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Limited transcriptional response of ovine microglia to prion accumulation

James B. Stanton^{a,*}, Donald P. Knowles^{a,b}, Douglas R. Call^a, Bruce A. Mathison^a, and Timothy V. Baszler^a

^aDepartment of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA 99164

^bUnited States Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, Pullman, WA, USA 99164

Abstract

The conversion of normal cellular prion protein to disease-associated prion protein (PrP^{Sc}) is a fundamental component of prion disease pathogenesis. The molecular mechanisms contributing to prion conversion and the impact of PrP^{Sc} accumulation on cellular biology are not fully understood. To further define the molecular changes associated with PrP^{Sc} accumulation in cultured cells, the transcriptional profile of PrP^{Sc}-accumulating primary ovine microglia was compared to the profile of PrP^{Sc}-lacking microglia 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 point of near maximal PrP^{Sc} accumulation levels as measured by ELISA. The array analysis revealed only 19 upregulated genes and 30 downregulated genes in PrP^{Sc}-accumulating microglia. The results support the hypothesis that chronic PrP^{Sc} accumulation in cultured microglia results in a limited transcriptional response.

Keywords

ovine; microglia; prion; scrapie; microarray

Introduction

Transmissible spongiform encephalopathies (TSE, prion diseases) are fatal, transmissible, neurodegenerative diseases, including scrapie in sheep and goats, and Creutzfeldt-Jakob disease in humans. Many of the underlying molecular mechanisms associated with the posttranslational conformational change of prion protein and the resulting cellular response are either unknown or poorly understood [1]. Although there are no known natural rodent prion diseases, previous studies into the transcriptomics associated with prion diseases have primarily employed the use of rodent models with only two studies using a sheep model [2, 3]. Neither of these sheep model experiments used cell cultures, but rather they used whole tissues. This prevents the determination of the cell type(s) responding to PrP^{Sc} and an inability

*Corresponding author. Mailing address: 402 Bustad Hall, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA 99164-7040, Phone: 1-509-335-3725, Fax: 1-509-335-8529, jstanton@vetmed.wsu.edu.

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to determine if the response is directly or indirectly related to PrP^{Sc}. 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) [4]. Sheep scrapie is a logical experimental model for the investigation of a natural prion disease in a natural host. Currently there are no studies that have investigated the response of primary natural TSE-host cells to PrP^{Sc} accumulation.

Importantly, the results of previous rodent-based cell culture transcriptomic studies have conflicted. Not only are different transcripts often differentially regulated in these studies, but the overall transcriptional response varies from a robust response [4,5] to a limited response [6,7]. In light of these conflicting results and because PrP^{Sc} is a misfolded host protein, and not a foreign protein [1], the following study tests the hypothesis that microglia have a limited response to PrP^{Sc} accumulation. Additionally, this study extends the prion transcriptomic studies into sheep, the host of the prototypical prion disease, and focuses on the activation of microglia, which are likely contributors to prion-induced neuropathology [8].

Materials and Methods

Inoculation of primary microglia with PrP^{Sc}

Primary mixed glial cultures were obtained from a previously confirmed homozygous VRQ/VRQ [9] ovine fetal brain and cultured using a previously described technique [10]. VRQ inoculum derived from mechanical lysates of inoculated (Rov9^{Sc}) and uninoculated (Rov9^C) Rov9 cells [11] was used, as previously described [10].

Primary microglia were passed into 6-well plates, grown to approximately 60% confluency, and treated in triplicate. Briefly, microglia were rinsed with PBS, overlaid with 200 μ l 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 I Reduced Serum Medium (Invitrogen, Carlsbad, CA), incubated for six hours, and then 200 μ l of maintenance medium [10] were added to each well. After two days of incubation, 0.5 ml of maintenance medium was added, and microglia were incubated for four days at which time they were expanded into 25-cm² tissue culture flasks. Microglia were fed every three to four days as necessary and serially passed 1/5.

As previously described [10], at selected passages following trypsinization, 4/5 of the microglial cell suspension from a 25-cm² tissue culture flask was analyzed for PrP^{Sc} by commercial ELISA (HerdChekTM Scrapie Antigen Test Kit ELISA, IDEXX, Westbrook, ME) following the manufacturer's instructions.

RNA Collection, preparation, and microarray hybridization

Microarray experiments were performed in biological triplicates, each with its own set of technical triplicates. Near the maximal PrP^{Sc} levels, each biological replicate was passaged into three 75-cm² 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, shredded (QIAshredder, Qiagen, Valencia, CA), and RNA was purified using RNeasy mini spin columns (Qiagen). Total RNA quantity and quality were determined by spectrophotometry and agarose gel electrophoresis, respectively. Samples for InocB and MockB were collected and analyzed by microarray at passage 8 post-PrP^{Sc} inoculation, and samples for InocA, InocC, MockA, and MockC were collected and analyzed at passage 6 post-inoculation.

Biotinylated cRNA targets were generated from total RNA using the One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA), fragmented, and then hybridized to the Affymetrix GeneChip Bovine Genome Array (representing approximately 23,000 transcripts). Arrays

were processed on an Affymetrix GeneChip Fluidics Station 400 and scanned on a Gene/Array Scanner 2500A (Agilent, Santa Clara, CA). These steps were performed at the Laboratory for Biotechnology and Bioanalysis 1 at Washington State University (Pullman, WA). Complete microarray data is available online, Gene Expression Omnibus Accession Number **GSE12688**.

Microarray 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 R programming language-based [12] Bioconductor [13] package “affy” [14]. 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.

The number of transcripts detected in this study was compared to previous reports that used ovine or bovine mRNA on the Affymetrix GeneChip Bovine array. 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>]). Only those transcripts that were present in all three biological replicates were considered “detected”.

For determining differentially expressed transcripts, microarray data were preprocessed using the Micro Array Suite 5.0 (MAS5) [15] algorithm. Probe sets 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 interquartile range were analyzed further. Hierarchical clustering using the hclust function (Euclidean metrics, complete linkage) demonstrated a batch effect, which was corrected using the empirical Bayes-based ComBat algorithm [16]. Values for the technical replicates were averaged to attain a single value for each biological replicate. Differential transcriptional levels were determined by fitting the LIMMA linear model [17] to each probe set and correcting for multiple comparisons by controlling the false discover rate via the Benjamini and Hochberg method [18] with $\alpha < 0.05$.

The results of this study using the Bovine Genome Array were compared to previous mouse and human model-based prion studies. The mouse and human homologues of the differentially expressed transcripts were determined via the annotations provided by Affymetrix NetAffx Analysis Center (accessed July 23, 2008 <http://www.affymetrix.com/analysis/index.affx>). Probe sets representing the differentially expressed transcripts were also annotated to 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. RNA samples were treated with DNase (DNA-free kit, Ambion, Austin, TX) followed by Dnase Inactivation Reagent (Ambion) and centrifugation at 10,000 × g for 1.5 min. Samples were pooled for each set of technical replicates within a biological replicate and each reaction was conducted in triplicate. One microgram of RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). Quantitative real-time PCR was performed in an iCycler iQ (Bio-Rad, Hercules, CA). The 20 µl reaction mix contained 1× SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), 200 nM of each specific primer, 8 µl of 1/100 diluted cDNA (1 µg), 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 followed immediately by a melt curve. GAPDH primers for sheep have been previously reported [19]; all other gene specific primers (Table 1) were designed by using

PrimerQuest (Integrated DNA Technologies [<http://www.idtdna.com/Scitools/Applications/Primerquest/>]). 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 $\Delta\Delta CT$ method with normalization to GAPDH [20].

Results

Primary sheep microglia were inoculated with PrP^{Sc} to determine their transcriptional response to PrP^{Sc}. Accumulation of PrP^{Sc} was monitored over time with a commercially available ELISA for PrP^{Sc} that was previously shown to correlate with PrP^{Sc} immunoblots [10].

Evaluation of Bovine Genome Array and verification of microglial cultures

Before identifying genes differentially regulated following PrP^{Sc} exposure, it was determined if the ovine mRNA used in this study yielded detectable transcripts when hybridized to the Affymetrix Bovine Genome Array. For each treatment group, over 13,000 (55%) transcripts were detected in all three of the biological replicates. This was consistent with previous reports of using ovine mRNA [21] and bovine mRNA [22] on the Affymetrix Bovine Genome Array.

While the cell isolation procedure used in this study has been previously described in sheep and shown to result in cultures of microglia [10], the transcript levels of five genes commonly used to identify microglia were analyzed to further characterize the cells. Toll-like receptor 4, CD68, and cathepsin K were detected in all technical replicates for each of the six biological groups. CD14 and CD163L1 were detected in the majority of technical replicates for each of the biological groups. The possibility of significant contamination of the cultures with astrocytes and endothelial cells was further excluded by the lack of transcripts for glial fibrillary acidic protein and von Willebrand factor, respectively. Based on these transcript features, the primary cell cultures were classified as uniformly sheep microglia and it was demonstrated that the Bovine Genome Array provides useful data from ovine samples.

Identification of differentially regulated genes associated with PrP^{Sc}

To determine which transcripts were significantly upregulated or downregulated in PrP^{Sc}-accumulating microglia, the array data were subjected to statistical analysis with threshold values for significance defined as a Benjamini and Hochberg (BH) corrected $P < 0.05$. Using this criterion, nineteen genes were significantly upregulated in PrP^{Sc} accumulating microglia (Fig. 1A, Supplementary Table 1). Of these 19 genes, six had BH corrected $P < 0.01$. The maximum fold upregulation was observed for platelet factor 4 (PF4, CXCL4), which was upregulated 2.2-fold. Only one other transcript, milk fat globule-EGF factor 8 protein (MFGE8, lactadherin), was detected with at least a twofold increase. Thirty transcripts were considered significantly (BH corrected $P < 0.05$) downregulated in the PrP^{Sc} treated group (Fig. 1B, Supplementary Table 2). None of the transcripts in the PrP^{Sc} treated group were decreased more than twofold.

Functional categorization of differentially regulated genes

To functionally categorize the differentially regulated transcripts and identify patterns in the cellular response to PrP^{Sc} accumulation, the gene ontology (GO) categories were clustered to select GO parent categories (nodes) and then graphed as the percentage of probe sets within a node (Fig. 2). Although there was a slight trend towards microglial activation (cytokine activity, chemotaxis, cell communication, proteolysis in the upregulated transcripts), no definitive pattern of microglial response is evident.

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 [23]. The complete pathogenesis leading to this neurodegeneration and limited inflammation is poorly understood. Previous studies investigating the transcriptional response of murine neuronal cells have yielded conflicting results [4-7], and it can not be assumed that the ovine microglial response would be similar to the murine neuronal response. Thus, to further understand the microglial responses to PrP^{Sc} accumulation in a natural host we investigated the transcriptional response of primary ovine microglia to determine if microglia have a limited response to PrP^{Sc} accumulation.

The transcriptional response by the primary microglia was limited with only a total of 49 of approximately 13,000 transcripts being differentially regulated with relatively small magnitudes of change (largest absolute value fold change = 2.2) and no distinct pattern of activation. This is markedly different than what is found when microglia or macrophages are activated in culture either by lipopolysaccharide (LPS) [24] or when infected by viruses [25] or bacteria [26], in which 200–600 genes are upregulated with 10-fold to 100-fold changes in transcript levels. In light of the conflicting results from previous studies that investigated the transcriptional response of neuronal cell cultures to PrP^{Sc} [4-7], the limited transcriptional response using sheep microglial cultures support the more recent findings [6,7] and indicate that PrP^{Sc} fails to induce a significant transcriptional response in neuronal cells [6,7] and microglia (current study).

The minimal transcriptional response to PrP^{Sc} in cultured neurons and microglia contrasts with the robust transcriptional response from whole brain tissue in which hundreds of transcripts have been shown to be differentially regulated [27-31]. This discrepancy is likely multifactorial. Contributing factors to this discrepancy include, but are not limited to, lack of multiple cell types and associated cell to cell signaling (e.g. neurons signaling microglia), lack of normal cellular functions in culture conditions, and time of disease progression (often months to years in organismal studies). It is speculated that the *in vivo* transcriptional response likely represents the response to neurodegeneration as much, if not more than, the response specifically to PrP^{Sc}.

While the overall transcriptomic response was limited, there were several specific genes that warrant further study to determine their relevance to prion diseases based on previous prion-based studies, or studies relating to the molecular functions of the transcripts. Three transcripts differentially regulated in the current study (CCL2, SGK1, and AASDHPPT) were differentially regulated in a manner similar to previous reports using rodent-adapted scrapie (CCL2: [27], SGK1: [29,30] and AASDHPPT: [4]). To identify additional transcripts of interest, each differentially regulated gene was searched via Pubmed (July 30th, 2008) and The Database for Annotation, Visualization and Integrated Discovery (DAVID; July 29th, 2008) for associations with Alzheimer's disease (another amyloid plaque neurological disease). It was found that CCL2 [32,33] and MFGE8 [34] were also upregulated in studies of Alzheimer's disease. Interestingly, the MFGE8 gene encodes the lactadherin protein, whose homologue in yeast (SED1) increases heterologous protein secretion [35]. MFGE8 is also present on exosomes of dendritic cells [36] and in the PrP^C enriched exosomes of Mov cells [37]. PrP^{Sc}

is also 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 [38]. Additionally, MFGE8 functions in the phagocytosis of Alzheimer's disease-associated amyloid β -peptide [34]. 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.

Taken together, these data show that like two previous reports using neuronal cell cultures [6,7], but unlike two other earlier reports [4,5], the transcriptional response of primary sheep microglia to PrP^{Sc} is limited both in number of genes differentially expressed and the magnitude of change. This limited response is in stark contrast to microglia and monocyte cultures exposed to LPS [24], viruses [25], and bacteria [26]. It is proposed that this limited response is consistent with the unique pathogenesis and limited inflammation of prion diseases. Additionally, based on previous information concerning CCL2 and MFGE8, further investigation into the specific role(s) these genes play in the pathogenesis PrP^{Sc} accumulation is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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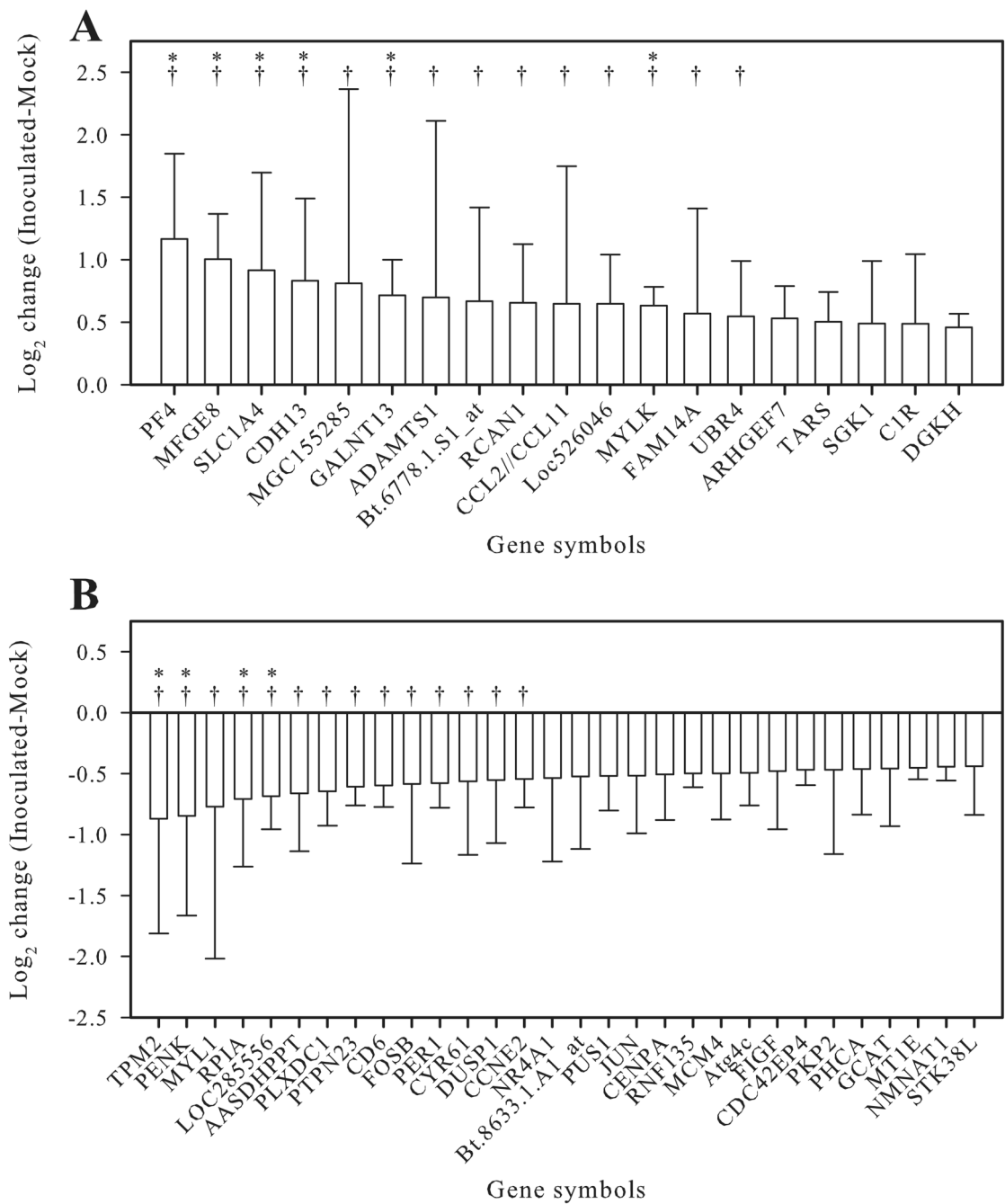


Fig. 1. Transcripts upregulated (A) and downregulated (B) in PrP^{Sc}-accumulating primary sheep microglia as compared to mock-treated microglia. Error bars represent one standard deviation based on the three pairwise comparisons between biological replicates; * = BH corrected $P < 0.01$, † = fold change greater than 1.5.

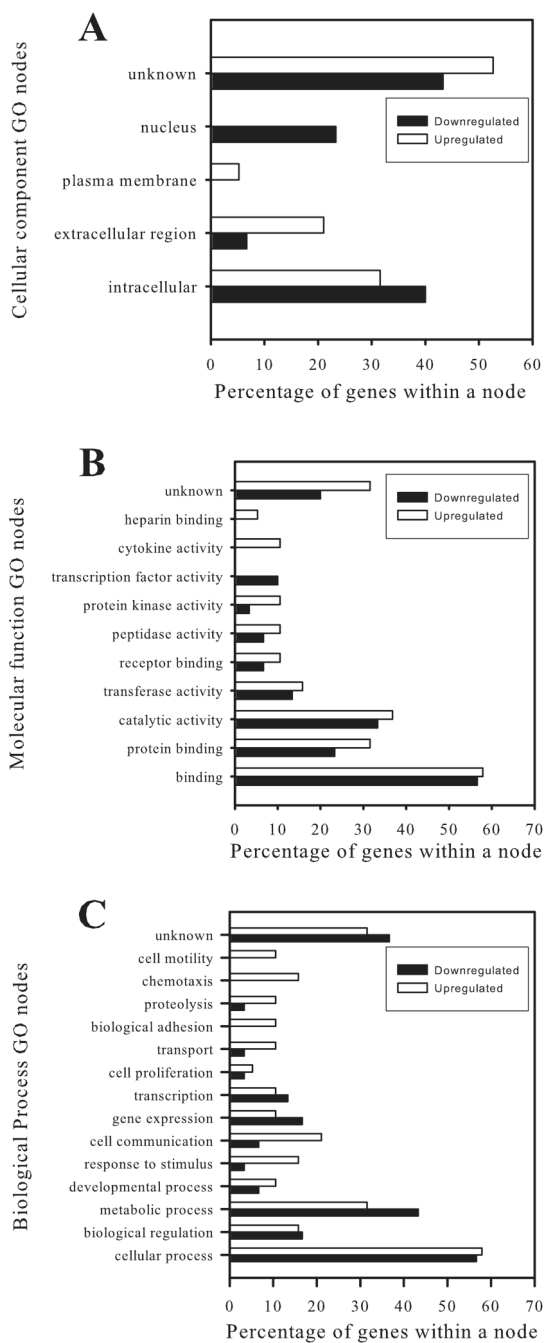


Fig. 2. Functional categorization of differentially expressed transcripts using gene ontology (GO) annotations. Differentially expressed transcripts were annotated to GO categories for cellular component (A), molecular function (B), and biological process (C) using NetAffx. Transcripts without functional annotations are listed as unknown.

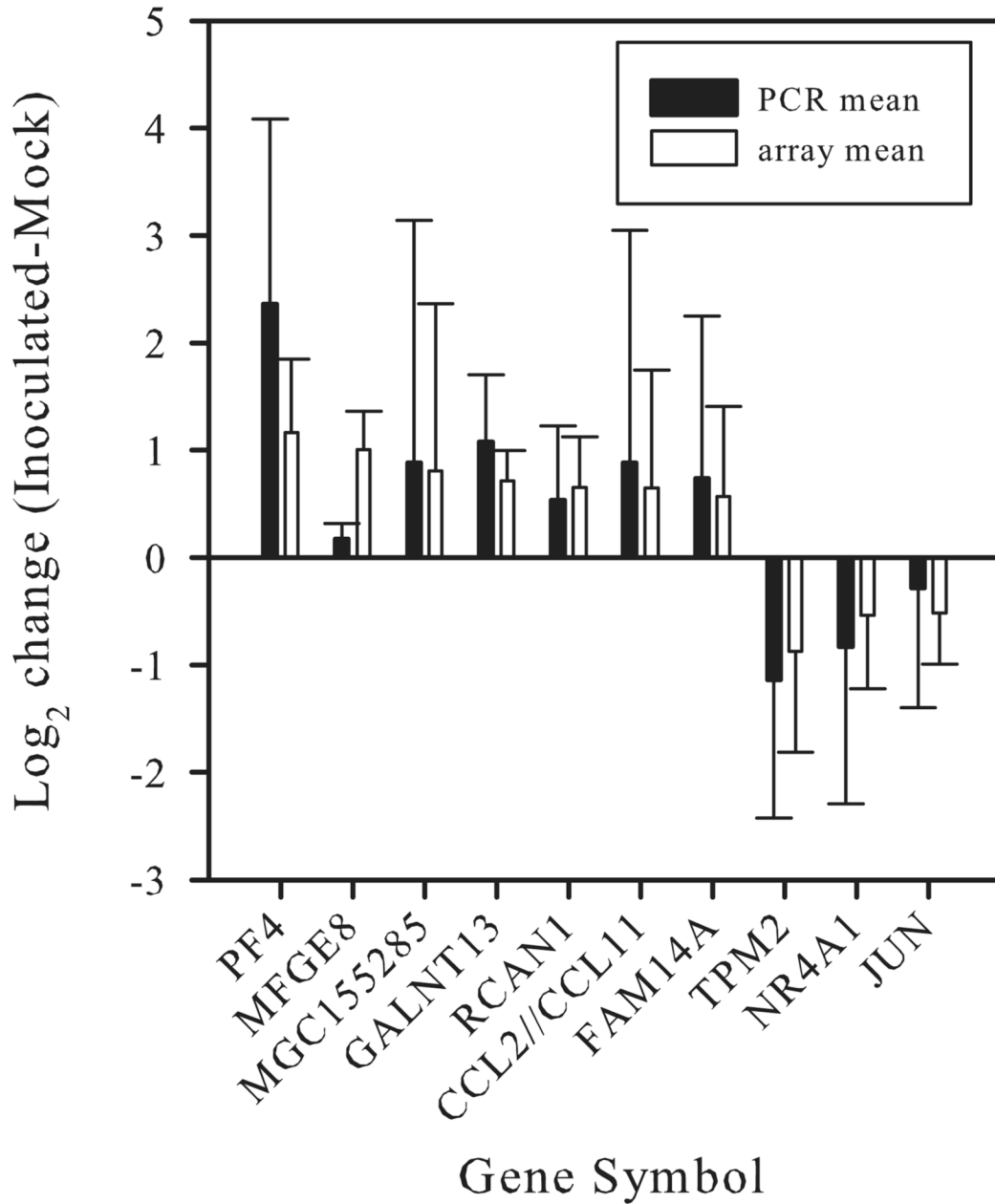


Fig. 3.

Verification of microarray analysis by quantitative RT-PCR (qRT-PCR). qRT-PCR results for each gene were normalized to GAPDH, and the log₂ 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.

Table 1

qRT-PCR primer information

Gene Symbol	Forward primer sequence	Reverse Primer Sequence	Amplicon size (bp)	Reference sequence
PF4	AATGCGTGTGCCTGAAGACCACTT	TTCCTGCTTTCAGCGTAGCTATCA	159	P30035 ^a
MFE8	TCTCTGGCACCCAGTCTCAA	AAAGTCAACCCAGGCTCTGTACTT	107	NA
GALNT13	TAGACAACAATGGGCCGCAAGGAAA	ATCCAAGCACAAATGTCATCGGTTCCG	125	NA
RCAN1	ATTACACCTGTTGTGCACCTTGGG	ATTAAATGGGAAAAGGCACATGCACCC	184	AY205232
CCL2	AGAAGATCACACCACAGCAAGTGT	TTGAGATGGTTTATGGCGTCTCTGG	119	[39]
FAM14A	AAGCAACCAAAAGGGACCACATCAG	AGATTCTCTGTTCTGGTGAAGCCCA	175	BN000255
MGC155285	GTTTGGGCTCAGCAAATCCACTGT	CTGCTCCTTCGGATTAAAGGTTAC	132	NA
TPM2	ACCGAGCTATGAAGGATGAGGA	CTCTCCTTCCAGGATCACCAAGTTT	131	NA
NR4A1	CACACACACCCCATGCTGTAAAT	AAGGAGGCAGGAAAGAGAGCAAGTA	138	NA
JUN	AACAGGTGGCCAGCTTAAACAGA	TTGCAACTGCTGCGTTAGCATGAG	80	NA

^a A protein sequence reference is supplied for PF4 as there is no nucleotide sequence available for ovine PF4. The reference protein sequence was manually aligned with the bovine sequence to determine areas of amino acid similarity, and inferred nucleotide similarity. NA, not applicable and microarray probe sequences used to design primers.