Spring 2010

Effect of Membrane Cholesterol Levels and Allelic Variation on Prion Conversion

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Effect of Membrane Cholesterol Levels and Allelic Variation on Prion Conversion

Submitted in partial fulfillment of the requirements for graduation with honors from the Department of Natural Sciences at Carroll College, Helena, MT

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May 5, 2010
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May 5, 2010
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Abstract

Chronic Wasting Disease (CWD) is caused by an accumulation of misfolded prion proteins (PrP\textsuperscript{sc}) and subsequent plaque formation in the central nervous system. CWD is horizontally transferable; misfolded prions from one animal can enter another and cause normal prion proteins (PrP\textsuperscript{c}) to misfold. This misfolding process is termed prion conversion.

In natural deer populations an allelic variation in the prion gene is thought to confer resistance to CWD. Wild type mule deer are serine (S) homozygotes at codon 225. Mule deer that are Serine/Phenyalanine (S/F) heterozygotes exhibit resistance to prion infection. In this study the F encoding allele was cloned into a bacterial vector. From this stage a recombinant virus can be made and used to determine the biochemical mechanisms responsible for disease resistance.

A further area of study focused on membrane microdomains or cholesterol rafts. Prion proteins are concentrated to these lipid raft regions. This may affect the conversion process. Using insect cells cultured in cholesterol depleted medium, lipid raft reduction occurred. Conversion assays can now be performed on cholesterol depleted cells to determine if lipid raft reduction influences prion conversion.
Introduction

The causative agent of Chronic Wasting Disease (CWD) is believed to be a misfolded prion protein (PrPsc) (Spraker et al. 1997) that is then capable of misfolding the normal prions (PrPc) of the host (reviewed in Prusiner 1991). The normal prion has no confirmed functions, but the misfolded protein is capable of causing plaques in brain tissue and neuron cell death (DeArmond et al. 1985). Many prion diseases show limited transmissibility, especially in wild populations (Williams and Young 1992). Chronic Wasting Disease, however, shows exceptional horizontal transmissibility within species of wild populations of cervids (Miller and Williams 2003). Mule deer (Odocoileus hemionus) in particular develop copious amounts of misfolded prions within months rather than years after first contracting the disease, and succumb to neurodegeneration (Fox et al. 2006). Furthermore, PrPsc proteins have been detected in the lymph nodes (Fox et al. 2006), pancreas (Fox et al. 2006; Sigurdson et al. 2006), adrenal glands (Fox et al. 2006; Sigurdson et al. 2006), blood (Mathiason et al. 2006), and skeletal muscle (Angers et al. 2006) of diseased O. hemionus.

Effect of an Allelic Variation

The prion gene in O. hemionus is polymorphic, with some forms encoding a serine (S) at amino acid 225 and others a phenylalanine (F). O. hemionus heterozygous (SF) at codon 225 do not show detectable amounts of PrPsc in the central nervous system until 482 days post inoculation, versus 189 days for the more common 225SS genotype.
(Fox et al. 2006). This delay in misfolding kinetics could be due to a decreased production of prion protein, or alternatively, to a change in the shape of the protein.

To determine if the F form is inherently expressed at low levels, or if it resists misfolding, the phenylalanine encoding allele was cloned and inserted into a virus. This virus can be used to infect insect cells in vitro. The amount of protein produced can be compared with that for the previously cloned serine allele. These expressed proteins can subsequently be used to examine their susceptibility to misfolding. Because it is unlikely that this amino acid change will affect protein production or stability, I hypothesized that equal amounts of prion protein would be produced by cell lines with the F allele as compared to those expressing the S allele. I further predicted that prions from cells expressing the phenylalanine allele would be less susceptible to misfolding.

**Effect of Membrane Cholesterol**

The PrP^C protein is anchored to membrane lipid raft microdomains by a carboxy-terminal linked glycoposphatidyl inositol (GPI) anchor (Stahl et al. 1987). Thus, prion proteins tend to be densely concentrated in these regions. Dispersion of these lipid rafts may affect the kinetics of the misfolding event by distributing prion proteins across the cell surface.

These microdomains are rich in membranous cholesterol, and depletion of cholesterol destroys these microdomains (Rothberg et al. 1990). The cholesterol synthesis inhibitor, Lovastatin, has been shown to deplete membrane cholesterol and to reduce the formation of PrP^Sc (Taraboulos et al. 1995). Lovastatin accomplishes this by inhibiting 3-hydroxy-3methylglutaryl-coenzyme A reductase (Alberts et al. 1980). This
also halts the production of isoprenoids (Goldstein et al 1990). Isoprenylation of proteins affects protein-protein interactions, protein-membrane interactions, and numerous other cellular processes (Perez-Sala 2007). So, the misfolding could have been inhibited by a removal of cholesterol and reduction of lipid rafts, or alternatively, by blocking isoprenylation events.

In the present study insect cells will be used to determine if cholesterol plays a role in prion conversion. These cells normally produce isoprenoids, but fail to fully synthesize cholesterol, and are therefore cholesterol auxotrophs (Cleverley et al. 1997). Still they remain viable when cholesterol is depleted from their growth medium (Cleverley et al. 1997). Thus, in this study, two cell lines have been developed, one in cholesterol depleted medium, and the other in medium containing cholesterol. An in vitro conversion assay can then be used to determine the effects of cholesterol and microdomains on PrPsc formation. I hypothesized that reducing cholesterol in the medium of these cells would reduce lipid raft levels, which would correlate with a reduction in conversion to PrPsc.
Materials and Methods

Cloning the Phenylalanine-encoding Allele

A diagram illustrating the cloning strategy is shown in Figure 1.

**Polymerase Chain Reaction:** Genomic DNA from an *O. hemionus* S/F heterozygote was used to amplify the prion gene via the polymerase chain reaction (PCR). Five PCR tubes were made; each contained: 1, 2, 3, 0.1, or 0.05 μL DNA (a gift from Jean Jewel, University of Wyoming), one μL of five picomolar mule deer forward primer (MDF-1) and the same amount and concentration of mule deer reverse primer (MDR-1), and 22.5 μL of Pfx supermix (Invitrogen). The PCR initiation step was 95°C for five minutes. The amplification sequence was 30 cycles of 95° for 30 seconds, 54° for 30 seconds, and 72° for 59 seconds. The termination step was 72° for seven minutes.

- **MDR-1 sequence** 5′- AGA AGA TAA TGA AAA CAG GAA - 3′
- **MDF-1 sequence** 5′ - CAC CAC ACC CTC TTT ATT TTG CAG - 3′

The PCR products were separated using 1% agarose gel electrophoresis. An agarose gel extraction was performed to remove impurities (Millipore Ultrafree-DA Cat #42600) with the following details. To make a 1% agarose gel, seven μL of ethidium bromide were added to 75 mL of modified TAE buffer (made according to manufacturer’s recommendation).

The PCR product recovered from the agarose was precipitated. Nine μL of 3M sodium acetate and 225 μL of 70% ethanol were added to 90 μL of product recovered from the gel extraction. This mixture was incubated for 30 minutes at -20 °C, and spun at 21,130 g for 15 minutes. The supernatant was removed and 500 μL of ice cold 70%
ethanol was added to wash the pellet. The tube was spun at 21,130 g for 10 minutes and the supernatant was discarded. The tube was left open overnight and then 15 µL of sterile, deionized water was added to resuspend the pellet. Five µL of ethanol precipitated DNA, 10 µL un-precipitated DNA, and 8 µL of 1kb Biorad DNA ladder were run on a one percent gel to determine the relative recovery.

Cloning into the pENTR vector: Purified PCR product was then inserted into the pENTR TOPO cloning vector and then used to transform chemically competent E. coli cells (Invitrogen pENTR Directional TOPO Cloning Kit) with the following modifications. Reaction mixture 1 (RM1) contained 0.5 µL of purified DNA, while RM2 used 4 µL. Four vials of competent cells were transformed (3 µL RM1, 1 µL RM1, 3ul RM2, 1 µL RM2). Following heat shock transformation, 84 µL from each tube of cells were spread over 12 LB agar plates containing 50 µg/mL kanamycin. The plates were incubated overnight at 37°C.

Cracking gel analysis of plasmids: To identify colonies containing the gene insert, microfuge tubes containing 400 µL each of LB liquid broth plus kanamycin were inoculated and incubated overnight at 37°C with one E. coli colony per tube. From each tube, 100 µL were removed and spun at 21,130 g for two minutes. The supernatant was removed and the pellet was resuspended in 60 µL of cracking buffer (0.05M NaOH, 0.5% SDS, 5mM EDTA, 0.5 mg/mL bromophenol blue, 0.08 g/mL sucrose). The tubes were placed in a 50°C water bath for 30 minutes and were flicked to mix them every 10 minutes. They were then spun at 21,130 g for 15 minutes to pellet the viscous genomic DNA. The pellet was removed and discarded. Twenty µL of the supernatant from each
tube were then loaded and run on a one percent agarose gel. Samples testing positive for insert containing plasmid were cultured overnight in 7mL of LB-Kanamycin broth.

Restriction digest analysis: Cells from overnight cultures were pelleted by centrifugation at 1380 g. The supernatant was discarded and DNA was extracted from the cell pellet using a Qiagen spin miniprep kit. Ten µL of purified DNA was digested with BamH1 and EcoR1. The digestion mixture was incubated at 37° for 2 hours. Digested samples were then run on 1% agarose gels. Samples testing positive for the F-encoding prion gene were re-cultured overnight in 7mL of LB-Kanamycin broth. In addition, each sample was used to inoculate three extra 7mL cultures.

Prion gene sequencing: Cells cultured overnight were pelleted and DNA was extracted using the procedure above with the exception that the DNA was eluted with sterile water instead of buffer EB. Twenty µL of each sample was then run on agarose gels (1%) and compared to a 100 bp DNA ladder (Gelpilot) to estimate plasmid concentration. Samples pF225RH1 (RH1), pF225RH2 (RH2), pF225RH3 (RH3) had their prion gene inserts sequenced by the Bionexus corporation using M13F and M13R primers. Two forward and two reverse reactions were performed for each sample.

To analyze the data, nucleotide sequences were translated to amino acid sequences using the translation program from the Expasy website. The amino acid sequences were aligned with wild-type O. hemionus prion sequence using the BLAST program (available via the National Institute of Health). A consensus sequence was developed for each of the three clones; figure 5 shows the consensus sequence for RH1.
Generating Cholesterol Depleted Medium

The strategy for examining the effect of cholesterol on prion conversion is outlined in Figure 2. Fetal Bovine Serum (FBS) was depleted of lipoproteins using the following protocol. Four and one half grams of fumed silica were combined with 150 mL of FBS. The mixture was stirred briskly at room temperature until the silica dissolved. It was then left at 4 degrees for 48 hours at a slow stir. The FBS was then centrifuged at 2,357 g for 15 minutes, and the supernatant was filtered through a Buchner funnel with VW&R filter paper #28320-100. The solution was filtered again through a sterilizing filter. Fifty mL of cholesterol depleted FBS, and 5ml of Pen-Strep were added to 445 mL of Grace’s media under sterile conditions. This produced the cholesterol depleted media. Cholesterol containing media was formed in the same way, except the FBS was not depleted of lipoproteins.

Cell Growth and Prion Protein Production

High 5 cells (Invitrogen) were cultured and passaged in either cholesterol containing (CH+) or cholesterol depleted (CH-) media. Three 25 cm² flasks of CH- and three of CH+ cells were subcultured after 45 and 46 passages respectively. Media was removed from each confluent flask and discarded. Flasks were then infected with 20 μL of Baculovirus containing the wild-type prion gene and incubated in 1 mL of the appropriate medium. Flasks were rocked for one hour at RT. Then, two additional mL of media were added to each flask, and they were placed in a 27 C incubator.
Forty-two hours later the cells were harvested by dislodging them in their own medium, and pooling CH+ or CH- into different centrifuge tubes. Cells were pelleted at 1380 g and the media were discarded. The cells were then washed twice with 6mL of phosphate buffered saline (DPBS). After the final spin cells were resuspended in 9mL of PMCA buffer at pH of 7.4 (0.015 g Saponin, 0.3 mL Triton X-100, 0.044 g EDTA, 0.26 g NaCl, and 3 tablets CPI, brought to 30 mL with DPBS). Samples were added to a Tenbroek homogenizer and homogenized with twelve passes. CH+ and CH- samples were then centrifuged for 2 minutes at 1470 g. The supernatants containing isolated CH+ and CH- membranes were stored in 250 μL aliquots at -80C.

Imaging Lipid Rafts

To determine if lipid raft reduction had occurred in CH- cells, a lipid raft labeling kit was used (Vybrant Kit, Molecular Probes). The kit uses recombinant cholera toxin subunit B (CT-B) conjugated to a fluorescent molecule which has a fluorescence excitation maximum of 495 nM and a fluorescence emission maximum of 519 nM. CT-B binds to the pentasaccharide chain of plasma membrane ganglioside (Gm1) and is crosslinked with an anti-CT-B antibody. Because Gm1 is localized in lipid rafts, fluorescently labeled CT-B can be used to effectively mark lipid rafts of living cells. The lipid raft labeling procedure was performed on both CH+ and CH- cells following the manufacturer’s recommendations.

The cells were visualized using a Nikon TE200 fluorescent microscope and a Qimaging QICAM 12bit Mono Fast 1394 cooled camera. The camera was set for a monochrome capture with 8 bit capture depth and fluorescence setting at maximum and
light setting at ½ maximum. A variety of exposures were performed that varied from 8 ms to 3 seconds using 100X magnification. The acquired images were then viewed and magnified digitally with QCapture software to optimize analysis.

Prion Protein Immunoblot

To assess the recovery of prion protein from CH+ and CH- cell membranes, an SDS PAGE/Immunoblot was performed on the membranes isolated from CH+ and CH- cells using the following protocol. Sample A contained 9.75 μL membrane isolate, 3.75 μL NuPAGE sample buffer, and 1.5 μL reducing agent. Sample B contained 3.25 μL of membrane isolate, 6.25 μL sample buffer, 2.5 μL reducing agent, and 13.0 μL DPBS. Samples were boiled for 10 min, loaded on a 12% gel, and run at 200V for one hour in NuPAGE MOPS buffer (upper buffer contained 0.5 mL antioxidant and 200 mL MOPS). A transfer was then made to a PVDF membrane using 30V for 1.25 hr in NuPAGE transfer buffer.

The membrane was then placed in a blocking buffer solution (5% nonfat dry milk in TBST), and rocked for one hour. TBST was at a pH of 7.5 and contained 2.4 g Tris, 8.2 g NaCl, and 1 ml Tween20 per liter of water. Following this, the membrane was washed for one minute in 10 ml TBST, then placed in a Seal-Pak pouch with 4 mL of primary antibody (20 μL of SAF-32 anti-prion monoclonal antibody, and 0.04 g of nonfat dry milk in 4 ml of TBST). The membrane was incubated overnight at 4 C. The following morning, the membrane was washed 3x with 20 ml TBST for ten minutes each. Then the membrane was incubated in secondary antibody for one hour. The secondary antibody consisted of 2 μL alkaline phosphatase conjugated anti-mouse IgG and 0.75 g
nonfat dry milk in 15 ml TBST. A sequence of three, ten minute washes in TBST came next. The membrane was washed a final 2x in 25 mL of assay buffer (Applied Biosystems). Three ml of CDP-Star substrate (Tropix) was added onto the top of the membrane, soaking it for 5 minutes. The finished membrane was subjected to chemiluminescent imaging for four 15 minute exposures using a Kodak Gel Logic 1500 Imaging System.
Results

Analysis of pENTR plasmids following directional cloning

The DNA from two hundred colonies was analyzed in order to ultimately isolate the RH1, 2, and 3 clones. Electrophoresis of DNA isolated using cracking buffer revealed seven samples that electrophoresed at the same rate as a previously cloned plasmid with the serine allele (Fig. 3). This indicated that they were likely plasmids with prion gene inserts. Cells containing these seven plasmids were grown in order to isolate their DNA and analyze it via restriction digestion.

Substitution of F for S at 225 creates an EcoR1 restriction enzyme recognition site, and the pENTR plasmid contains a BamH1 site (Bankhead 2009). Upon treatment with these two enzymes, only plasmids with the F225 prion insert will be cut twice, resulting in two linear fragments. Double digestion of the seven samples identified by cracking gels showed that three of those plasmids had been cut with both EcoR1 and BamH1 producing a characteristic 500 bp fragment (Fig. 4). These three plasmids were designated pF225RH1, pF225RH2, and pF225RH3.

Analysis of the consensus sequence from the four sequencing reactions performed on plasmid RH1 showed that, all RH1 clone sequences differed from the wild-type sequence by only replacing the S at 225 with an F (Fig. 5). Thus, RH1 encodes the F allele and will be used to generate the recombinant virus encoding a prion protein with F at AA225.
Results of Cholesterol Depletion

The following general observations were made concerning CH+ and CH- cells. CH- cells began forming consistent monolayers after fourteen passages in CH- medium. CH+ cells reached this same stage after fifteen passages in CH+ medium. CH+ cells tend to adhere to the flask more tightly than CH- cells. Some CH- cells contain what appear to be lipid droplets within their cytoplasm (Gretch unpublished observations).

Results of Fluorescence Microscopy of Lipid Rafts

Fluorescence microscopy was used to evaluate the effect of cholesterol depletion on lipid raft formation in the insect cells used in these experiments. Lipid rafts were visualized by incubating cells with fluorescent cholera toxin B which binds to the pentasaccharide chain of plasma membrane ganglioside G_{M1} which partitions into cholesterol-rich lipid raft microdomains. Cross-linking the cell surface bound CT-B molecules allows them to be visualized using fluorescence microscopy. Figure 6, panel (a) clearly shows the appearance of lipid raft domains in CH+ cells as evidenced by discrete regions of fluorescence on the cell surface. This punctuate pattern of fluorescence demonstrates that there are well defined regions of CT-B localization suggesting the presence of detectible lipid raft regions that are present when cells are grown in normal cholesterol containing medium.

Conversely, when CH- cells were incubated with the fluorescent CT-B molecules, the pattern of fluorescence was much more diffuse and distributed widely across the cell surface (panel b).
**Immunoblotting.**

Immunoblotting showed that the CH+ membranes had a prion concentration several orders of magnitude higher than the CH- cells (Fig. 7). At very high levels of applied sample, higher MW smearing and minor lower MW degradation products were observed in addition to the three bands corresponding to mono, di, and unglycosylated prion.
Discussion

Allelic Discussion

PCR amplification was an effective method to amplify the prion gene. The MDF-1 and MDR-1 primers ensured amplification of the true prion gene instead of the pseudogene. Furthermore, the primers allowed for directional cloning of the prion gene into the pENTR vector. Agarose gel extraction allowed removal of PCR byproducts that could interfere with the transformation process.

Cracking Gel treatment proved to be an efficient method for high volume processing of plasmid DNA. Most transformed cells contained self-annealed plasmids. With cracking gel analysis these cells could be identified and eliminated from further study. Cracking gel treatment avoided the alternative process of performing DNA extraction with the Quiagen spin miniprep kit on a high volume of samples. This saved both time and resources.

Of the plasmids found to contain an insert, there was an equal likelihood the insert encoded a prion protein with either an S or F allele. This is because the PCR amplification used DNA from an S/F heterozygote. Double digestion with BamH1 and EcoR1 allowed discrimination of plasmids containing F encoding alleles from those with S alleles. This is because the switch from S to F at 225 creates an EcoR1 restriction site.

Four sequencing reactions per clone were needed to ensure that differences were not the result of sequencing errors. Comparing these four sequencing reactions to one another allowed the creation of a consensus sequence. The consensus sequence for the RH1 clone was identical to the wild type except for the switch to F at 225.
Cholesterol Discussion

Maintaining vitality of cells in CH- medium was a major obstacle. Previous attempts failed to culture cells in cholesterol reduced medium. The cause of this was thought to be the use of amphotericin B as an antifungal agent (Gretch unpublished). Amphotericin B binds cell membrane cholesterol, so the depletion of membrane cholesterol from CH- cells could have made them more sensitive to the drug. For this reason both the CH- and CH+ media lacked amphotericin B. Because High 5 cells were able to be transitioned directly into CH- medium and remain viable, it appears that amphotericin B was the cause of the earlier inability to culture viable cells.

Fluorescence microscopy showed a punctuate pattern of fluorescence in the CH+ cells and a diffuse fluorescence in the CH- cells. CT-B conjugated to a fluorescent dye binds to G_{M1}, which localizes to lipid rafts. The variance in fluorescence pattern between the CH- and CH+ cells indicates that growing insect cells in cholesterol depleted growth medium results in a decrease of cell surface lipid rafts. These cholesterol depleted cells therefore will be an effective tool for studying whether decreasing cellular cholesterol (and therefore decreasing plasma membrane lipid rafts) results in variation in prion protein conversion efficiency.

Immunoblotting revealed that membrane isolates from CH+ cells contained a much higher concentration of prion protein than those from CH- cells. This discrepancy could be due to several causes. The first possibility is that more CH+ cells were used for infection and membrane isolation. Alternatively, CH- cells could have reduced
production or lower stability of their prion proteins. Reduced transport of the prion protein to the cell membrane could also produce the results observed. Repeating the membrane isolation and immunoblot in addition to Bradford assays and whole-cell immunoblotting should elucidate the cause of these current results. Bradford assays will show total protein present in CH+ and CH- cultures. Total protein will correspond directly to the amount of cells in each culture. Prion immunoblots on whole-cell lysates will reveal if CH- cells are producing less prion protein or are experiencing reduced transport of prion proteins to the plasma membrane.
Allelic Studies Strategy:

1. **PCR Amplify Prion Gene**

2. Clone PCR product into pENTR vector

3. Transform vector into *E. Coli* cells and isolate plasmid

4. Use cracking gels to identify plasmids with the prion gene.

5. Analyze prion plasmids with restriction digest

6. Sequence plasmid gene from samples that test positive during digest

7. Recombine prion gene from plasmid into a Baculovirus

8. Infect High 5 cells with virus

9. Analyze prion production and conversion through use of Western Blots.
Cholesterol Studies Strategy:

Deplete cholesterol from growth media

Culture Hi 5 cells in reduced cholesterol medium and unreduced medium

Passage cells in appropriate medium for several generations

Use fluorescence microscopy to visualize lipid rafts

Use SDS PAGE/ immunoblotting to analyze the amount of prion protein produced by CH- and CH+ cells

Perform prion Conversion Assay on prions from CH+ and CH- cells
Figure 3 Cracking Gel Analysis. In this representative gel, four samples ran at the same rate as the control, indicated that these samples contained a DNA insert in their plasmid.
Figure 4 Double Digestions. Three of the samples (lanes 6, 7, & 9) show the double bands characteristic of cutting by both BamH1 and EcoR1. The lower band is a 500bp fragment. Production of this fragment by cutting with both enzymes identifies that the plasmid contains the F allele insert.

Lane 1: MW ladder  
Lane 2: Plasmid with S insert (-control)  
Lane 5: Plasmid without an insert  
Lanes 3-4, 6-10: Seven samples being tested
Figure 5

Consensus Sequence

Query is wild-type allele, Subject is RH1

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Figure 6 Fluorescent labeling of lipid rafts: Cells were incubated with fluorescently labeled cholera toxin subunit B (CT-B) which binds to cell surface markers localized in lipid rafts. The CT-B is cross-linked with anti-CT-B antibody to maintain its cell surface location prior to analysis via fluorescence microscopy.

Panel a) : CH+ cells  
Panel b) : CH- cells
Figure 7 Recovery of prion protein from CH+ and CH- cells. An immunoblot comparing amount of prion protein produced by CH+ and CH- cells.

a. 6.3 µL Prestained MW marker (Benchmark)
b. CH+ Sample A
c. CH+ Sample B
d. CH- Sample A
e. CH- Sample B
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Acknowledgments

I thank Dr. Dan Gretch for his knowledge, assistance, and support for the duration of this research undertaking. I thank my readers Dr. Jeffery Morris, and Dr. Murphy Fox for combing this work to ensure grammatical accuracy. I also thank Dr. Gerald Shields for his advice on thesis writing and poster creation. I thank Carroll College for use of the laboratory space, and the E. L. Wiegand Foundation for providing an excellent research facility. This project was supported by NIH Grant Number P20 RR16455-04 from the INBRE-BRIN Program of the National Center for Research Resources, and from a new faculty research award from the Murdock Charitable Trust to Dr. Dan Gretch.