$ infection and demonstrate a much weaker transcriptional response as compared to microglia exposed to other infectious agents.

Gene ontology terms were used to functionally categorize the differentially regulated transcripts. Similar to the limited overall number of differentially regulated transcripts, few GO nodes are represented. Overall, though, a limited activation of the microglial cells' immune function is suggested by the GO terms. The limited activation of microglia in vitro by $PrP^{Sc}$ is consistent with the limited in vivo inflammation that is a characteristic of prion diseases (30). .

**Analysis of genes previously associated with $PrP^{Sc}$ accumulation.** Three transcripts differentially regulated in the current study (CCL2, SGK1, and AASDHPPT) were differentially regulated in a manner similar to previous reports. The probe set Bt.2408.1.S1_s_at (bovine CCL2) was annotated by NetAffx to be orthologous to mouse CCL12, which is upregulated in brain samples of mice infected with both the ME7 and RML mouse-adapted scrapie strains (93). Bovine serum/glucocorticoid regulated kinase 1 (SGK1) (probe set: Bt.16123.1.S1_at) was upregulated in the current study, and is upregulated in brains of mice infected with ME7 (14, 75, 78), 79a (14, 78), 139A (64) and 22a (78) mouse-adapted scrapie strains. The probe set Bt.3939.2.A1_at is not annotated; however, it is listed by NetAffx as having a "strong similarity" to human AASDHPPT and is nearly identical (BLASTn: 98–99% identity, 0.0 E value) to two sequences (XM_001250765.2 and XM_864079.3) for the predicted bovine AASHDPPT gene.

Human AASDHPPT is downregulated in the brains of people with sporadic CJD (sCJD) (92) and in the human neuroblastoma cell line SH-SY5Y exposed to prion peptide 106–126 (56).

Three additional transcripts (MT1E, NR4A1, PKP2) with altered levels in previous studies were also identified in this current study, but in a manner opposite to the previous reports. Plakophilin 2 (PKP2) is upregulated in mice infected with the 301V strain of BSE (69). This contrasts with the downregulation of this transcript (Bt.21547.1.A1_at) in the present study. Similarly, nuclear receptor subfamily 4, group A, member 1 (NR4A1) is upregulated in the hippocampus of preclinical ME7 infected mice (16), but downregulated in our study (Bt.23373.1.S1_at). Based on the NetAffx annotations, the probe set Bt.23042.1.S1_at (bovine MT1E) is orthologous to murine Mt2, which is downregulated in both bovine (69) and scrapie infected mice brains (25).

Previous work demonstrates upregulation of various activator protein-1 (AP-1) family members (FOS, FOSB, FOSL1, FOSL2, JUNB, and JUND) in the brains of mice infected with $PrP^{Sc}$ (16, 69, 73), but in our study JUN (Bt.11159.1.S1_at) and FOSB (Bt.11018.1.S1_at) were downregulated. Interestingly, in a study using primary microglial cultures isolated from the brains of mice infected with the Fukuoka-1 strain, FOSL1 is downregulated (5), which is similar to our study, and dissimilar from the organismal studies. The conflicting data between the whole brain studies and the studies using isolated microglia likely represent differences due to the cell types examined and a lack of input from other cells, specifically neurons and astrocytes, in microglial cultures.

Metallothioneins (MT) are a family of closely related proteins that often show differential transcriptional regulation in prion studies. In previous studies using mouse brains and murine neuronal cell cultures, MT1 (39, 64) and MT2 (25, 65, 69) are upregulated. Similarly, human

MT1F is upregulated in the brain of sCJD patients (92). In contrast, MT1E was downregulated in the current study, suggesting that the upregulation of metallothionein in brain tissue is not a response by microglia specifically to $PrP^{Sc}$ accumulation. Similar to the response of the AP-1 family members, it is possible that the upregulation of metallothionein found in brain studies is a neuron-specific response to $PrP^{Sc}$ accumulation, or that it is a secondary effect of one cell type (e.g. microglia or astrocytes) in response to cell signals produced by a second, $PrP^{Sc}$-infected cell type (e.g. neurons).

Additional similarities between this current study and previous studies were also identified. For example, it has been demonstrated that nicotinamide nucleotide adenylyltransferase 2 (NMNAT2) is downregulated in the brains of patients with sCJD (92). In the current study bovine NMNAT1 (Bt.14046.1.A1_at) was also downregulated in sheep microglial cells. Both bovine NMNAT1 and human NMNAT2 encode a nicotinamide-nucleotide adenylyltransferase (EC: 2.7.7.1) and function in the same step of the nicotinate and nicotinamide metabolic pathway (Kyoto Encyclopedia of Genes and Genomes [KEGG] path: rn00760), thus indicating a similar response to $PrP^{Sc}$ accumulation by primary sheep microglial cultures and human sCJD-affected brains. In the same study, dual specificity phosphatase 6 (DUSP6) is also downregulated in the brains of sCJD patients. DUSP1 (Bt.1658.1.S1_at) was downregulated in the current study and encodes a transcript for the same enzyme (EC: 3.1.3.16 and 3.1.3.48) as DUSP6. Another similar findings, is the upregulation of GalNAc-T10 (GALNT10) in the brains of scrapie infected mice (75) and the upregulation of GALNT13 (Bt.24402.2.A1_at) in the primary sheep microglial cells in the current study. Again, both genes encode transcripts for the same enzyme (EC: 2.4.1.41), which in this case functions in O-Glycan biosynthesis (KEGG path: map00512).

**Analysis of genes previously associated with neurodegenerative diseases.** Each gene was searched via Pubmed (July 30th, 2008) and The Database for Annotation, Visualization and Integrated Discovery (DAVID July 29th, 2008) for associations with other neurological diseases and neurodegeneration. CCL2, which is upregulated in a previous study of prion diseases (see above) is also upregulated in Alzheimer's disease (45, 76), the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's diseases (61), and other neurological conditions (71, 72, 84). Other genes upregulated in our study that are upregulated in Alzheimer's disease include complement component 1, r subcomponent (C1r) (94), ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS1) (59), MFGE8 (12), and regulator of calcineurin 1 (RCAN1) (31). RCAN1 also increases the sensitivity of neurons to oxidative damage-induced neurodegeneration (62). The upregulation of SGK1, which has been demonstrated in the current study and several other prion studies (14, 64, 75, 78), is also found in the MPTP mouse model of Parkinson's disease (80). Finally, dual specificity phosphatase 1 (DUSP1) was downregulated following $PrP^{Sc}$ accumulation in this study, and is similarly downregulated in familial amyloidotic polyneuropathy (60).

**Other genes of specific interest.** Two upregulated genes in this study are also interesting findings considering the known information for these two genes. The MFGE8 gene encodes the lactadherin protein, whose homologue in yeast (SED1) increases heterologous protein secretion (88). MFGE8 is also present on exosomes of dendritic cells (83) and in the $PrP^{C}$ enriched exosomes of Mov cells (immortalized neuroglial cells isolated from mice expressing ovine PRNP) (34). $PrP^{C}$ (33, 66) and $PrP^{Sc}$ (85) are found in exosomes released from various cell types, and it is thought that these exosomes are at least one possible mechanism of $PrP^{Sc}$ transfer from cell to cell (85). Additionally, MFGE8 functions in the phagocytosis of

amyloid β-peptide (a key component of the senile plaques found in Alzheimer's disease) (12). The colocalization of MFGE8 with $PrP^{C}$ in exosomes, its ability to stimulate protein expression from yeast, and the data indicating a specific role for MFGE8 in the phagocytosis of amyloid β-peptide suggest a possible role in the pathogenesis of prion disease; however, further experiments are required to test any hypotheses concerning the role of MFGE8 in $PrP^{Sc}$ accumulation.

Another result that needs further examination is the upregulation of probe set Bt.19378.1.A1_at. While this probe set remains unannotated, its closest similarities are to bovine surfeit 4 (SURF4) and to the predicted bovine SURF2 (BLASTn: 87% identity, 3.-24 E value). While the specific functions of these housekeeping genes are unknown, there have been previous associations of SURF2 with $PrP^{Sc}$ accumulation. SURF2 is weakly downregulated in the brains of scrapie infected mice (91), in contrast to the upregulation of the Bt.19378.1.A1_at transcript in this study. Additionally, using a protein microarray, Satoh et al. demonstrated that SURF2 is a $PrP^{C}$-interacting protein (68). Given the limited information available for this probe set and the likely gene it represents, further investigation is warranted to determine if either SURF2 or SURF4 interact directly with $PrP^{C}$ or $PrP^{Sc}$ during the disease state.

Taken together, these data show that the transcriptional response of primary sheep microglia to $PrP^{Sc}$ is limited, but suggests a small degree of microglial activation. This limited response is similar to previously described studies of murine neuronal cell cultures, but is in stark contrast to microglia and monocyte cultures exposed to LPS, viruses, and bacteria. It is proposed that this limited response is consistent with the unique pathogenesis and limited inflammation of prion diseases. While it is recognized that the full transcriptional response of the sheep microglial cells to $PrP^{Sc}$ accumulation can not be fully evaluated due to the use of the bovine

microarray with ovine mRNA samples and the lack of full annotation of the bovine genome, the results described herein were obtained with available reagents and are consistent with previous reports. Specifically, several differentially regulated genes identified in this study have been previously associated with $PrP^{Sc}$ accumulation by similar transcriptomic studies (e.g. CCL2, SGK1, and AASDHPPT) and other transcripts identified in this study (MFGE8) colocalize with $PrP^{C}$ and $PrP^{Sc}$ in previous studies. Further investigation into the specific role(s) these genes play in the pathogenesis $PrP^{Sc}$ accumulation is warranted.

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**TABLES**

TABLE 1. qRT-PCR primer information

| Gene Symbol | Forward primer sequence | Reverse Primer Sequence | Amplicon size (bp) | Reference sequence |
|---|---|---|---|---|
| PF4 | AATGCGTGTGCCTGAAGACCACTT | TTCCCGTCTTCAGCGTAGCTATCA | 159 | P30035[a] |
| MFGE8 | TCTCTGGCACCCAGTCCTCAA | AAAGGTCACCCACGCTCTGTACTT | 107 | NA |
| GALNT13 | TAGACAACATGGGCCGCAAGGAAA | ATCCAAGCACAAGTCATCGGTTCG | 125 | NA |
| RCAN1 | ATTCACACCTGTTGTGCACTTGGG | ATTAATGGGAAAGGCACTGCACCC | 184 | AY205232 |
| CCL2 | AGAAGAGTCACCACCAGCAAGTGT | TTGAGATGGTTTATGGCGTCCTGG | 119 | (28) |
| FAM14A | AAGCAACCAAAGGGACCACATCAG | AGATTCCTGTTCTGGTGAAGCCCA | 175 | BN000255 |
| MGC155285 | GTTTGGGCTCAGCAATTCCACTGT | CTGCTCCTTCGGATTAAAGGTTCAC | 132 | NA |
| TPM2 | ACCGAGCTATGAAGGATGAGGA | CTCTCCTTCCAGGATCACCAGTTT | 131 | NA |
| NR4A1 | CACACACACCGCCATGCTGTAAAT | AAGGAGGGCAGGAAGAGAGCAAGTA | 138 | NA |
| JUN | AACAGGTGGCCCAGCTTAAACAGA | TTGCAACTGCTGCGTTAGCATGAG | 80 | NA |

[a]no nucleotide sequence is available for sheep. The reference sequence is a protein sequence which was manually aligned with the bovine sequence to determine areas of amino acid similarity.

TABLE 2. Gene expression of microglial-associated genes

| Gene Symbol | Probe set | InocA | InocB | InocC | MockA | MockB | MockC |
|---|---|---|---|---|---|---|---|
| CD14(52) | Bt.4285.1.S1_at | +[a] | -[c] | ++ | ++ | ++ | ++ |
| | Bt.13789.1.A1_at | ++[b] | ++ | ++ | ++ | ++ | ++ |
| TLR4(37) | Bt.9030.1.S1_at | ++ | ++ | ++ | ++ | ++ | ++ |
| CD68(39) | Bt.2334.1.S1_at | ++ | ++ | ++ | ++ | ++ | ++ |
| CTSK(18) | Bt.23218.1.S1_at | ++ | ++ | ++ | ++ | ++ | ++ |
| CD163L1(48) | Bt.25985.1.A1_at | ++ | ++ | ++ | ++ | + | ++ |
| | Bt.20506.2.S1_at | + | ++ | ++ | ++ | ++ | ++ |
| | Bt.213.1.S1_at | ++ | ++ | ++ | ++ | ++ | ++ |
| GFAP(10) | Bt.21127.1.S1_at | - | - | - | - | - | - |
| VWF(90) | Bt.12823.1.S1_at | - | - | - | - | - | - |
| | Bt.18361.1.A1_at | + | - | + | - | - | - |
| | Bt.25505.1.A1_at | - | - | - | - | - | - |
| | Bt.12823.2.S1_at | - | - | - | - | - | - |

[a]present in two of three technical replicates

[b]present in all three technical replicates

[c]present in either one or zero technical replicates

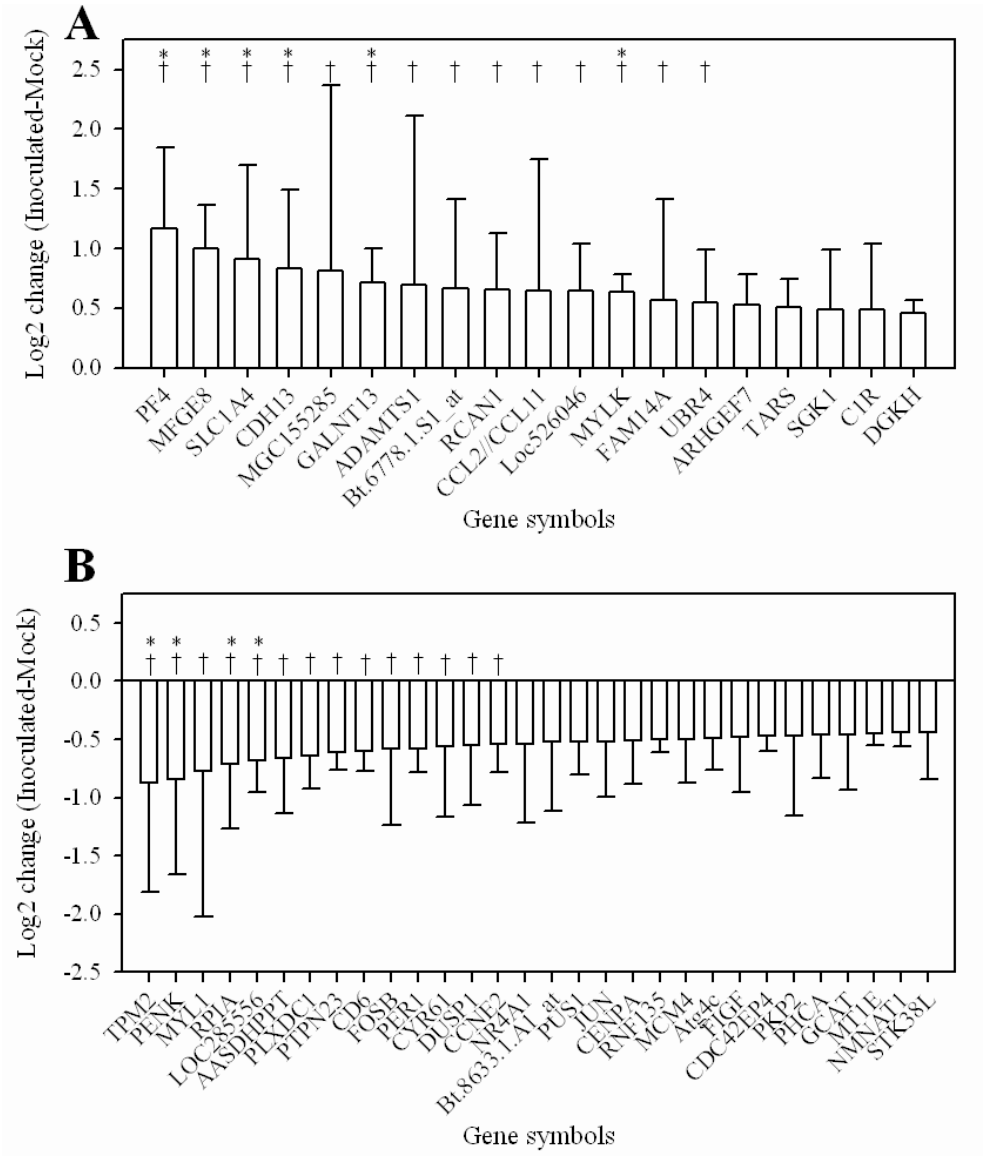


**Figure. 1.** Transcripts upregulated (A) and downregulated (B) in $PrP^{Sc}$-accumulating primary sheep microglia as compared to mock-treated microglia. Three biological replicates of primary sheep microglia were inoculated with either $PrP^{Sc}$-containing (Inoc) or $PrP^{Sc}$-lacking (Mock) cell lysates derived from the $PrP^{Sc}$ positive or negative, respectively, Rov9 cell lines (86) and then serially passaged. RNA samples from three technical replicates per biological replicate were collected at the time of maximal $PrP^{Sc}$ accumulation, as determined by ELISA, and subjected to commercial microarray analysis. Probe set intensities were normalized via the MAS5 algorithm, and genes were filtered out by two criteria, 1) absent in both treatment groups and 2) those with less than a 0.5 $\log_2$ change within the 25%–75% interquartile range. Remaining genes were tested for differential transcriptional regulation with a Benjamini and Hochberg corrected cutoff of $P < 0.5$. Differentially regulated genes are listed along the horizontal axis and graphed as the $\log_2$ change in transcript levels (vertical axis). Error bars represent one standard deviation based on the three pairwise comparisons between biological replicates. ; * = $P < 0.01$, † = fold change greater than 1.5.

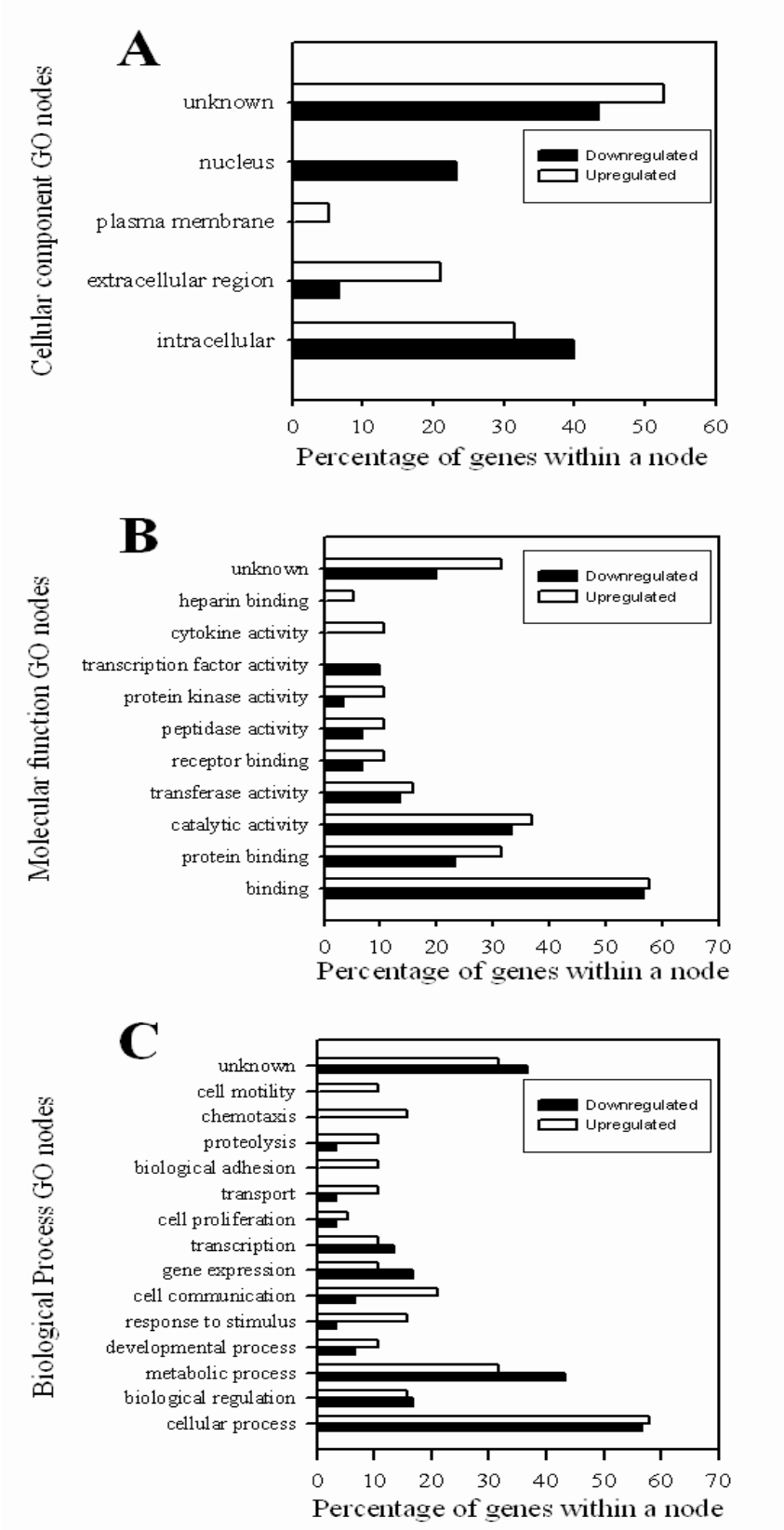
A
Cellular component GO nodes
unknown
nucleus
plasma membrane
extracellular region
intracellular
Downregulated
Upregulated
0 10 20 30 40 50 60
Percentage of genes within a node
B
Molecular function GO nodes
unknown
heparin binding
cytokine activity
transcription factor activity
protein kinase activity
peptidase activity
receptor binding
transferase activity
catalytic activity
protein binding
binding
Downregulated
Upregulated
0 10 20 30 40 50 60 70
Percentage of genes within a node
C
Biological Process GO nodes
unknown
cell motility
chemotaxis
proteolysis
biological adhesion
transport
cell proliferation
transcription
gene expression
cell communication
response to stimulus
developmental process
metabolic process
biological regulation
cellular process
Downregulated
Upregulated
0 10 20 30 40 50 60 70
Percentage of genes within a node

**Figure 2.** Functional categorization of differentially expressed transcripts using gene ontology (GO) annotations. Differentially expressed transcripts were annotated to GO terms for cellular component (A), molecular function (B), and biological process (C) using NetAffx. Select gene ontology nodes are present on the vertical axis and graphed with the percentage of the upregulated (unfilled bars) and downregulated (black bars) genes annotated to that node (horizontal axis). Transcripts without annotations are listed as unknown.

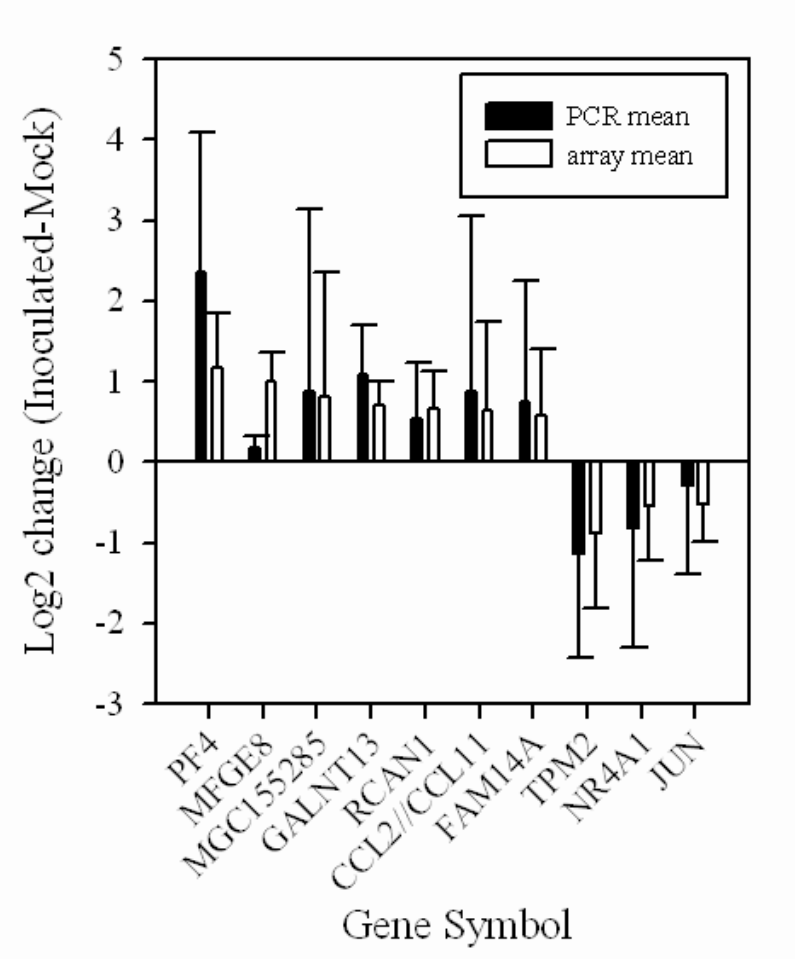


**Figure 3.** Verification of microarray analysis. RNA from aliquots used for the microarray was tested for differential transcriptional regulation of seven upregulated genes and three downregulated genes by quantitative RT-PCR (qRT-PCR). qRT-PCR results for each gene were normalized to GAPDH, and the $\log_2$ change in transcript levels between Inoc cells and Mock cells was calculated using the $\Delta\Delta C_T$ method (black bars). The microarray data (unfilled bars) from Figure 1 are included for comparison. Error bars represent one standard deviation of the biological replicates for each treatment group.