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Award Number: DAMD17-03-1-0321

TITLE: Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

PRINCIPAL INVESTIGATOR: George A. Carlson, Ph.D.
Leroy E. Hood, M.D., Ph.D.

CONTRACTING ORGANIZATION: McLaughlin Research Institute
Great Falls, MT 59405

REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual (1 May 2004 - 30 Apr 2005)

4. TITLE AND SUBTITLE
Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

5. FUNDING NUMBERS
DAMD17-03-1-0321

6. AUTHOR(S)
George A. Carlson, Ph.D.
Leroy E. Hood, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
McLaughlin Research Institute
Great Falls, MT 59405

E-Mail: gac@po.mri.montana.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The first goal is to identify changes in mRNA in blood cells and in serum glycoprotein concentrations that are induced by prion infection in mice. Using a panel of mouse strains and genetically modified mice inoculated with two different prion strains we have found 57 genes that are differentially expressed in the brains of four host-agent combinations. Hundreds of genes are differentially expressed in any single host-agent combination, illustrating the power of analyzing multiple combinations. Some of these genes are differentially expressed as early as 4 weeks post-inoculation, well before signs of neurological disease are apparent, making them candidates for early diagnostic markers. One of these early genes is differentially expressed in blood cells. Application of a novel mass spectrometry-based proteomics technology is underway to identify quantitative changes in serum glycoproteins resulting from prion infection. The second goal is to determine whether CNS stem cells can provide an in vitro model for prion infection. We have successfully infected cell lines from transgenic and non-transgenic mice with scrapie prions, providing the ability to analyze the genetics of prion susceptibility in cell culture. These results also offer the possibility of an in vitro bioassay for prions.

14. SUBJECT TERMS
Prion, diagnostic, mice, RNA, gene expresión, blood, cell culture

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Annual Report 2
DAMD17-03-1-0321
Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

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Early Host Responses to Prion Infection: Development of In Vivo and In Vitro Assays

George A. Carlson, Ph.D., Principal Investigator
Leroy E. Hood, M.D., Ph.D. Co-investigator

INTRODUCTION

A misfolded form of prion protein (PrP^{Sc}) is the functional component of infectious prions and is derived posttranslationally from a benign cellular isoform of unknown function (PrP^{C}). A long incubation time is a hallmark of prion disease and during this preclinical phase of infection prion replication occurs without any obvious harm to the organism or to the infected cells. Advances in genomics and proteomics provide the opportunity to search for specific patterns of change in gene and protein expression that occur after prion infection. We hypothesize that a specific constellation of changes in mRNA and protein expression will prove to be a more sensitive indicator of prion exposure than current assays exclusively focused on PrP. We are measuring changes in mRNA in brain, spleen, and blood cells and in serum glycoprotein concentrations that are induced by prion infection in mice. A prion-specific signature would form the basis for a blood-based screen for prion infected individuals. We also have had success in developing mouse CNS stem cell-containing neurospheres as genetically tractable models for prion infection. Neurosphere cultures are capable of supporting prion replication and may serve as test systems for infectious prion particles from a variety of species.

BODY

Progress towards completing each specific sub-task in the Statement of Work are indicated in **bold type** below:

Task 1. Determine whether there are specific changes in mRNA and protein expression profiles in the blood of prion-infected mice. (Months 1-30)

a. Expand our colonies of mice to provide sufficient numbers of C57BL/6J, B6.I-1, FVB-*Prnp*^{tm1}, (FVB x FVB. *Prnp*^{tm1})F1, FVB/NCr, and FVB-Tg(MoPrP-A)B4053 to provide sufficient numbers for the experiment. There will be 14 groups of mice with 20 mice per group—a total of 280 mice. (Months 1-6) **Successfully completed FY 01.**
b. Establish reproducibility of mRNA isolation from blood cells and glycoproteins from serum using 10 inbred C57BL/6J mice. (Months 1-6) **Successfully completed FY 01.**
c. Inoculate these genetically defined mice with one of two different prion strains and analyze at regular intervals (7, 14 or 28 days) throughout the pre-clinical incubation period and after clinical signs appear. (Months 6-24) **In progress, nearing completion.**
d. Perform DNA microarray and serum glycoprotein analyses, analyze the data and determine whether there are expression profiles unique to each host-agent combination and/or whether a genotype-independent and agent-independent profile specific to prion exposure can be detected. Each timepoint will consist of 4 replicates

per group. (Months 6-36) **In Progress. Problems with reproducibility led us to switch from custom arrays to Affymetrix chips and to include analysis of brain and spleen in addition to blood. Differentially expressed genes common to multiple mouse strain-prion strain combinations have been identified.**

e. Changes in mRNA expression suggestive of specificity for prion infection will be re-evaluated using larger volumes of blood pooled from relevant groups of mice. Up to 200 mice will be available for this purpose. (Months 12-36) **In Progress. We also have begun fractionation of blood cells to enhance our ability to detect differentially expressed genes in different subsets of cells.**

Task 2. Using existing mouse neurosphere lines, determine whether CNS stem cells can provide an in vitro model for prion infection.

1) Import and establish cultures of mouse CNS neurosphere lines provided by our collaborators at StemCells, Inc. and the Salk Institute. (Months 1-2) **Successfully completed, FY 01.**
2) Assess levels of PrP^C expression by immunoblotting and immunofluorescence in the neurosphere lines in comparison to N2A cells that can be infected with prions. (Months 2-8) **Successfully completed, FY01.**
3) Infect PrP^C-expressing neurosphere lines in culture with RML scrapie isolate. Assess viability, proliferation and differentiation at each passage (approximately every 2 weeks). Harvest cultures, prepare protein isolates and determine whether proteinase K-resistant PrP^{Sc} increases over the amount present immediately after infection. (Months 6-24) **Successfully completed. We have expanded this aspect of the project and produced neurosphere lines from the same mouse strains used for our global gene expression analysis of prion-inoculated mice.**
4) Determine whether prions are propagated in infected neurosphere cultures by incubation time analysis in mice. Up to 250 mice will be available for these studies. (Months 12-36). **Completed for prion-infected neurospheres from Tg4053 mice. These cells produce prions that are transmissible to Tg4053 and FVB/N mice. These studies are continuing.**
5) Using mRNA expression profiling and quantitation of secreted or released glycoproteins identifies a subset of prion-specific changes identified in blood. (Months 18-36). **Recently initiated.**

Task 1. To identify a gene expression signature specific to prion-infected individuals, we used Affymetrix Genechip Mouse Genome 430 2.0 arrays to search for changes in mRNA expression that showed overlap in different mouse strain-prion strain combinations. A summary of our results is presented in Figure 1. The differentially expressed genes (DEGs) in brain that are common to all host-agent combinations are candidates for prion disease-specific markers. However, most of the genes expressed after disease is apparent in the mouse reflect the astrocytic gliosis accompanying the spongiform change; similar results have been seen by others. Unfortunately, DEGs reflecting gliosis and neurodegeneration are not unique to prion disorders and, for

example, there is considerable overlap between prion DEGs and DEGs in mouse models for Alzheimer's disease. However, the combinations of genes expressed in each model could be unique.

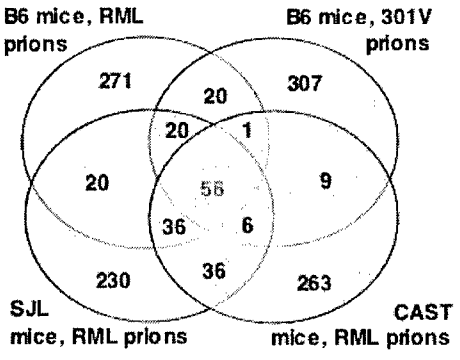


Figure 1. Differentially expressed genes in prion disease: analysis using three mouse strains and two prion strains. The four ovals represent differentially expressed genes in brains from each of 4 mouse strain-prion strain combinations when disease was present. Incubation times differ for each host-agent combination. RML = Rocky Mountain Lab isolate of mouse-adapted scrapie, 301V = Mouse adapted BSE. Numbers show differentially expressed genes in each combination and in overlapping sets. The genes of greatest interest as potential markers for disease are the 56 (indicated in red) that are differentially expressed in all 4 combinations.

Genes expressed during the preclinical phase of the incubation period are more likely to have value as components of a disease-specific signature. Figure 2 illustrates a time course of differential gene expression in brain following infection of SJL/J mice with the RML isolate of scrapie prions. The DEGs can be divided into three clusters, those differentially expressed late in the incubation period (panel A), regulatory genes whose expression changes early (panel B), and early responding homeostatic genes (panel C).

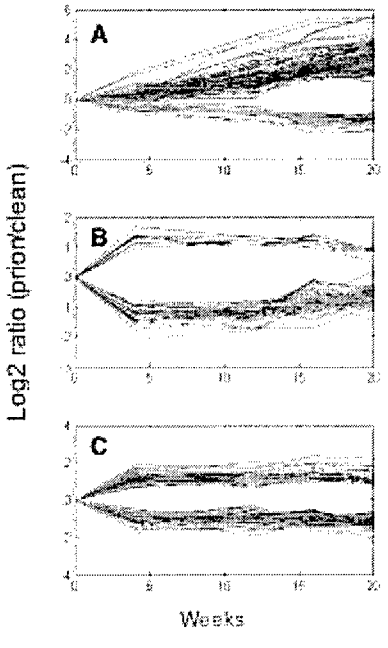


Figure 2. Time course of differential gene expression in the brains of SJL/J mice following inoculation with RML scrapie prions. Log_2 of the difference in expression between mice inoculated with RML isolate and those inoculated with a normal brain homogenate are shown for samples (three mice each) analyzed at 4, 12, 16, and 20 weeks post-infection. Panel A indicates genes differentially expressed late in disease, Panel B indicates regulatory genes with differential expression early in disease, and Panel C indicates homeostatic early response genes.

Similar clusters of up-regulated and down-regulated genes were also seen in a parallel time course with RML inoculated CAST/Ei mice, which have longer incubation times than SJL/J mice. Of the genes differentially expressed early during the course of infection only 9 were shared between SJL/J and CAST/Ei mice. Ongoing studies will determine whether these same genes are shared among other mouse strain-prion strain combinations. Several of these genes, including *C3ar1* (complement 3a receptor 1), *C1qb* (complement component 1qb), and *H2K1* (histocompatibility) likely reflect the onset of astrocytic gliosis and neurodegenerative changes. The roles of others

(*Cyst7*, *Cebpa*, *Lgals3b*, *Ctsz*, *Ptprc*, and *Ccl6*) in prion replication or neurodegeneration are unknown. *Ccl6* (chemokine ligand 6) is differentially expressed in the blood cells of clinically ill mice; experiments are underway to determine whether early changes in *Ccl6* are included in prion-specific DEG signatures detected in blood.

Dr. Inyoul Lee, who is Dr. Hood's lead on this project at the Institute for Systems Biology is also making excellent progress on looking at differential expression at the protein level in plasma. Figure 3 shows a four-fold difference in expression of a glycosylated protein in prion-infected mice.

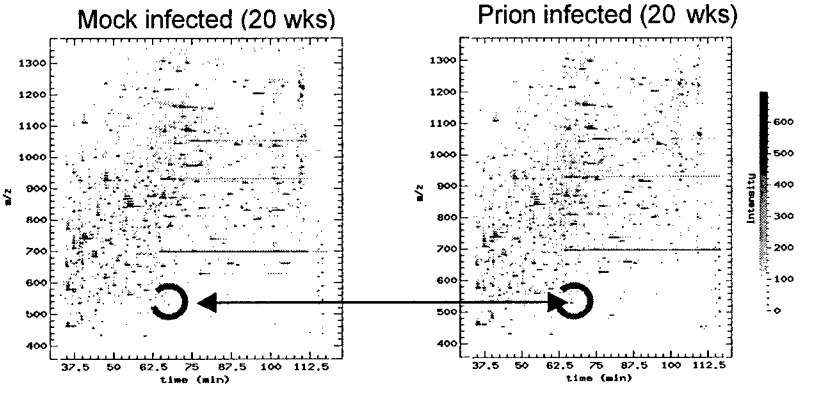


Figure 3. Differential expression of a plasma protein in prion-infected versus uninfected C57BL/6 mice. Results from Pep3d images (2-D density plots) of two LC-MS data sets of plasma gycopeptides are shown.

Task 2. We have established neurosphere lines from several strains and transgenic lines of mice. A prerequisite for infection of neurosphere cultures with prions is that they express PrP. Figure 4 illustrates immunofluorescent staining with anti-PrP and anti-nestin (a marker present on CNS-stem cells) of cultures from FVB-Tg(MoPrP-A)4053 transgenic mice, which overexpress PrP, and from FVB.129-*Prnp*^{0} mice, which lack PrP. In our experiments to infect CNS-SC in vitro, neurosphere cell lines from FVB/N, FVB-Tg(Mo-PrP)4053, and FVB.129-*Prnp*^{0} micewere exposed to 30 μl of a 1:10 dilution of a 10% brain homogenate from mice clinically ill from the RML scrapie isolate. As detected by the presence of PK-resistant PrP^{Sc} in cell blots, dot blots, and western blots, the inoculum persisted for 2 weeks, even in the *Prnp*^{0} neurospheres (data not shown). By 5 weeks after infection, increased PrP^{Sc} was clearly evident in the PrP-overexpressing transgenic line Tg4053; PK-resistant PrP^{Sc} persisted in the FVB cells, but was undetectable in cells from *Prnp*^{0} mice. As shown in Figure 5, clear replication of PrP^{Sc} in two independent cultures of Tg4053 cells was clearly evident 119 days and multiple passages after infection. Importantly, cell lysates from these cultures transmitted disease to Tg4053 mice with incubation times of 75 and 80 days for 1:10 and 1:100 dilutions and to normal FVB/N mice with 158 and 171 day incubation times for the same dilutions. It is clear that neurosphere cultures produce infectious mouse prions. The misfolded PrP^{Sc} isoform appears to accumulate as granular deposits within the cytoplasm of infectedcells, but additional work is needed to confirm this.

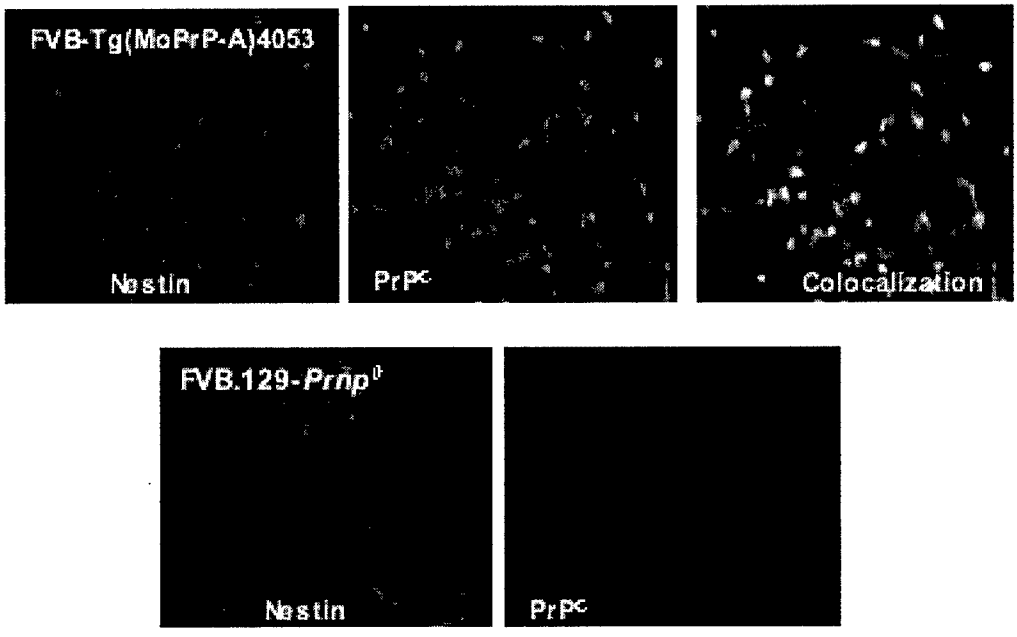


Figure 4. Expression of PrP^C in CNS-stem cell neurosphere cultures from mice expressing PrP. PrP detected with D18 F(ab) is indicated by green fluorescence and nestin is indicated by red fluorescence. The cells were pemeabilized before staining to reveal both membrane and intracellular antigens. The lack of PrP staining in cells derived from FVB.129-*Prnp*^{0} mice that lack PrP demonstrates the specificity of the PrP antibody.

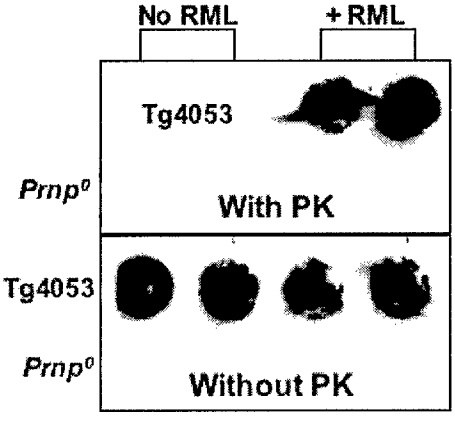


Figure 5. Prion replication in Tg(Mo-PrP)4053 neurospheres 119 days after infection. Two independent Tg4053 neurosphere lines and two lines from *Prnp*^{0} mice were exposed to 30 μl of RML scrapie isolate (see text) for two days; controls were incubated with vehicle. Cells were transferred to nitrocellulose; one filter was digested with PK prior to immunostaining with D18 anti-PrP F(ab).

KEY RESEARCH ACCOMPLISHMENTS

Identified 56 DEGs in brains common to three strains of mice, one of which was infected with two distinct prion strains. Over 500 DEGs ($P < .05$) are found in each individual mouse strain-prion strain combination.

Determined that DEGs are detectable in brain as early as 4 weeks post-inoculation, long before clinical signs of illness are apparent.

Identified 9 genes differentially expressed early after infection shared between the two mouse strains for which complete time course data are available.

Demonstrated differential gene expression in the blood of prion-infected mice using microarray and proteomic approaches.

Produced CNS stem cell lines from the same strains of mice used in our animal studies. Analysis of differential expression in these cell lines may allow discrimination of proteins specific to prion replication from those involved in neurodegeneration in general.

Demonstrated that CNS stem cell (neurosphere) lines can produce proteinase K resistant PrP^{Sc}.

Demonstrated that infected CNS stem cell lines produce infectious prions that can transmit disease to mice.

REPORTABLE OUTCOMES

CNS stem cell/neurosphere lines have been produced from FVB/NCr, FVB-Tg(MoPrP)4053, FVB.129-*Prnp*^{0}, C57BL/6J, B6.I, SJL/J, and CAST/Ei mice.

In addition, some of this work was reported at the Keystone Symposium on Molecular Mechanisms of Transmissible Spongiform Encephalopathies (Prions Diseases) held in Utah, January 11-15 2005 and at the Symposium on Systems Biology held in Seattle in April 2005. The abstract of the Keystone presentation and the poster for the Systems Biology presentation are included with this report.

CONCLUSIONS

Based on our results thus far, it is likely that we will be able to identify a prion specific signature of DEGs in blood prior to the onset of clinical signs. These studies will be greatly aided by our demonstration that CNS stem cell lines can be infected with prions. Neurosphere lines hold great promise as a sensitive tissue culture bioassay for infectious prions from a variety of species, including bovids and humans.

Note: Work on this project began July 1 2003, rather than May 1, 2003, to allow recruitment of the personnel and postdoctoral fellows needed for execution of the project.

Keystone Symposium on Molecular Mechanisms of Transmissible Spongiform Encephalopathies (Prions Diseases). January 11-15, 2005, Snowbird Utah

Dissection of Genetic Susceptibility to Prion Disease *In Vivo* and *In Vitro*

George A. Carlson, Christine Ebeling, William Miller, Anna Gibson, Rajeev Kumar, and Ranjit Giri. McLaughlin Research Institute, Great Falls, Montana, USA 59405

Allelic variants of the prion protein (PrP) gene dramatically affect prion incubation time and disease susceptibility in mice and humans. However, we and others have shown that genes other than *Prnp* also profoundly affect prion incubation time in mice (Stephenson et al. 2000. Genomics **69**:47; Lloyd et al. 2001. Proc Nat Acad Sci **98**:6279). We now report a strong prion incubation time modifier linked to *Prnp* on mouse Chromosome 2 in genetic crosses between C57BL/6J (B6) and MA/MyJ (MA) mice; the amino acid sequence of PrP encoded by these two strains is identical. B6 mice die 143 ± 4 days after inoculation with the RML isolate of murine scrapie, MA mice at 190 ± 5 days, while their F1 hybrids survive to 215 ± 3 days. Survival of F2 intercross offspring ranged from 93 to 267 days after prion inoculation. Quantitative trait analysis of age at death revealed highly significant linkage (LOD = 6.77) to an interval on Chromosome 2 that contains the *Prnp* gene. Levels of PrP or its regional distribution in the brain can affect prion incubation time, but other genes in this genetic interval might also influence prion disease susceptibility. For example, mutations in the Attractin gene, located near *Prnp*, cause spongiform degeneration (He et al. 2001. Nature Genet **27**:40). The mechanisms responsible for genetic modulation of prion disease by *Prnp* or by other genes have been difficult to address given the lack of genetically tractable *in vitro* models. We have established neurosphere/CNS stem cell lines from various inbred strains and transgenic lines of mice. These cultures are susceptible to prion infection, providing a new system for mechanistic analysis of the bases for genetic differences in prion susceptibility.

Supported by NINDS program project grant NS419997 and by a grant from the National Prion Research Program of the U.S. Department of Defense.

On following page, poster from:
Systems Biology Symposium, 14-15 April, 2005, Seattle Washington

The Prion Study at ISB

Inyoul Lee, Daehee Hwang, Eugene Yi, Brianne Ogata, David Baxter, Hyuntae Yoo, Hui Zhang, Shizhen Qin, Doug Spicer*, Rajeev Kumar*, George A. Carlson*, and Leroy Hood

Institute for Systems Biology, Seattle, WA. *McLaughlin Research Institute, Great Falls, MT.

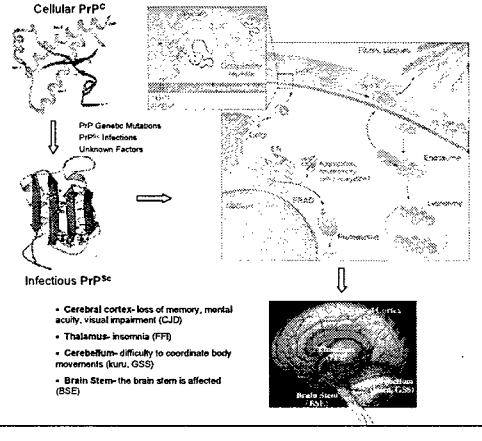
Abstract

We present a network-based framework that can identify potential non-invasive diagnostic markers in any disease by integrating molecular signatures detected at different cellular levels (mRNA, proteins and their interactions) across multiple tissues and the circulation system. This approach was demonstrated using prion infected mouse. We harvested brain, spleen, blood cells and measured mRNA levels to identify differentially expressed genes (DEGs) between infected and healthy mice in these tissues. Also, plasma protein expressions were profiled using LC/MS and LC/MS/MS analyses to identify differentially expressed proteins (DEPs). These distinct molecular signatures were integrated by developing network models for individual tissues, and these networks were then combined with DEPs detected in plasma. The results show that genes/proteins commonly detected as DEGs/DEPs across tissues and plasma play critical roles in determining prion-related disease phenotypes. Also, we observed several important module communications to achieve specific functions in brain, such as neurogenesis, neurodegeneration, and immune response. These network-driven hypotheses are being validated.

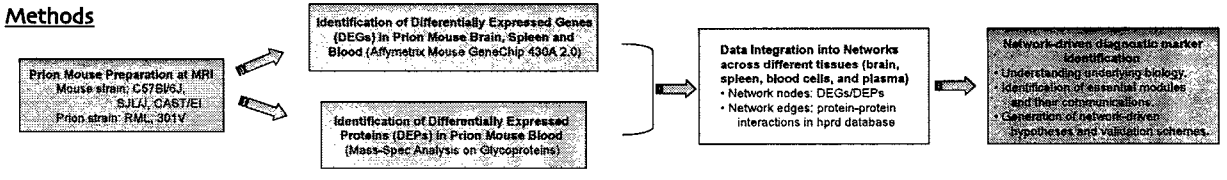
Background

'Prion' is an infectious protein responsible for several neurodegenerative diseases found in mammals, including humans. The most common form of prion diseases is scrapie, found in sheep and goats. Afflicted animals lose coordination and eventually become so incapacitated that they cannot stand. The other animal prion diseases are transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, and bovine spongiform encephalopathy (BSE, better known as mad cow disease). One of the human prion diseases is Creutzfeldt-Jakob disease (CJD), a fatal neurodegenerative disease, which can lay dormant for decades without being detected. The most common subtype CJD usually affects older people (1 case per million per year) and characterized by a rapidly progressive dementia. In contrast to classical CJD, new variant CJD affects predominantly young people. It is generally thought to be caused by the consumption of BSE infected products and first ten cases were identified in 1996, approximately ten years after the outbreak of the BSE in the UK.

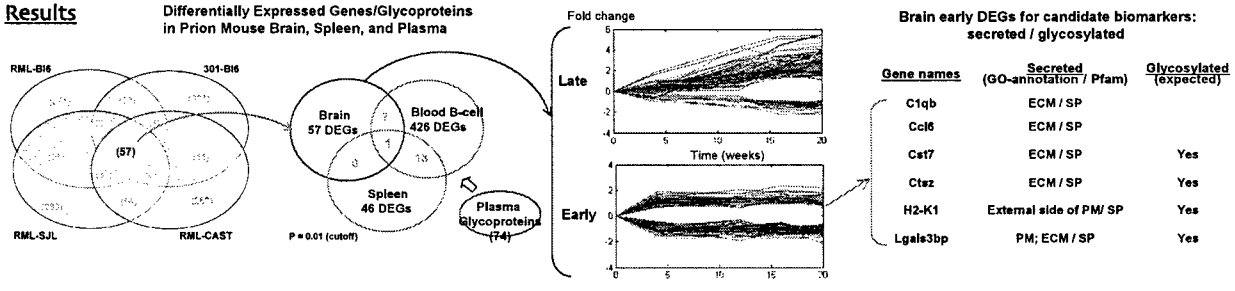
Currently, prion disease is diagnosed by clinically and confirmed by post-mortem histopathological examination of brain tissue. The only reliable molecular marker for prion diseases is PrP(Sc), the pathological conformer of the prion protein that accumulates in the central nervous system and, to a lesser extent, in lymphoreticular tissues. New diagnostic techniques aimed at increasing sensitivity and specificity of PrP(Sc) detection in body fluids and at identifying novel surrogate markers are under development in many groups. Therefore, we are aiming to identify a set of diagnostic and prognostic markers using a network-based approach in a prion mouse model.



Methods



Results

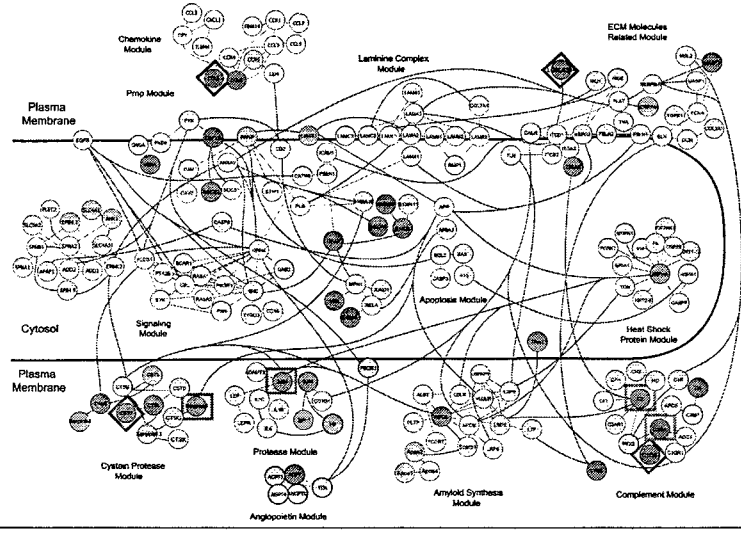


Brain early DEGs for candidate biomarkers: secreted / glycosylated

Gene names	Secreted (GO-annotation / Pfam)	Glycosylated (expected)
C1qb	ECM / SP	
Ccl6	ECM / SP	
Cst7	ECM / SP	Yes
Ctsz	ECM / SP	Yes
H2-K1	External side of PM/ SP	Yes
Lgals3bp	PM; ECM / SP	Yes

Gene Expression Profile & Glycoprotein : Mouse models of prion disease from combination of two prion strains(RML and 301) and three mouse strains (Bl6, SJL, and CAST) were analyzed for their gene expression profile and plasma glycoproteins. Among 57 common differentially-expressed genes (DEGs) in mouse brain, 25 genes were differentially expressed at early time points as can be seen in the bottom graph with clustering gene expression levels along the time points from 4 weeks to 20 weeks. Among them, 6 genes/proteins have been determined to be secreted, and 4 of them is expected to be N-glycosylated. Thus, these genes/proteins are good candidates for early diagnostic markers in serum for prion disease by glycoprotein-isolation and mass spectrometry techniques. In our preliminary study on mouse plasma glycoproteins, 74 glycoproteins were successfully identified.

Network Modeling: We developed a network model to understand underlying relationships among differentially expressed genes identified in both brain and spleen using protein-protein interactions in hprd database (www.hprd.org). In the networks, genes increased, decreased, and not changed in expression levels were denoted in red, green, and yellow, respectively. Also, proteins involved in the same functional pathways were clustered as modules, each of which was represented by either purple or green background depending on its overall change in expression levels. Finally, four overlapping genes/proteins in brain and plasma were denoted by purple squares while four of the early differentially-expressed genes/proteins that are also secreted were denoted by green diamonds.



Brain Network:
Network analysis allowed us to identify:
- known prion-related modules (prion, laminine, heat-shock protein modules);
- new modules potentially related to prion disease, such as complement, chemokine, cystein protease, protease, and amyloid synthesis);
- several module communications to achieve 1) neurogenesis (laminine, S100, Gfap, Vim); 2) neuro-degeneration (Amyloid, protease, heat-shock protein); and 3) immune response (complement, chemokine, apoptosis)