Drexel University

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2014 – December 31, 2014

Formula Grant Overview

Drexel University received $1,401,259 in formula funds for the grant award period January 1, 2013 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role of MeCP2 in Pain and its Regulation by microRNAs – The goal of this research is to understand the role of methyl CpG binding protein 2 (MeCP2) in both normal and aberrant nociception. We hypothesize that the reduced pain sensitivity observed in Rett syndrome (RTT) patients results from a decrease in MeCP2 protein, and that a decrease in microRNAs (miRNAs) that bind and repress translation of MeCP2 will cause an increase in MeCP2 levels and thus contribute to pain. By integrating the two epigenetic mechanisms of DNA methylation and miRNAs, studies described here will render insight on how MeCP2 can bring about global gene regulation in a pain state. Our studies can lead to the identification of novel targets, a better understanding and thus innovative approaches for treating pain.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Decreased pain perception is commonly reported in children with Rett syndrome (RTT), with nearly all cases of RTT caused by mutations in the MECP2 gene. There is a potential relationship between pain severity and specific mutations in the MECP2 gene, with 65% of RTT patients reporting decreased pain and 75% of the patients experiencing abnormal sensitivity. These data from RTT patients suggest normal MeCP2 function is important in modulating pain. This phenomenon of diminished pain perception in RTT patients offers the "proof of concept" for how the methylation marks "read" by MeCP2 protein and the ensuing gene regulatory consequences upon its binding to genomic DNA is critical in pain perception. Several MeCP2 mouse models including those with RTT-associated mutations have been generated.

Note: This project is funded, in part, under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

The objectives of this research are to:
1) Characterize changes in MeCP2 mRNA, protein and miRNAs in DRG from a mouse model of inflammatory pain and naïve T158A knock in (KI) mice
2) Assess mechanical and thermal sensitivity in MeCP2 T158A KI mice at two different ages (pre-symptomatic 6 weeks and post-symptomatic 9 weeks of age) to evaluate pain sensitivity
3) Validate miRNA binding to the 3'UTR of mouse MeCP2 and in vitro confirmation for one chosen miRNA for its ability to regulate endogenous MeCP2 expression
4) Identify regions of the genome differentially bound by MeCP2 in DRG from a pain model and control (or T158A KI and control as an alternate strategy) by ChIP sequencing.

By utilizing rodent models of pain and RTT, we believe our approach can provide mechanistic insight on a) how miRNAs mediate MeCP2 expression in pain and 2) how binding/occupancy of MeCP2 on genomic DNA in DRG bring about gene regulatory consequences mediating downstream gene expression changes resulting in pain. These studies will investigate miRNA mediated regulation of MeCP2 expression and could provide insight on therapeutic utility of miRNAs. Various aspects of previously unexplored avenues in pain research investigated here will increase our understanding of the molecular mechanisms and could lead to better treatment options for patients suffering from pain.

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Expected Research Outcomes and Benefits

Present treatment options for pain are limited to NSAIDS, opioids, anticonvulsants and antidepressants providing relief to only about 50% of RTT patients, clearly highlighting the unmet medical need for such a common and widespread ailment. The pain field can benefit from new approaches rendering novel perspectives on how we treat pain. Though disease states are often the result of altered gene expression caused by mutations within a gene, aberrant epigenetic modifications of the chromatin surrounding the gene can also result in profound changes in gene expression. Epigenetics encompasses heritable alterations in gene expression and chromatin without accompanying changes in the DNA sequence. Epigenetics has been predicted to play a key role in pain and analgesia both in terms influencing pro- and antinociceptive gene expression and in modulating pharmacodynamics or pharmacokinetic properties of analgesics. Rett is an

unusual model to study pain. Our data indicating a miRNA mediated regulation of MeCP2 in rodent models of pain led to the formulation of the hypothesis linking an increase in MeCP2 to pain. Studies during this project will render insight on how a master regulator can bring about global gene regulatory changes under pain state. Our studies also integrate functional consequences of two different epigenetic mechanisms, DNA methylation and miRNA mediated gene silencing underlying pain. By utilizing rodent models of pain and RTT, we believe our approach can provide mechanistic insight on the role of miRNA mediated MeCP2 expression and its role in pain. Binding/occupancy studies of MeCP2 on genomic DNA in neuronal tissue will help elucidate the gene regulatory consequences mediating downstream gene expression changes resulting in pain.

Summary of Research Completed

Aim 1 Characterize changes in MeCP2 mRNA, protein and miRNAs in dorsal root ganglion (DRG) from a mouse model of pain and naïve T158A transgenic mouse.
Progress: We performed miRNA profiling of DRG from Mecp2 null mice and prioritized it over T158A mice and SNI model mice to investigate whether the loss of MeCP2 modulates miRNA expression in DRG. Since we had identified 19 miRNAs to be altered in MeCP2 KO mice, we pursued qPCR for selected miRNAs of interest in SNI model of neuropathic pain (Figure 1). We observed differences in miRNA expression between SNI model in mice and our previously published study in rat model of SNL (spinal nerve ligation) model of neuropathic pain. Species difference, different surgical procedures used in the generation of these models and data normalization methodology could have contributed to this difference.

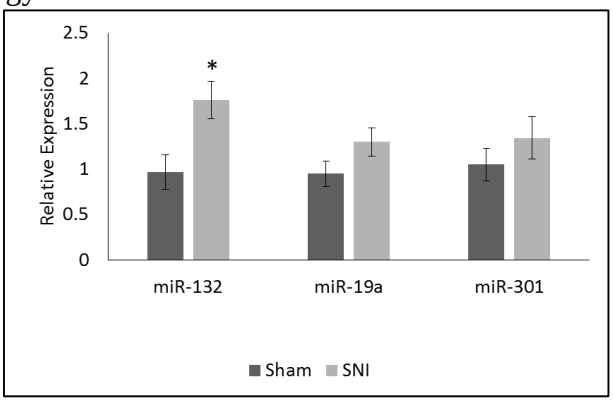
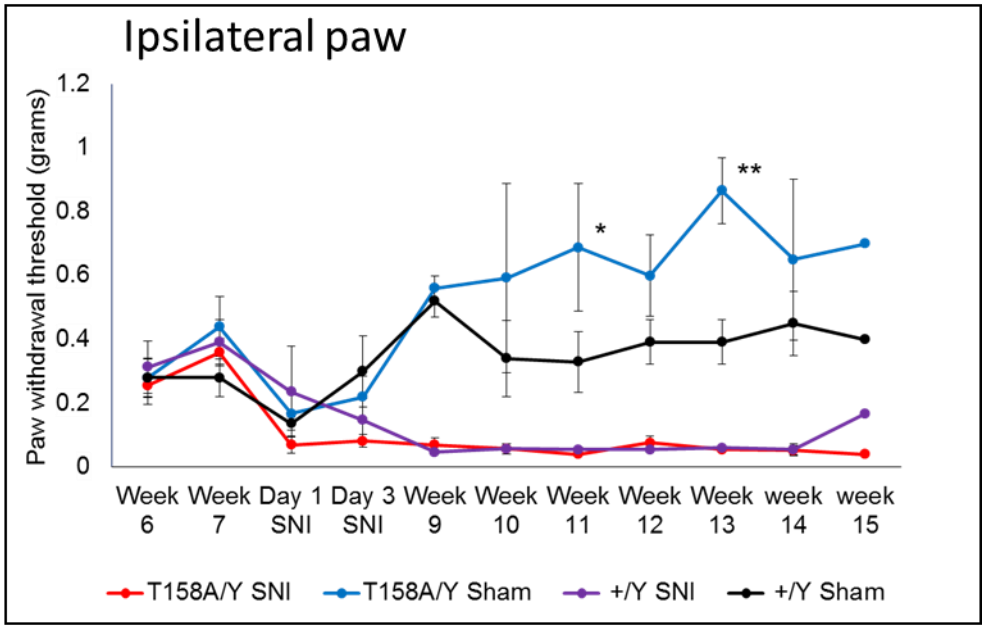


Figure 1 Taqman analysis of selected miRNAs in mice DRG from SNI model of neuropathic pain. Total RNA isolated from DRG four weeks after SNI surgery was used to quantify miR-132, miR-19a and miR-301. U6 was used as the normalizer.

Aim 2 Assess mechanical and thermal sensitivity in MeCP2 T158A KI mice.
Progress: To investigate the role played by MeCP2 in nociception and to characterize the effect of MeCP2 mutation on the development of neuropathic pain, SNI model of neuropathic pain was generated before the development of RTT-like symptoms in Mecp2 T158A mice and in littermate controls. The SNI model was generated as described previously. Briefly, mice were anesthetized under isoflurane; the common peroneal and tibial nerves of the left paw was ligated and sectioned distal to the ligation. The distal nerve stump (2 to 4 mm) was removed. The sham

surgery was identical to that performed on the SNI group, but without ligation. Mechanical sensitivity was measured before and after SNI using von Frey filaments at different time points. We conducted SNI surgery before the development of decreased pain sensitivity to evaluate the role of MeCP2 in the development of pain. Our experiments included the Mecp2 T158A knock-in SNI model and sham surgery controls, and wild-type littermates SNI model and sham surgery controls. Pain behavior was evaluated by comparing the SNI groups with the sham groups and also by comparing the contralateral with the ipsilateral side of the same mouse (Figure 2).

Our study again confirmed that T158A/y sham mice have decreased mechanical sensitivity. However, after inducing nerve injury, the sensitivity on the ipsilateral or injured side of Mecp2 T158A knock-in was comparable to that of the ipsilateral paw of littermate control mice that underwent SNI surgery. This data suggests that the motor function is intact in Mecp2 T158A knock-in mice.



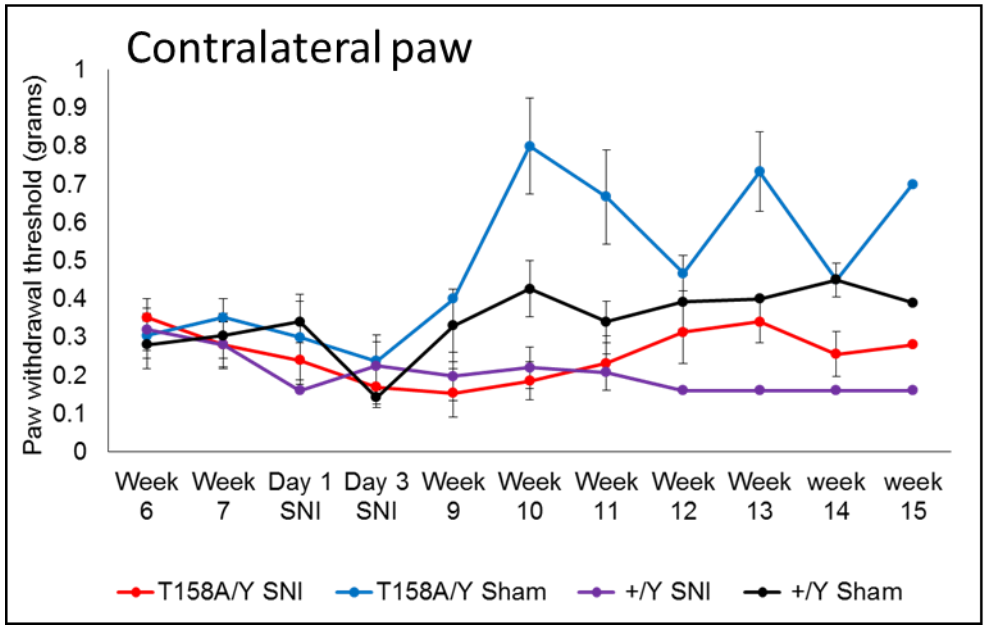


Figure 2 Mecp2 T158A KI mice do not have altered sensitivity compared to wild type mice in the SNI model of neuropathic pain conducted before the development of reduced pain sensitivity. Animals were tested once per week, n=3-5. Statistically significant difference from control was calculated using Student t-test, P value ** <0.01, *** <0.001.

Aim 3a Validate miRNA binding to the 3'UTR of mouse MeCP2 and confirm miRNA alterations can modulate endogenous MeCP2 expression in vitro.
Completed and reported in the previous progress report.

Aim 3b To investigate if miRNAs can regulate endogenous MeCP2 expression, we will perform in vitro overexpression and knockdown studies for one miRNA, potentially miR-132.

Progress: Completed and reported in the previous progress report.
During this period, we investigated the functional consequence of miRNA alterations by studying changes in Bdnf. MeCP2 is a known modulator of Bdnf and it increase in DRG in rodent models of pain. Hence we examined changes in Bdnf transcripts after transfection of Neuro2aa cells with miRNAs miR-132, miR-19a and miR-301. Figure 3 show that these miRNAs significantly reduced Bdnf transcripts 48 h after transfection. We also observed that MeCP2 transcripts were not altered (but the protein decreased significantly; reported in the previous progress report) suggesting that regulation of MeCP2 by these miRNAs can be due to translational repression.

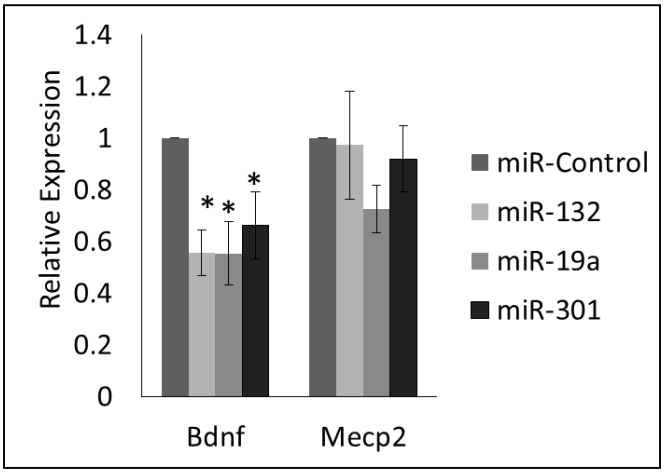


Figure 3 miRNAs targeting MeCP2 can lead to changes in Bdnf but not MeCP2 mRNA. Neuro-2a cells were transfected with miRNA precursor plasmids for 48h (n=3) and qPCR analysis were performed for Bdnf and Mecp2 transcript levels. Data was normalized to Gapdh.

We then extended our studies to DRG from Mecp2-KO mice and confirmed that there is a decrease in Bdnf transcripts in the absence of MeCP2 protein (Figure 4). This decrease in Bdnf could be one of the factors contributing to decreased pain sensitivity in RTT patients and MeCP2 knockout mice.

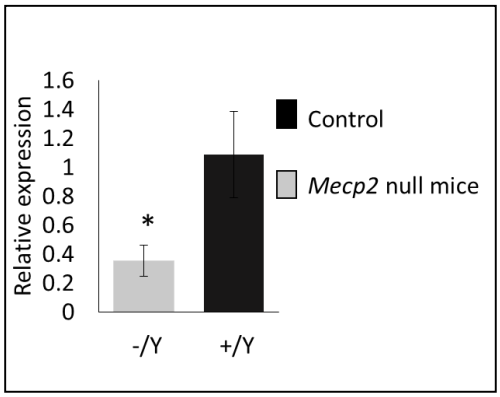


Figure 4 Taqman analysis of Bdnf in DRG from Mecp2 null mice. Data normalized to Gapdh. Statistically significant difference was calculated using a Student t-test * p value <0.05.

Aim 4 Identify regions of the genome differentially bound by MeCP2 in DRG from a pain model and control by chromatin immunoprecipitation (ChIP) sequencing.
Progress: To confirm that the increased MeCP2 binding can influence gene expression, we performed qPCR using RNA isolated from DRG obtained from SNI model of neuropathic pain. Our qPCR analysis of selected genes with enriched MeCP2 binding showed that the transcripts for three genes were significantly upregulated while the others showed a trend towards increased expression (Figure 5).

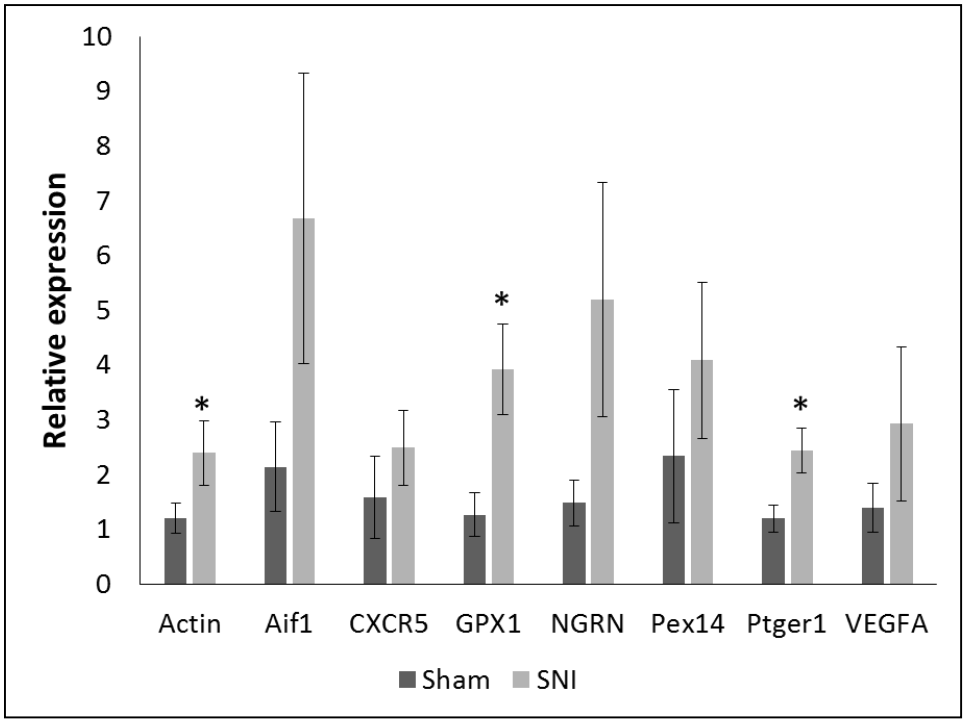


Figure 5 Taqman analysis of genes in DRG identified to have increased MeCP2 binding. Data normalized to Gapdh. Statistically significant difference was calculated using a Student t-test * *p* value <0.05.

We measured mmu-miR-126 in DRG from SNI and sham model four weeks after nerve injury using TaqMan MicroRNA Reverse Transcription kit reagents and protocol (Applied Biosystems) with gene specific RT primers for miRNA targets. For each qPCR assay 2 μL of RT reaction, 5 μL of Universal PCR master mix II, 2.5 μL of nuclease-free water, and 0.5 μL of the 20x TaqMan assay were used. Ct analysis was performed with SDS v2.3 software, and average and standard deviations were calculated. Our analysis showed that miR-126 decreased in SNI model (Figure 6). MeCP2 can both suppress and activate the expression of genes and our data suggests that increased MeCP2 binding could have contributed to the repression of miR-126.

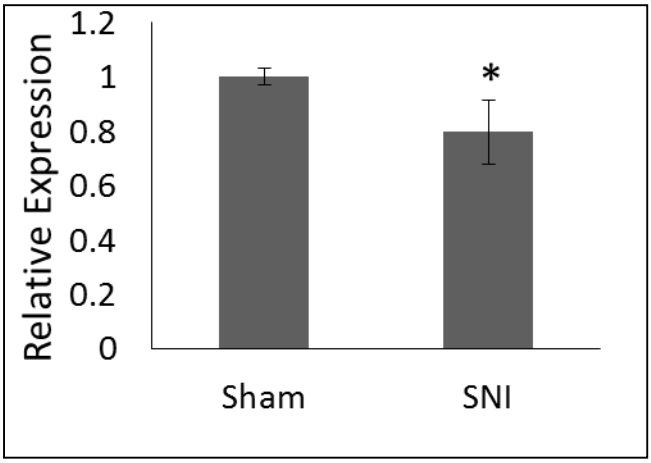


Figure 6 Taqman analysis of mmu-miR-126. Total RNA isolated from DRG four weeks after SNI surgery was used to quantify miR-126. U6 was used as the normalizer. Statistically significant difference was calculated using a Student t-test * p value <0.05.

Thus our ChIP-seq studies for MeCP2 showed that there are miRNAs and genes with enriched MeCP2 binding in the DRG under chronic pain state. Overall binding profile is indicative of a large number of potential binding sites suggesting a global regulatory role for MeCP2. Both novel genes and regions previously reported to be bound by MeCP2 in the brain were identified in our study. Our results provide insight on the molecular mechanisms on how increased binding of MeCP2 could lead to changes in gene expression leading to chronic pain.

Research Project 2: Project Title and Purpose

Making a Mouse to Study Hereditary Spastic Paraplegia – The purpose of this project is to generate and characterize a new transgenic mouse that expresses a human pathogenic mutant form of the SPG4 gene, which encodes for a protein called spastin. Mutations of this gene are the chief cause of Hereditary Spastic Paraplegia. The new mouse will be a key tool for studies on the etiology of the disease and the development of therapies for treating it.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Hereditary Spastic Paraplegia (HSP) is a debilitating constellation of neurodegenerative disorders that dramatically diminish the quality of life for patients. Mutations of *SPG4* are the most common cause, accounting for over 40% of HSP cases. *SPG4* codes for a protein called spastin, which is an enzyme that physiologically severs microtubules (MTs). Severing of MTs is important for mobility within the MT array of the axon, and for regulating MT number as well as

the distribution of plus ends of MTs in the shaft of the axon and the synapse. In theory, too little or too much MT severing could be greatly detrimental to the vitality of the axon. Genetic analyses of the broad spectrum of mutations associated with *SPG4* have led to the predominate view that haploinsufficiency is the molecular mechanism of the disease. In this view, a reduction in the levels of active spastin results in MT severing insufficient to keep the adult axon vital. However, a haploinsufficiency model would not explain why the degeneration is specific to the corticospinal tracts, nor is it consistent with the lack of developmental disorders in the patients, as MT severing is particularly important for growing axons.

We propose a toxic gain-of-function model, in which axonal degeneration results not from inadequate MT severing but rather from toxic effects of the mutated spastin molecules that accumulate in the axons of afflicted neurons. Spastin has two start codons, and hence produces a full-length isoform called M1 and a slightly shorter isoform called M87. The shorter isoform is found throughout the nervous system during development and in the adult, but M1 is present in appreciable amounts only in the adult spinal cord. The hypothesis is that the mutant form of M1 is toxic and accumulates, while the mutant form of M87 is not so toxic and rapidly degrades. The goal of these studies is to generate a transgenic mouse expressing the M1 form of a pathogenic mutant form of human spastin, so that studies can be performed on the biochemical basis of its toxic properties and so that potential therapies can be tested.

Specific Aim 1. Generate a transgenic mouse model for *SPG4*-based HSP.
Specific Aim 2. Conduct behavioral analyses, focusing mainly on walking behavior.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

The expected outcome is the production of a new transgenic mouse that can be used as a model for Hereditary Spastic Paraplegia. This mouse will enable future studies on the mechanism of the disease and allow potential therapies to be tested.

Summary of Research Completed

We propose a toxic gain-of-function model, in which axonal degeneration results not from inadequate MT severing but rather from toxic effects of the mutated spastin molecules that

accumulate in the axons of afflicted neurons. Spastin has two start codons, and hence produces a full-length isoform called M1 and a slightly shorter isoform called M87. The shorter isoform is found throughout the nervous system during development and in the adult, but M1 is present in appreciable amounts only in the adult spinal cord. The hypothesis is that the mutant form of M1 is toxic and accumulates, while the mutant form of M87 is not so toxic and rapidly degrades. The goal of these studies is to generate a transgenic mouse expressing the M1 and M87 forms of a pathogenic mutant form of human spastin, so that studies can be performed on the biochemical basis of its toxic properties and so that potential therapies can be tested.

Progress Specific Aim 1:

Mutations in more than 50 distinct loci and more than 20 mutated gene products have been identified in patients with hereditary spastic paraplegias, a diverse group of neurologic disorders characterized predominantly but not exclusively by progressive lower limb spasticity and weakness resulting from distal degeneration of corticospinal tract axons. Mutations in the *SPG4* gene that encodes the microtubule-severing protein called spastin are the most common cause of the disease. The etiology of the disease is poorly understood, but partial loss of microtubule-severing activity resulting from inactivating mutations in one *SPG4* allele is the most prevalent explanation. However, decreased severing of microtubules may not fully explain the disease. The presence of two translation initiation codons in *SPG4* allows synthesis of two spastin isoforms, each of which has functional specificity. M87, the shorter isoform, is more abundant in both neuronal and non-neuronal tissues. M1, the longer isoform, is only readily detected in adult spinal cord, which is where nerve degeneration mainly occurs in the disease. The microtubule-severing activity of M87 may be important not only for regulating features of the microtubule array, but also for endosomal trafficking. Due to its hydrophobic N-terminal domain, M1 may insert into the endoplasmic reticulum and play a role in the morphogenesis of this organelle. Although some mutated spastins may act in dominant-negative fashion to further lower microtubule-severing activity, others do not behave in this fashion and yet still have detrimental effects. Detrimental effects on microtubule dynamics, axonal transport, endoplasmic reticulum, and endosomal trafficking may be due to diminished microtubule-severing and/or other functions of spastin, but also may be due to neurotoxicity caused by mutant spastin proteins, chiefly mutant M1. Some large deletions in *SPG4* can also affect adjacent genes, further complicating the disease. Thus, while identifying spastin as a microtubule-severing protein was an important mechanistic breakthrough, it seems certain that insufficient microtubule severing is not an adequate explanation for HSP-*SPG4.*

To evaluate the significance of spastin loss in mammals, two spastin knockout mouse models were previously generated and analyzed by other research groups [Tarrade et al., 2006 (Human Molecular Genetics 15: 3544-58); Kasher et al., 2009 (Journal of Neurochemistry 110: 34-44)]. In the first model, spastin exons 5-7 were deleted via homologous recombination. As a result of the accompanying frame-shift, a termination codon was created 29 base pairs downstream from the new exon 4-8 junction. Interestingly, truncated spastin transcripts lacking exon 5-7 were readily detected in various tissues including brain, indicating that the PTC did not trigger NMD. Truncated spastin protein, however, was not detected in brain. In the second mouse model, exon 7 of spastin was deleted as a result of point mutation in the splice donor site. Sequencing revealed that the mutated transcription product would encode truncated spastin with 50 novel C-

terminal amino acids followed by a termination codon. Again, the presence of PTC did not trigger NMD, but truncated protein was not detected in mouse brain. Neither of these spastin mutations generated in mice led to developmental abnormalities. Compared to many human HSP-*SPG4* patients carrying just one copy of mutated *SPG4*, homozygous *SPG4* knockout mice that did not express any active spastin had only mild motor defects and heterozygous mice did not exhibit any detectable gait abnormalities. The most prominent phenomenon observed in both mouse models was the presence of focal axonal swellings filled with organelles and filaments, observed in both descending and ascending tracts of the spinal cord. Interestingly, sections of spinal cord from two HSP-*SPG4* patients also revealed the presence of axonal swellings containing neurofilaments and mitochondria. These findings might best be explained by perturbations of axonal transport, and indeed selective reduction of anterograde transport was found in cultured neurons derived from one of the mutated mouse models. On this basis, it was proposed that spastin depletion results in misregulation of microtubule dynamics, leading to increased cargo stalling, possibly by disturbing cargo loading on microtubules. This explanation, however, is not entirely satisfactory for various reasons. First, axonal swellings have not been observed in cultured neurons depleted of spastin by siRNA. Second, it has been shown that kinesin-1, the motor protein responsible for most of anterograde organelle transport in the axon preferentially moves on stable microtubules rich in acetylated and detyrosinated □-tubulin and such modified microtubules seem to be not less but more numerous in spastin depleted cells. It has also been shown that ER extension (sliding) along microtubules is facilitated by tubulin acetylation. Finally, while severing increases the number of microtubule plus-ends that could then undergo polymerization and depolymerization, there is no indication that the rate of microtubule polymerization or depolymerization, within itself, is affected by WT spastin. Indeed, no decrease in microtubule polymerization has been found in spastin-depleted cultured neurons. As microtubule-modifying drugs have been suggested as a potential therapy for HSP-*SPG4*, further studies and clarification on these issues is of great practical importance.

No mouse model previously existed to test potential toxic gain-of-function effects of mutant spastin proteins. Given that haploinsuffiency does not appear to be a satisfactory explanation for the disease and given that only so much can be done in cell culture or in *Drosophila*, we set forth to make a novel gain-of-function mouse model for *SPG4*-HSP.

Experimental approach:

To develop a versatile new mouse designed to test the hypothesis that mutant spastin proteins produce cytotoxic gain-of-function effects, we wished to knock the human spastin gene into mice, with the gene bearing a pathogenic mutation. Given that over 200 different mutations of different types have been identified in patients, choosing just one mutation to be broadly representative was a difficult and perhaps impossible task. We chose the C448Y mutation because it definitely does not create a truncation, and because we studied it extensively in our recently published work, in which we knocked human mutant *SPG4* into *Drosophila* [Solowska et al., 2014 (Journal of Neuroscience 34: 1856-67)]. We originally proposed to knock in mutant M1 specifically, but ultimately chose instead to knock in the entire gene such as the mutant M87 and mutant M1 would be synthesized together, to better reflect the situation in human patients.

We investigated various options from companies that specialize in making such animals. We found the most appealing option to be one that offered great flexibility in experimental design. GenOway, a company in France, provided us the following in formation, which is quoted directly from their communication with us:

"Generation of a Knock-in line by transgene insertion into a permissive locus.
In order to avoid transgene positional effect and to bypass the time consuming validation of several lines deriving from random insertion based models, we offer generating transgenic models by directed transgenesis. The transgene could be inserted either at the *Hprt* or *Rosa 26* loci. Both loci have been studied for their ability to sustain transgene expression when inserted at these sites. These loci are both neutral and described as open chromatin regions. The site directed transgenesis is performed using one of our ready-to-use *Hprt* or *Rosa26* targeting vectors. These vectors have already been extensively validated. Having access to these ready-to-use vectors will reduce the development time of your model while securing the expression of your transgene. We strongly recommend using this approach instead of any approaches based on random insertion as the expression of the transgene is secure, the model is of higher quality (no risk of transgene instability or segregation) and the validation of the line is straightforward (to be conducted on one single line in contrary to the random insertion-based strategies which requires 3 to 5 lines). This approach is often economically more appropriate. Features of *Hprt* and *Rosa 26* targeting strategies:

- *Hprt* gene being localized on the X chromosome, it is therefore preferable to analyze the expression of your transgene in male mice or homozygotic females while the *Rosa26* locus is autosomal and located on chromosome 6.
- The *Hprt* targeting requires the use of 129 Ola ES cells, whereas the *Rosa 26* targeting can be performed using either 129 or C57BL/6 ES cells.
- From studies we have conducted, *Hprt* gene targeting is well adapted for both tissue specific and ubiquitous promoters, whereas *Rosa26* is recommended for ubiquitous expression.

Then and as evoked in your discussion, you would be interested in the generation of a conditional tissue specific mouse line. Such model could be obtained using the conditional *Hprt / Rosa26* gene targeting approach. The conditional activation is based on the presence of a *loxP* site flanked Stop signal between the ubiquitous promoter (CMV early enhancer/chicken beta actin (CCAG) promoter) and the cDNA of interest. This construct has no activity and the transgene over-expression is silenced by the presence of a Stop signal. The activation of this construct is achieved by introducing the Cre recombinase which catalyses recombination between the two integrated *loxP* sites which removes the Stop signal, thus activating the transgene expression. Then, such conditional expression model will thereafter have to be crossed with the Cre recombinase expressing line of your choice for the activation of your transgene in the tissue of interest. For the development of your mouse model we would highly recommend the conditional *Rosa26* gene targeting technology. Indeed the conditional expression strategy would bypass any colony management issue due to potentially severe phenotypes due to mutated spastin expression (impaired fertility or altered breeding performance due to paraplegic phenotype). Subsequently, our offer can be summarized as follows:

Generation of the model via conditional Rosa26 gene targeting technology This offer requires that you provide genOway with your cDNA of interest. genOway is then in charge of the model development from the insertion of the cDNA within the conditional expression *Rosa26* targeting vector (that is already containing the pCCAG promoter and a transcriptional STOP cassette) up to the obtainment and characterization of F1 heterozygous conditional expression *Rosa26* mutant animals. *Development cost: $49,250 using C57Bl/6 ES cells - The genetic background of the model will be pure (F1: 100% C57Bl/6) Development time: 35 weeks*

⇨ The deliverable will be a mouse line containing the "pCCAG – loxP – STOP – loxP – cDNA" construct. Then and as mentioned above, such line would then have to be crossed with the Cre recombinase expressing line of your choice in order to enable the activation of your transgene in the tissue of interest."

We chose this approach because it would enable us to control the tissues in which the human mutant spastin is expressed by crossing the mouse line prepared by the company with various Cre lines. This way, we can express the mutant protein, for example, throughout the CNS, or in a limited population of neurons in the CNS, etc. The mouse corticospinal tracts are not as well developed as the human corticospinal tracts, and the mouse does not walk upright as humans do. We hope that walking behavior can be used as the preferred behavioral test, because walking is the behavior most notably impaired in humans, but it may be that we have to turn to a different type of behavior, based on mouse anatomy and physiology. Thus, having the power to express the pathogenic protein in different populations of cells is an incredibly powerful option.

We provided GenOway with the DNA construct for human spastin with the relevant mutation, the same as we had used in our *Drosophila* work. Once ordered, it took roughly 10 months to generate the "locked" mutant spastin founder mice. These founder Cre-locked mutant spastin animals (Spastin+/- mice on a B6 background) were bred to control B6 mice obtaining offspring, 50% of which contain the cre-locked mutant Spastin. This colony has provided the Cre-locked spastin +/- animals bred to Cre expressing mice to examine phenotype. We have now established the colony in at Drexel University, under the direct supervision of Dr. Terry Heiman-Patterson.

Specific Aim 2 Progress:

In order to produce mutant spastin expressing mice for behavioral analysis, these mice (Spastin +/-) are mated to the ubiquitous expressing CMV Cre +/- mice (B6) to generate a colony of mice in which ¼ offspring are Spastin +/-, Cre +/-. In addition, ¼ offspring will be Spastin -/- and Cre -/- providing control animals for the behavioral and pathologic studies. We chose a ubiquitously expressing Cre mouse to maximize our chance of observing phenotype. The remaining offspring will be Spastin +/- Cre -/- or spastin -/-, Cre +/- and can be used for futher colony expansion of the heterozygous Cre colony and the heterozygous locked Spastin colony .

The mice that are heterozygous for the both the mutated spastin gene (Spastin +/-, Cre +/-) will ubiquitously express the mutant spastin isoforms since the stop codon is removed by the action of Cre Recombinase allowing the expression of the mutant spastin gene in all tissues. We are

continuing to mate our founder Spastin+/- mice to the ubiquitous expressing Cre +/- mice (i.e. mating of double heterozygotes (Spastin +/-, Cre +/-) to generate litters of mice in which ¼ offspring will be Spastin +/-, Cre +/-. In addition, ¼ offspring will be Spastin -/- and Cre -/- providing control animals for the behavioral studies.

We now have 4 litters of animals (heterozygous ubiquitous Cre expressing mice x "locked" heterozygous mutant spastin mice) that contain animals that are Cre-/Spastin -, Cre+/Spastin- or Cre+/Spastin+ in a ratio of 1:2:1. The animals are being evaluated for motor deficit and phenotype expression and range in age from 91 to 105 days. Assessments for onset of disease have been started at 40 days of age and we are examining the animals 2 times weekly. Assessments include weights, splay, tremor and ladder behavior. The weight is measured to assess any changes with onset. We also assess motor behaviors. Onset for splay, ladder down and ladder up is judged on a scale from 0 to 3. For splay, 0 is a perfect leg splay resulting on raising the hindlimbs while a 3 is the inability to spread hind legs at all. For ladder behavior the animal is placed at the base of the ladder and encouraged to climb up and then down the ladder. For ladder up a 0 refers to climbing up the ladder with no slippage, 1 indicates 1 or 2 slips going up or not alternating legs, 2 indicates slipping with a prolonged (>20 seconds) to climb up the ladder and 3 indicates the mouse falls down the ladder on attempting to climb up. For climbing down the ladder a 0 is normal descent with alternating hind legs and no slippage, a 1 is indicates either one or two slips or not alternating hind limb, a 2 indicates that the mouse slips down most of the ladder and does not alternate its hindlimbs and a 3 indicates that the mouse falls down the ladder. Onset is the first day of when the animal scores 1 for two consecutive assessments and the animal never again scores 0. In addition we examine for tremor, which is a correlate of clonus or upper motor neuron involvement. Onset by tremor is determined by the persistence of tremor on two consecutive examinations and dated by the first appearance. The animals' attention to hygiene and other activity is also observed.
We have one mouse that developed a tremor at 46 days and weakness at 74 days but we are in the process of verifying the genetic characterization and performing further studies to verify that this animal is in fact expressing mutant spastin. The remaining animals are between 91 and 105 days without definite phenotype.
Survival is determined by the point at which the animals can no longer move around the cage and obtain nutrition. While traditionally we have used the inability of an animal to right themselves after 10 seconds as a survival endpoint, in our preliminary results the affected mouse appeared active and healthy despite hindlimb paralysis. Once we obtain phenotype we will characterize affected and control mice pathologically for evidence of neurodegeneration along with biochemical studies for expression of mutant spastin isoforms.
If we are successful in developing this model of motor system illness, this mouse can be used to study spastin interaction with microtubules, test therapeutic agents directed at microtubule stability and explore the role of microtubules in motor neuron and other disorders in which microtubule function is implicated. This would have widespread importance to cell motility and cell proliferation.

Research Project 3: Project Title and Purpose

Irreversible HIV-1 Inactivation for AIDS Intervention and Prevention – Agents have been discovered at Drexel University that can irreversibly inactivate HIV-1 before host cell encounter. These virucidal agents have the potential to prevent HIV-1 infection and spread by treatment at early stages of human exposure to the virus. In addition, viruses from already-infected individuals can be isolated, inactivated by these agents and then used as therapeutic vaccines in the infected donors. This project will examine the breadth of virucide action on different virus tropisms and subtypes, determine inactivation potency in cellular environments, determine the immunoreactivity of the inactivated virus and test the neutralization activity following inactivated virus immunization.

Duration of Project

1/1/2013 – 12/31/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.pa.gov/MyRecords/Health-Research/CURE.

Research Project 4: Project Title and Purpose

Phenotypic Diversity of Neurons Modulating Executive Function in ADHD – The purpose of this project is to examine the phenotypic relationship between the mammalian prefrontal cortex (PFC), the area of the brain responsible for executive function, and the locus coeruleus (LC), one of the major brainstem modulatory centers that regulate functional operations in the PFC, in both normal and ADHD rats. This research will inform the development of drugs that are potential treatments for attention disorders.

Duration of Project

1/1/2013 – 12/31/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.pa.gov/MyRecords/Health-Research/CURE.

Research Project 5: Project Title and Purpose

Investigating Nervous System Structure and Function by 2 Photon Laser Scanning Microscopy – A fundamental goal in neuroscience is to understand how neural circuits are formed and maintained in development, and throughout the life of the animal. Proper connectivity is critical to learning and memory processes in the healthy brain, and to functional recovery following injury or disease. Understanding the mechanisms regulating neural circuitry and connectivity is critical to developing therapies for treating neurological dysfunction as a result of injury or disease. This project aims to investigate the cell-cell interactions between astrocytes and dendritic spines, or synapses, in vivo. The results from this project will provide novel and important insight into the cellular and molecular mechanisms underlying astrocyte regulation of synaptic connectivity.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

This project examines the role of astrocytes, the most abundant glial cell type in the CNS, in regulating the establishment and maintenance of dendritic spines during postnatal development, and in the mature brain. Spines are the principal sites of excitatory synapses, and serve as good indicators of synaptic function. In this project, we will use transgenic mice in which the molecular signaling pathway, Sonic hedgehog (Shh), has been targeted for disruption selectively in astrocytes. In Aim 1, we will examine whether Shh signaling in astrocytes is required for the development of dendrites and spines during early postnatal development. The morphological properties of dendrites and spines of Layer V neurons will be examined in the cortex at various stages during postnatal development, and in the adult brain. In Aim 2, we will use chronic, in vivo imaging by 2P LSM to examine the role of Shh signaling in astrocytes in the dynamic turnover of cortical spines. The rate of spine turnover will be examined in the cortex of adult mutants and controls to determine whether astrocytic Shh signaling is required for the structural reorganization of synapses. Together, these data will provide valuable insight into astrocyte-synapse interactions, and will facilitate future studies on the molecular and cellular mechanisms mediating synaptic plasticity.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This project aims to address the role of astrocytes in regulating the formation and maintenance of synapses *in vivo*. A more complete understanding of the mechanisms regulating neural circuitry and connectivity is critical to developing therapies for treating neurological disorders, and increasing evidence points to a critical role for astrocytes in establishing and maintaining neural circuits. In Aim 1, we will examine whether disruption of Shh signaling in astrocytes impairs cortical synaptogenesis during early postnatal development. If astrocytic Shh signaling is required for the establishment of normal synaptic connections in the cortex, then we expect to observe a concomitant disruption in the number and/or morphology of dendritic spines. In Aim 2, we will examine whether Shh signaling in astrocytes plays a role in the dynamic turnover of spines. If astrocytic Shh signaling is required for normal spine dynamics, then we expect to observe disturbances in the rate of spine addition and/or elimination following disruption of Shh signaling in astrocytes. The results from this project will provide new knowledge of how astrocytes contribute to the establishment of cortical circuits during postnatal development. Moreover, we will gain insight into the role of astrocytes in regulating the rearrangement of synapses in the living brain. Taken together, these results will provide the foundation for further studies into the cellular and molecular mechanisms regulating astrocyte-synapse interactions, and will serve as preliminary data for future extramural grant support.

Summary of Research Completed

<u>Progress Aim 1</u>
Previous studies have established a role for non-canonical Shh signaling within neurons in synapse formation between Layer II/III and Layer V cortical neurons. To rule out any non-specific effects of Smo deletion in neurons, we examined the spine densities of Layer V cortical neurons in the somatosensory cortex of *CamKinase2αCre;Smo$^{fl/fl}$;Thy1GFPM* mice (referred to here as CK2α Smo CKO). CK2α is expressed exclusively in pyramidal neurons, thus enabling us to interrogate whether Smo deletion in neurons has an effect on spine density. In contrast to GfapCre Smo CKO mice, CK2α Smo CKO mice show no signs of reactive gliosis (**Fig. 1**). The number of cortical astrocytes expressing GFAP is comparable between CK2α Smo CKO mutants and littermate controls, and astrocytes in CK2α Smo CKO cortex show no signs of cellular hypertrophy. We examined the spine density of apical dendrites from Layer V cortical neurons in the somatosensory cortex of CK2α Smo CKO mutants and controls and observed no difference in spine density (**Fig. 1**). This suggests that Smo-mediated Shh signaling in postnatal neurons is not required for spine development or long term maintenance, and further supports a specific role for astrocytic Shh signaling in regulating the synaptic architecture of Layer V cortical neurons. Taken together, these data support a role for Shh signaling in astrocytes in regulating the long term maintenance of cortical circuits. Aim 1 is complete.

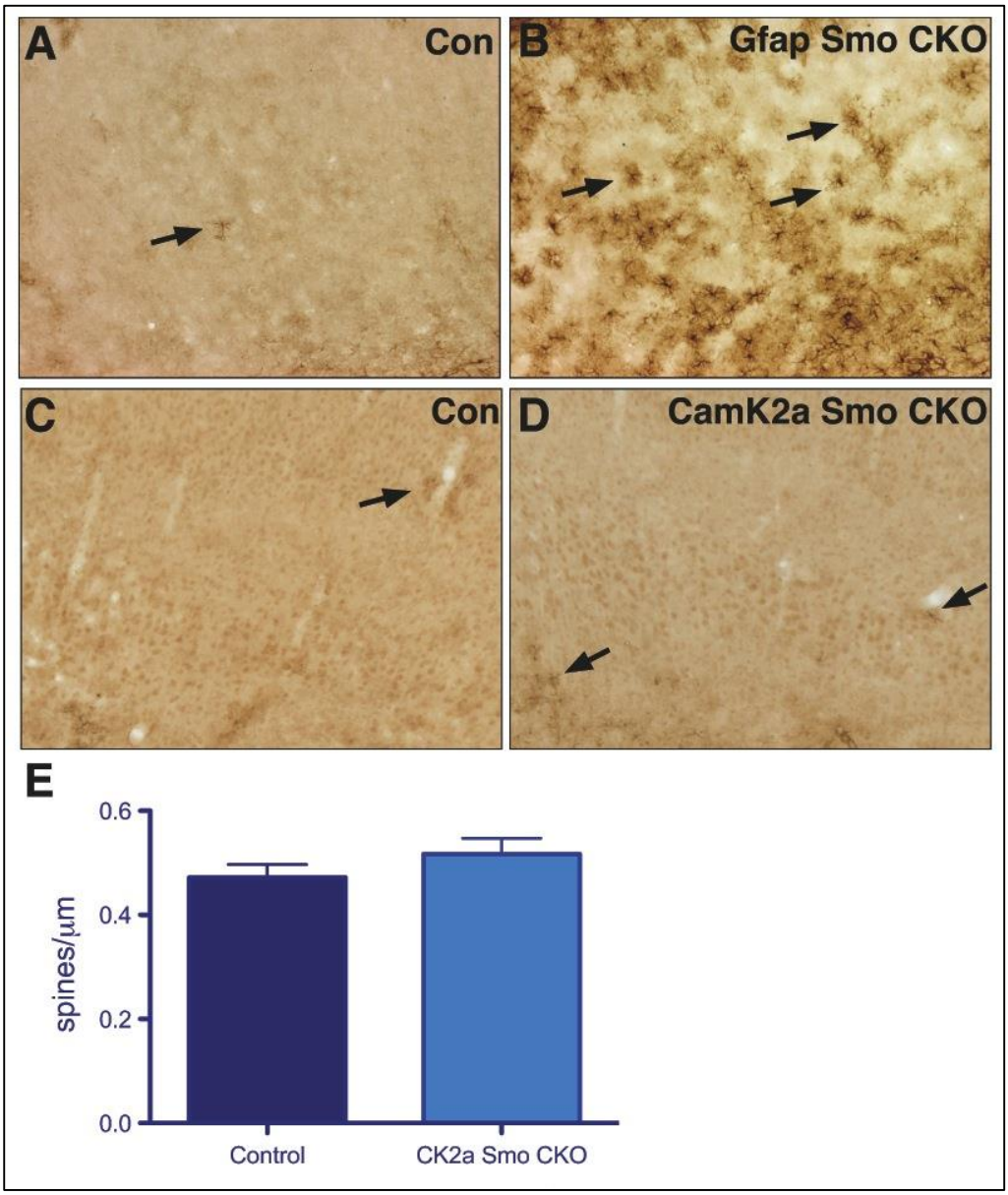

Figure 1. Targeted deletion of Smo in pyramidal neurons does not produce reactive gliosis and shows no difference in spine density compared to controls. (A-D) Brightfield immunohistochemistry for glial fibrillary acidic protein (GFAP) in astrocytes of the somatosensory cortex of adult controls (A, C), GfapSmo CKO (B), and CK2a Smo CKO (D) mice. (E) Spine density in apical dendrites of Layer V cortical neurons.

Progress Aim 2

We have further examined spine dynamics in an adult CK2α Smo CKO mouse to determine whether loss of function of Smo in pyramidal neurons has an effect on spine dynamics.

Consistent with our results in GfapSmo CKO animals and their controls, spine elimination was greater than formation in the somatosensory cortex of an adult CK2α Smo CKO mouse (**Fig. 2**). Interestingly, the overall proportion of spines eliminated and formed in the CK2α Smo CKO mouse was lower than that observed in GfapSmo CKO animals and their controls. Further experiments are underway with more animals and with appropriate controls to confirm these results, and to determine whether Smo disruption in pyramidal neurons versus astrocytes has a differential effect on spine organization.

Finally, we have performed preliminary experiments in at least 1 mutant animal to examine whether astrocyte dysfunction plays a role in age related changes in spine dynamics. We have performed repeated imaging over 1 day intervals, at 3 and 6 months of age (**Fig. 3**). Preliminary analysis shows that while the fraction of spines formed over 1 day is comparable between 3 and 6 months old, spine elimination is drastically reduced at 6 months, compared to 3 months of age. Further experiments are under way in wild type controls to examine whether Shh signaling in astrocytes plays a role in age related decline in spine reorganization.

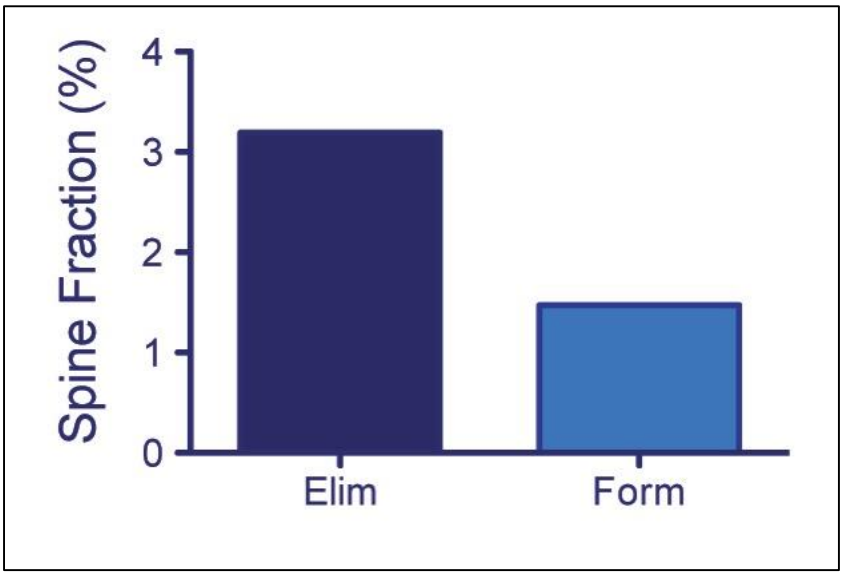

Figure 2. Spine dynamics of GFP-labeled spines in CK2a Smo CKO; Thy1 GFPM mice. Proportion of spines eliminated and formed over 2 days of imaging in the somatosensory cortex of an adult CK2a Smo CKO mutant mouse.

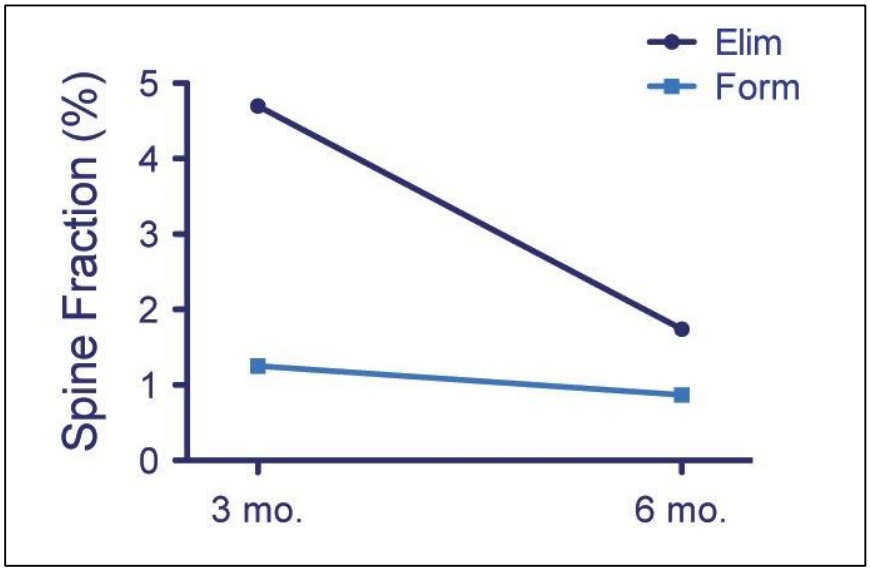

Figure 3. Age related decline in spine elimination. Proportion of spines eliminated and formed over 2 days of imaging in the somatosensory cortex of adult GfapCre Smo CKO mutant mouse at 3 months and 6 months old.

Research Project 6: Project Title and Purpose

Tumor Characterization in Breast Carcinoma Using Computerized Image Analysis – We have developed a computational method by which we can characterize lymph node metastasis status from histological images of primary breast tumor specimens. We propose to investigate the relationship between lymph node metastasis status and surrogate tumor markers, and to develop a novel machine learning technique designed to use histological images to predict tumor subtype, previously only possible with expensive molecular testing.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Histological methods have been shown to be a useful and cost-effective tool in determining pathologic stage. Regional lymph nodes status, primary tumor size, and distant metastasis are the three variables determining pathologic staging, as performed routinely for clinical cancer. Additionally, the Nottingham Prognostic Index utilizes tumor size, histologic grade, and lymph

node status to stratify patients and determine appropriate treatments. However, the main disadvantage of grading is its lack of consistency, which can only provide moderate reproducibility in some studies, and which can be affected by the experience of the pathologist issuing the pathology report. We have developed an automated procedure to objectively determine metastasis status via computerized image analysis of primary breast tumor histology. The generalizability of this procedure to other factors determining tumor status and sensitivity to therapeutic interventions has yet to be examined. The successful development of a reliable algorithm based on histological image analysis can potentially supplant molecular tools for the characterization of tumor subtypes and therapeutic strategies in breast carcinoma patients.

Specific Aim 1: To evaluate the predictive success of computerized histological image analysis using surrogate tumor markers. We will generate predictions of metastasis status from histopathological images of primary breast tumor specimens, and compare these predictions to immunohistochemical data commonly used to describe tumor aggressiveness and sensitivity. Our preliminary results indicate a strong correlation between predicted metastasis status and Ki-67, a measure of proliferation. We hypothesize that our image analysis paradigm provides information that can predict tumor status and guide treatment.

Specific Aim 2: To predict tumor subtypes via computerized image analysis of primary breast tumor histology. It has been shown that molecular diagnostic tools have a high degree of success determining tumor status and patient outcomes. We will develop an image analysis paradigm to use mRNA-defined tumor subtypes to train a multi-class machine learning classifier. We hypothesize that tumor subtypes can be predicted from histopathological image features, providing a cost-effective tool to define therapeutic groups.

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Expected Research Outcomes and Benefits

Breast cancer (BCa) is a very common carcinoma that affects 1,300,000 patients worldwide and is responsible for 450,000 deaths every year. BCa is a heterogeneous disease encompassing multiple morphologies and different outcomes. Most invasive mammary cancers are classified as invasive ductal carcinoma not otherwise specified (IDC NOS), whereas about 25% are defined as histological “special types”. These special-type BCas are categorized into at least 17 discrete

pathological entities. Current therapy decision-making is increasingly governed by the molecular classification of breast cancer. Currently, BCas are subclassified by global gene expression profiling into 4 major subtypes that inform therapy decision making: luminal A, luminal B, HER2-overexpressing and basal-like (triple negative). Recently, the Cancer Genome Atlas Network described how the phenotypic variation between BCa occurs within, but not across, these major subtypes, highlighting the importance of identifying each BCa for appropriate therapeutic decisions. Currently, there are two ways to characterize BCa into these subtypes: 1) Utilizing the gene expression profile and 2) Utilizing immunohistochemistry (IHC) for ER, PR, Ki-67, and HER2 as surrogate markers. Both of these solutions are costly and unsuitable for underdeveloped countries. We propose to develop an economical and reliable alternative that can classify specimens into these subtypes in order to provide adequate treatment to patients from rural and underserved areas in Pennsylvania.

Summary of Research Completed

Specific Aim 1: To evaluate the predictive success of computerized histological image analysis using surrogate tumor markers. We will generate predictions of metastasis status from histopathological images of primary breast tumor specimens, and compare these predictions to immunohistochemical data commonly used to describe tumor aggressiveness and sensitivity. Our preliminary results indicate a strong correlation between predicted metastasis status and Ki-67, a measure of proliferation. We hypothesize that our image analysis paradigm provides information that can predict tumor status and guide treatment.
Progress:

Our previous report demonstrated the significant predictive capacity of the developed algorithm, but also revealed that the prediction accuracy was not high enough for this to be a realizable model that could be utilized clinically. Our primary focus during this funding period, therefore, was to build upon our results to improve prediction accuracy. This first required that we identify the shortcomings of the algorithm design. The algorithm can be primarily divided into three stages: the identification of cell nuclei in histological images; the reduction of these images into a set of morphological features; and machine learning to generate predictions from the morphological features. We identified potential areas for improvement in the first two stages, which will be discussed in turn.

Nuclear identification and segmentation

The first stage of the algorithm consists of identifying cell nuclei and precisely determining each nucleus' boundaries so that the algorithm can proceed to make accurate morphological measurements for characterization and prediction. Manual review of the images produced from our initial attempt at this stage revealed two major shortcomings. First, nuclei were insufficiently identified across images, which naturally led to poor segmentation accuracy using our modified Watershed transform technique. We attribute this problem mostly to the inherent staining variability across images that often accompanies digital histological imaging. We developed a new algorithm to address this issue, which culminated in a recently submitted manuscript (J. Pathol. Informatic, submitted Nov 2014).

Second, in response to a manuscript that we submitted to Modern Pathology, one reviewer criticized the use of estrogen receptor (ER) stained images as the substrate for morphological analysis, citing the potential confound between ER positivity and nuclear detection. In order to remedy this concern, we decided to use a more "neutral" stain (H&E), which carries with it greater difficulty in nuclear identification and segmentation.

Given these two issues, we spent the majority of this funding period devising a technique to 1) accurately identify cell nuclei towards a more reliable segmentation stage by accounting for staining variability; and 2) tailor this technique to H&E images and their unique spatial and chromatic properties.

To accomplish this, we developed a procedure in which the computer identified the major histological structures present in the image and marked only the pixels that were classified as nuclei at a confidence level greater than an experimentally-derived threshold. This map of nuclei was then used as the input to the modified Watershed algorithm described in previous progress reports. Preliminary results of this algorithm were also discussed in the previous progress reports, but the algorithm was fully developed and completed during this funding period. In addition to the submitted manuscript, we have reported the results at the Pathology Visions conference (Oct 2014) and have an abstract accepted for platform presentation at the USCAP conference (Mar 2015). An abstract for presentation at the Pathology Informatics conference has also been submitted.

Algorithm details: Hematoxylin and Eosin (H&E) staining is ubiquitous in pathology practice and research. One of the major obstacles to quantitative analysis of H&E images is the high degree of variability often observed between different samples. In an effort to provide a substrate that can potentially mitigate this factor in quantitative image analysis, we developed a technique to project H&E images into an optimized color space more appropriate for our image analysis procedures. We used a decision tree-based support vector machine learning algorithm to classify 44 H&E stained whole slide images of resected breast tumors according to the histological structures that are present. This procedure takes an H&E image as an input and produces a classification map of the image that predicts the likelihood of a pixel belonging to any one of a set of user-defined structures (e.g. cytoplasm, stroma) (Figure 2). By reducing these maps into their constituent pixels in color space, an optimal reference vector is obtained for each structure, which identifies the color attributes that maximally distinguish one structure from other elements in the image (Figure 3). By aligning reference vectors derived from this technique, images can be transformed in a way that standardizes their color properties and makes them more amenable to image processing. We used this technique to automatically identify pixels likely belonging to cell nuclei, which is an essential first step for both our ROI detection procedure and our nuclear segmentation algorithms, the latter of which is described in the next section.

Zarella, M., Breen, D., Plagov, A., Garcia, F. *An optimized color transformation for the analysis of digital images of H&E stained slides*. Journal of Pathology Informatics. Submitted, 2014.

An improved architectural metric

Tumor architecture was previously characterized using stochastic geometry. This led to moderate accuracy rates in prediction performance, as described in the previous reports. However, this was a very computationally intensive procedure, as it required on the order of 10^9 iterations for a single image. Furthermore, the output of this procedure was in the form of distributions that needed to be further reduced (e.g. into medians) to be used in the machine learning procedure, which resulted in significant loss of information. Aided by summer researchers working under the ReTHINK fellowship program, we developed an alternative architectural metric using n-point statistics. This procedure is a computationally efficient algorithm that uses nearest neighbor distributions to characterize the spatial distributions of nuclei in an image. Support vector machine learning can then be applied to a multidimensional space represented by the span of N's (N = 1st nearest neighbor distance, 2nd nearest neighbor distance, and so forth). Preliminary results reveal significant predictive capacity of the technique (on metastasis data; we have not yet applied it to subtype prediction), and make it more amenable to inclusion into the overall framework. This work was presented internally in poster form.

Specific Aim 2: To predict tumor subtypes via computerized image analysis of primary breast tumor histology. It has been shown that molecular diagnostic tools have a high degree of success determining tumor status and patient outcomes. We will develop an image analysis paradigm to use mRNA-defined tumor subtypes to train a multi-class machine learning classifier. We hypothesize that tumor subtypes can be predicted from histopathological image features, providing a cost-effective tool to define therapeutic groups.
Progress: None.

Research Project 7: Project Title and Purpose

Innate Immunity and Bacterial Pathogenesis – The project aims to understand how innate immunity during viral-bacterial co-infections regulates susceptibility and pathogenicity and to establish models that allow the determination of important innate pathways that control infection. The studies proposed in this project will investigate important aspects of innate immunity in the context of bacterial infections. These studies will establish important models to understand the complex interaction between *N. gonorrhoeae* and HIV, and elucidate host factors that contribute to resistance to *L. pneumophila*.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

The foci of this project are to understand how innate immunity during viral-bacterial co-infections regulates susceptibility and pathogenicity and to establish models that allow the determination of important innate pathways that control infection.

AIM 1: Determine the effect of bacterial and viral co-infection on epithelial innate immune responses and susceptibility to infection. Using an in vitro cervical epithelial cell model of *N. gonorrhoeae* and HIV-1 co-infection, we will investigate inflammasome-mediated innate immune responses that impact pathogen transmission.

AIM 2: Establish a model to identify critical innate responses that protect from L. pneumophila. A. Identify the innate immune response components that are triggered by *L. pneumophila* in human macrophages. B. Using influenza virus infection of mice, which induces innate immune paralysis and increases susceptibility to secondary bacterial infections, we will determine the critical innate factors that confer resistance to *L. pneumophila.*

AIM 3: Determine whether innate immunity triggered by acALY18, an endogenous peptide activator of inflammasomes, can inhibit infection or enhance control of N. gonorrhoeae and L. pneumophila.

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Expected Research Outcomes and Benefits

Co-infections are becoming increasingly important with the spread of antibiotic resistance and the potential for co-infections with other classes of pathogen such as viruses. The innate immune response is a key process in determining host resistance or susceptibility to infection and regulates the initial host response to microbes. These responses will either prevent, control, or eliminate the infection. Not surprisingly, some pathogens have evolved strategies to evade detection by, or inhibit innate immunity by interfering with the detection system of the host. Indeed, it has been found that many of these evasion strategies are crucial for the virulence of the pathogen. Additionally, inhibitory or activating effects of a pathogen on the innate immune system can alter the susceptibility of the host to a different pathogen aiding in co-infections. Infection with one pathogen, by triggering or inhibiting the innate response can create a host environment that facilitates or promotes the co-infection with a different pathogen. With these studies, we expect to understand the host factors and interactions that control resistance and

susceptibility to bacterial pathogens. In addition, these studies will establish important models to understand the complex interaction between *Neisseria gonorrhoeae* and HIV, and elucidate host factors that contribute to resistance to *Legionella pneumophila*. Additionally, these studies will investigate the therapeutic potential of an inflammasome activator that has been shown to clear pathogen in simple infection models that will be tested in these novel models of complex infections.

Summary of Research Completed

Aim 1 Progress: These studies were made possible through our prior expertise (Krebs) in the use of a transwell model of the cervicovaginal epithelium. This model system, which replicates the polarized topology of the female reproductive tract (FRT), provides the opportunity to introduce sexually transmitted pathogens to the apical (outer) side of the epithelium and monitor resulting innate immune responses on the basolateral (inner) side of the epithelial layer, as well as changes in the barrier created by the polarized epithelial cells.

As a prelude to understanding the combined effects of the human immunodeficiency virus type 1 (HIV-1) and *N. gonorrhoeae* in this model system, experiments were designed to investigate the effects of *N. gonorrhoeae* infection alone, and to establish baseline measurements for future experiments in which HIV-1 and *N. gonorrhoeae* would be introduced concurrently.

In these studies, immortalized human ectocervical cells (Ect1/E6E7) were plated in transwell tissue culture inserts at a density of $1.5x10^5$ cells per well in serum-free media. The transwell tissue culture system includes an insert with a semi-permeable membrane (0.4 μm pore size) on which cells are grown. In this model of the cervicovaginal epithelium, the cells were maintained in the upper chamber in the presence of a cervicovaginal fluid simulant (CFS), which approximates the chemical content and pH (~4) of cervicovaginal fluid that bathes the apical side of the epithelium *in vivo*. The transwell insert is placed in a receiver plate (24-well format) containing media that comprises the lower chamber of the culture system. Cells were maintained for a period of approximately 16 days to permit cell growth into a confluent monolayer and the establishment of intercellular tight junctions. Cell confluence was monitored visually, while tight junction integrity was tracked by measuring trans-epithelial electrical resistance (TEER) between the upper and lower chambers. The epithelial barrier was considered established when TEER values had plateaued for at least three consecutive days. *N. gonorrhoeae* (strain FA1090) used for these experiments was cultured in overnight in GC Broth II and diluted to 0.3 OD_{600}, which yields a cell density of approximately $4x10^6$ colony forming units (CFU) per ml.

Semen samples used in these studies were obtained commercially (Lee Biosolutions, Inc.) from anonymous healthy donors. Samples from six donors were pooled to produce a sample volume representative of an "average" male. Whole semen was diluted to 25% (by volume) in these studies to mimic the degree of semen dilution that takes place during and after coitus.

Once the Ect1 cells had reached a TEER plateau, the CFS in each upper well was removed and replaced with approximately $4x10^4$ CFUs of *N. gonorrhoeae* suspended in either cell culture media or CFS. Alternatively, a mixture of *N. gonorrhoeae* and whole semen (25%) in media or

CFS was applied apically to the epithelial cells to simulate the simultaneous introduction of *N. gonorrhoeae* and semen during intercourse. EDTA, which causes a breakdown of tight junctions through chelation of divalent metal ions, was used as a positive control for reductions in epithelial barrier integrity. After incubation for 4 hours at 37°C, cells in the upper chamber were washed extensively and supplied with new media or CFS. Media samples were collected from the basolateral (lower) chambers for cytokine analysis, then replenished. TEER for each well was measured at 5-minute intervals from initial exposure (time = 0) through 30 minutes post-exposure, and at 1, 2, 3, and 4 hours post-exposure. Cells were also evaluated qualitatively throughout the experiment for reductions in cell numbers associated with *N. gonorrhoeae* exposure. Prior experiments have demonstrated that a 4-hour exposure to 25% seminal fluid results in negligible reductions in cell viability. Basolateral conditioned media samples from 4 hours post-exposure were analyzed using the Human TLR-induced Cytokines II: Microbial-induced Multi-Analyte ELISArray Kit (Qiagen), which assays the following cytokines and chemokines: TNFα, IL-1β, IL-6, IL-12, IL-17A, IL-8, MCP-1, RANTES, MIP-1α, MIP-1β, MDC, and eotaxin.

As previous experiments were conducted using cell culture media as the diluent for *N. gonorrhoeae* or *N. gonorrhoeae* combined with semen, we performed similar experiments in which *N. gonorrhoeae*, with or without semen, was diluted with CFS and introduced into the apical chamber. The purpose of this variation on the original experimental design was to examine the effect of CFS on changes in TEER induced by N. gonorrhoeae in the absence or presence of semen. Since CFS replicates the chemical content and pH of cervicovaginal fluid that bathes the apical side of the epithelium in the FRT, this change increases the physiological relevance of the model system.

In this experiment, the effect of *N. gonorrhoeae* on TEER was markedly different relative to the previous experiment (Fig. 1). Introduction of *N. gonorrhoeae* alone in the presence of CFS caused little or no change in TEER up to 1 hour post-exposure. Between 2 and 4 hours post-exposure, it was evident that TEER was increasing relative to mock-exposed cells. *N. gonorrhoeae* combined with semen caused a small reduction in TEER through 30 minutes post-exposure, followed by increases in TEER to approximately 150% of mock levels by 4 hours post-exposure. The small reduction in TEER following exposure to *N. gonorrhoeae* and semen, however, may not be significant, since a repeat experiment showed no change in TEER up to 15 minutes post-exposure and then a steady increase to approximately 170% relative to mock levels.

TLR-induced cytokines and chemokines accumulated in the basolateral chamber were also analyzed with respect to levels found in mock-exposed cells (Fig. 2). Cells in this experiment were exposed apically to *N. gonorrhoeae* or *N. gonorrhoeae* combined with semen and diluted with CFS. Like previous experiments performed using media as the apical diluent, IL-8 was again the dominant factor released by the epithelial cells in response to either *N. gonorrhoeae* or *N. gonorrhoeae* combined with semen. However, there were two clear differences between the results of this experiment and prior experiments in which *N. gonorrhoeae* or *N. gonorrhoeae* combined with semen were diluted with media. First, the IL-8 responses under both conditions (relative to mock exposed cells) were considerably higher (between 400-800% in this experiment compared to 200-300% in the experiment with the media dilution). Second, IL-8 was released at

higher levels (~774%) from cells exposed to both *N. gonorrhoeae* and semen in CFS compared to levels released from cells exposed to *N. gonorrhoeae* and CFS alone (~432%).

When introduced in the presence of CFS, *N. gonorrhoeae* had little or no detrimental effect on the epithelial barrier up to 1 hour post-exposure and a measurable positive effect on TEER with increasing post-exposure times. We conclude from a comparison of these results that the CFS is negating the effect of N. gonorrhoeae on epithelial cell integrity. One possible explanation for this finding is that one or more components of the CFS are interfering with N. gonorrhoeae-associated mechanisms that reduce TEER. Alternatively, since low pH is itself an innate antibacterial factor, it is possible that the CFS is reducing N. gonorrhoeae viability and thus its ability to reduce epithelial TEER. Both possibilities will be explored in future experiments.

Our efforts to make the model system more consistent with conditions found *in vivo* have also revealed a possible effect of cervicovaginal secretions that bathe the apical side of epithelial tissues in the FRT. With respect to the barrier function of the FRT epithelium, our results suggest that the cervicovaginal secretions within the FRT reverse reductions in epithelial integrity caused by the introduction of *N. gonorrhoeae*, even more so than the effect of semen. Our results also suggest that these secretions serve to significantly augment innate epithelial responses to *N. gonorrhoeae* and semen, resulting in a much greater pro-inflammatory response in the basolateral direction. These aspects of concurrent *N. gonorrhoeae* and HIV-1 infection will be explored in future investigations.

AIM 2 Progress:
A. Identify the innate immune response components that are triggered by *L. pneumophila* in human macrophages. B. Using influenza virus infection of mice, which induces innate immune paralysis and increases susceptibility to secondary bacterial infections, we will determine the critical innate factors that confer resistance to *L. pneumophila.*

Progress: Aim 2A This Sub-Aim was completed during the previous reporting period.
Aim 2B is designed to establish a novel bacterial-viral co-infection model *in vivo* in mice. After receiving approval for our new animal protocols, we conducted co-infection experiments in mice. Mice are first infected with Influenza virus strain PR8, and allowed to recover from weight loss before being challenged with *L. pneumophila* at a dose of $1x10^6$ intranasaly. The bacterial infection is allowed to proceed for up to four days, at which point the mice are sacrificed, and the outcome of infection is analyzed. We quantified bacterial burden by monitoring the weight of the animals during the infection, enumerating total lung colony-forming units (cfu's), assaying for dissemination by checking for liver cfu's and analyzing the cell population in lungs and in the bronchial-lavage fluid via FACS analysis.

Results show that in mice previously infected with influenza PR8, the lungs cannot support robust bacterial replication, as evidence by a significantly reduced bacterial burden recovered from co-infected mice compared to PBS control (Fig. 3). These results suggest that the lung inflammation caused by the PR8 infection may help restrict subsequent *L. pneumophila* infection if that infection is initiated while the mice are still actively sick with influenza. Detailed FACS analysis of lung samples from infected mice (Fig. 4) shows that in *L. pneumophila* infected mice

there is an elevated number of dendritic cells found in the lungs in the days follow the bacterial infection (Fig. 5). The elevated number of dendritic cells most likely is a result of recruitment of these cells to the site of infection, since the fast kinetics rules out proliferation as the source for these cells. Together, these results illustrate the importance of studying the underlying mechanism of viral-bacterial co-infection, and demonstrate that co-infection of mice with influenza and *L. pneumophila* can serve as a powerful tool to help uncover the processes that take place during co-infection.

AIM 3: Determine whether innate immunity triggered by acALY18, an endogenous peptide activator of inflammasomes, can inhibit infection or enhance control of N. gonorrhoeae and L. pneumophila.

Progress: This Aim was completed during the previous reporting period.

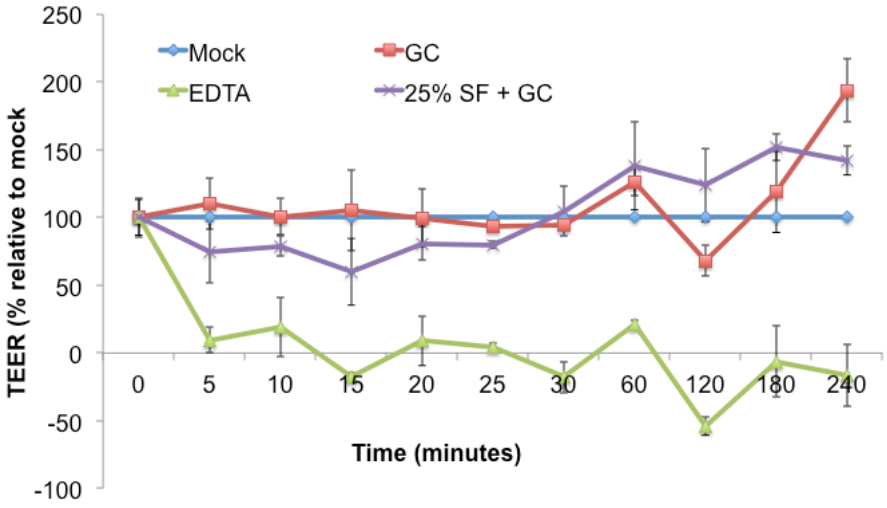


Fig. 1. The effect of *N. gonorrhoeae* on epithelial TEER in the absence or presence of seminal fluid is altered by cervicovaginal simulant in the apical chamber. Ect1/E6E7 cells cultured in a transwell tissue culture system were maintained in cervicovaginal fluid simulant (CFS) or exposed to either *N. gonorrhoeae* in CFS or *N. gonorrhoeae* combined with seminal fluid (25%) in CFS for 4 hours at 37°C. TEER values were recorded at the indicated post-exposure times. TEER is expressed as a percent relative to TEER values for CFS-only, mock-exposed cells. SF, seminal fluid; GC, *N. gonorrhoeae.*

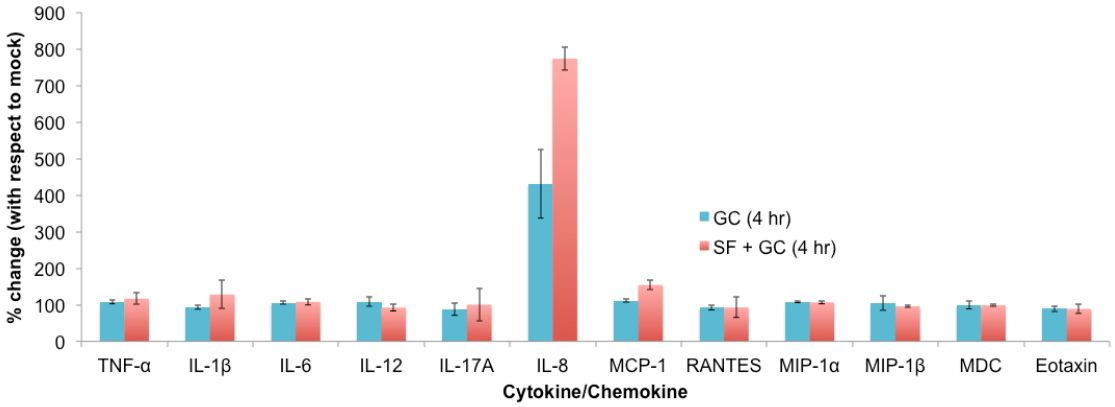
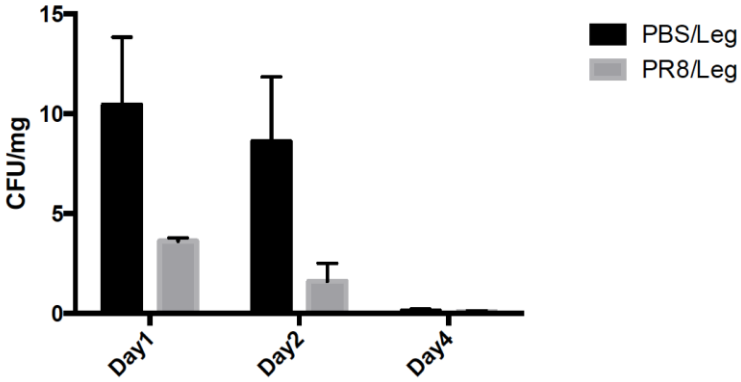


Fig. 2. IL-8 release subsequent to exposure to *N. gonorrhoeae* and seminal fluid is increased by dilution in CFS instead of cell culture media. Ect1/E6E7 cells cultured in a transwell tissue culture system were maintained in cervicovaginal fluid simulant (CFS) or exposed to either *N. gonorrhoeae* in CFS or *N. gonorrhoeae* combined with seminal fluid (25%) in CFS for 4 hours at 37°C. Conditioned media samples from the basolateral chambers were analyzed for cytokine and chemokine content at 4 hours post-exposure. Analyte concentration is expressed as a percent relative to analytes detected in conditioned media from CFS-only, mock-exposed cells. SF, seminal fluid; GC, *N. gonorrhoeae.*

Fig. 3. The bacterial burden in mice co-infected with *L. pneumophila* and Influenza is decreased. Mice were infected intranasaly with the influenza strain PR8, or a PBS control. 10 days after infection mice were intranasaly infected with ***L. pneumophila*** wild type strain JR32. Mice were sacrificed at days 1,2 and 4 after bacterial infection, and the lungs were harvested, homogenized, and bacteria were plated on solid media for enumeration.



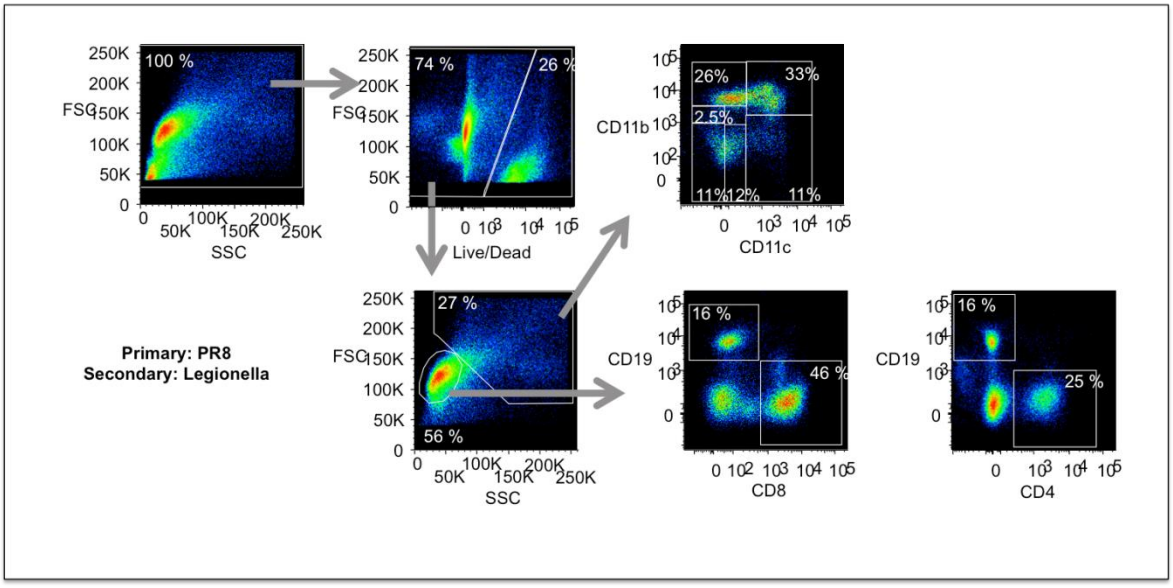
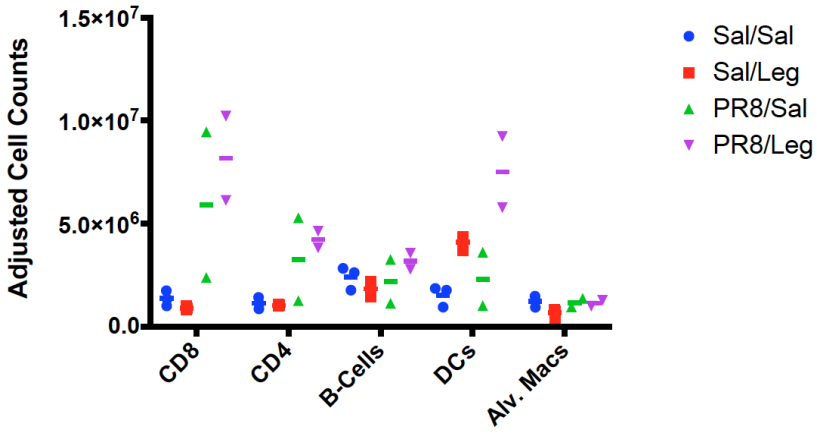


Fig. 4. Illustration of the FACS analysis done on samples of infected lungs. Shown are the data generated from a mouse co-infected with PR8 and *L. pneumophila*

Fig. 5. Adjusted cell count from the lungs of co infected mice analyzed by FACS. Data includes a complete experiment with four groups of 3 mice each, including PBS controls (Sal).



Research Project 8: Project Title and Purpose

Interaction of Interstitial Flow and ErbB2 Signaling in Breast Cancer Invasion – Interstitial fluid flow, which is the movement of fluid through tissues, increases in tumors and has been shown to promote tumor invasion, differentiation of stromal fibroblasts into myofibroblasts, increased cell motility, and activation of lymphatic endothelial cells. It is clearly evident that interstitial flow can have significant effects on tumor cells and their local microenvironment. However, there is a significant dearth of knowledge regarding how interstitial flow-induced cell signaling interacts with oncogenic signaling pathways to promote tumor invasion. Here, we propose a novel concept—that interstitial fluid flow mechanotransduction and ErbB2-activated signaling pathways cooperate to drive progression from premalignant to invasive cancer.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

Based on preliminary evidence, we propose that the response of ErbB2+ breast cancer cells to interstitial flow is potentially a critical trigger in the transition from DCIS to invasive breast cancer. The objectives of this project are to demonstrate that interstitial fluid flow induces invasion in ErbB2+ breast cancer cells, and determine what molecular factors mediate flow-induced invasion. *Our central hypothesis is that interstitial flow activation of chemokine receptors and phosphatidylinositol 3-kinase (PI3K) collaborates with ErbB2 signaling to enhance invasion of breast cancer cells.* We will utilize an *in vitro* three dimensional (3D) cell culture system developed in my laboratory that mimics DCIS phenotypes to unveil interstitial flow as a novel contributor to cancer progression and to test relevant potential therapeutic targets for blocking the invasion of breast cancer cells. To test this hypothesis, we propose three aims:

Aim 1—Determine the effects of interstitial fluid flow on ErbB2+ breast cancer cell invasion. We will characterize a flow invasion assay incorporating *in vivo*-like 3D cell structures and quantify the effects of interstitial fluid flow on breast cancer cells invasion.

Aim 2—Elucidate mechanisms of interstitial flow mechanotransduction in ErbB2+ breast cancer cells. Our initial focus will be on chemokine signaling and the role of PI3K. We will determine the role of chemokines in our system.

Aim 3—Measure changes of interstitial flow during tumor invasion in an *in vivo* breast cancer model. We will use MRI imaging to measure changes in interstitial flow to correlate with tumor progression in the mammary fat pad. These studies will begin to address the potential contribution of interstitial flow on cancer progression of ErbB2+ tumors *in vivo*.

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Expected Research Outcomes and Benefits

The hypothesis underlying the proposed work is extremely innovative in current breast cancer research. The hypothesis is paradigm shifting and suggests that there is a connection between interstitial flow and progression from premalignant to malignant invasive ErbB2 positive breast cancers. There is great interest in understanding how premalignant breast cancer, such as ductal carcinoma in situ (DCIS) can progress to invasive breast cancer and how to predict risk of progression for each patient. The potential of interstitial flow to promote progression to invasive breast cancer has to our knowledge not been considered. Demonstration that treatment of DCIS models in vitro with increased interstitial flow results in development of traits of invasive breast cancer and identification of signaling pathways involved would create a field of study concerning novel markers and potential targets for therapeutic intervention in breast cancer. Moreover, validation that increase in interstitial flow is associated with invasion and metastasis using in vivo model is novel and will be tested for the first time. Our work will yield important novel information on the potential contribution of interstitial flow to cancer associated signaling, as well as provide an intellectual basis for other researchers in the field of breast cancer signaling to examine relationships between interstitial flow and cancer invasion in their own systems related to understanding mechanisms of oncogenic progression and signaling.

Summary of Research Completed

Aim 1—Determine the effects of interstitial fluid flow on ErbB2+ breast cancer cell invasion. We will characterize a flow invasion assay incorporating *in vivo*-like 3D cell structures and quantify the effects of interstitial fluid flow on breast cancer cells invasion.
Progress:
-See last progress report.

Aim 2—Elucidate mechanisms of interstitial flow mechanotransduction in ErbB2+ breast cancer cells. Our initial focus will be on chemokine signaling and the role of PI3K. We will determine the role of chemokines in our system.
Progress:

Although it has previously been shown that interstitial fluid flow plays a role in cancer invasion, the molecular mechanisms underlying its effects are poorly understood. During this funding period, we have identified the role of key intracellular signaling pathways that mediate IFF-induced invasion. In all cell lines tested, IFF induced PI3K phosphorylation. The upstream activators and downstream mediators of PI3K however were identified to be EMT-dependent. In pre-invasive cells (pre-EMT NeuN), IFF induced PI3K activation in a chemokine-independent manner and downstream signaling via the p110α catalytic subunit, leading to increased invasion. In invasive EMT cells (NeuT, NeuN^{EMT}), IFF activated PI3K through CXCR4, leading to signaling through both p110α and β catalytic subunits and increased invasion. We hypothesize that EMT cells invade in response to IFF via CXCR4/CXCL12-dependent autologous chemotaxis similar to what was previously found. We proposed a mechanism of interstitial fluid flow in breast cancer depicting the separate signaling pathways that are activated in response to IFF in our cell model but all converge at the PI3K heterodimer (Fig. 1).

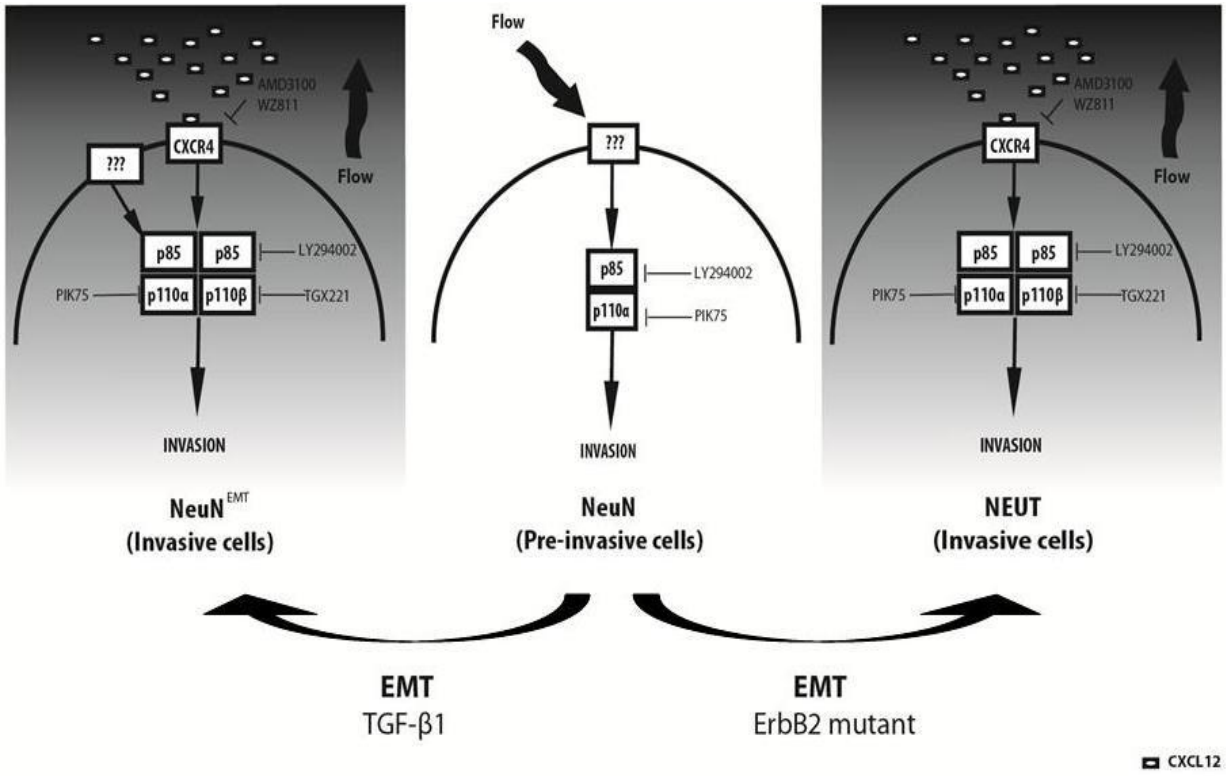

Figure 1: Proposed mechanism of interstitial fluid flow in breast cancer. Separate signaling pathways are activated in response to IFF in our cell model but they all converge at the PI3K heterodimer. In pre-invasive cells (NeuN) IFF activates PI3K activation through an unknown receptor leading to increased invasion via the p110α catalytic subunit. In invasive cells similar to those that have undergone EMT (NeuT, NeuN^{EMT}), IFF activates PI3K through CXCR4 which leads to increased invasion via both p110 catalytic subunits. This possibly occurs because IFF creates a CXCL12 gradient around the cells leading to invasion in the direction of IFF

Taken together, our results suggest for the first time that interstitial fluid flow increases invasion of cells via separate mechanisms depending on the stage of the cancer cells. The effect IFF plays

on separate stages of breast cancer had never been studied, especially in the context of how it may influence the early steps of tumor progression. We demonstrated that IFF increases the invasion of different types of human mammary breast cancer cells through activation of PI3K. Further studies will include identifying the PI3K effector in NeuN, visualizing IFF-induced CXCL12 gradients around NeuT, and validating these findings on HER negative cell lines and in three-dimensional acini structures. Understanding these cellular responses to interstitial fluid flow will revolutionize our understanding of how biophysical forces interact with molecular factors to drive breast cancer progression and contribute to resistance to targeted therapy.

Aim 3 Progress:

Measure changes of interstitial flow during tumor invasion in an *in vivo* breast cancer model. We will use MRI imaging to measure changes in interstitial flow to correlate with tumor progression in the mammary fat pad. These studies will begin to address the potential contribution of interstitial flow on cancer progression of ErbB2+ tumors *in vivo*.

The goal of this aim was to begin to measure changes in interstitial flow during tumor growth and invasion in vivo. Using transplanted mammary tumor epithelial cells (MTECs) derived from MMTV-*neu* transgenic mice into mammary fat pad we planned to measure changes in interstitial flow. Although initially we had made MTEC-Neu cells stably overexpressing luciferase that would allow us to monitor growth and spread in vivo, we quickly lost expression of luciferase during subsequent splitting of cells. We were unable to infect these mouse cells with high enough titer and make stable cells containing luciferase gene. Nevertheless, we injected 5 mice with these cells and only 2 out of 5 mice injected formed tumors (a 40% success rate compared to 90% success when we inject other breast cancer cells MDA-MB-231). Since we were able to make MCF10A-NeuT cells stably overexpressing luciferase, we also injected these cells into mammary fat pad. However, only 1 out of 5 mice formed tumor (a very small tumor). Thus we were unable to get a consistent tumor formation with these ErbB2-overexpressing cells. In future studies we will attempt to use other ErbB2/HER2 overexpressing cells including BT-474 or MDA-MB-453 to measure interstitial flow during tumor growth and spread.

Research Project 9: Project Title and Purpose

An Analysis of Mitochondrial Mutation and HIV Antiretroviral Therapy – This project will examine the potential for mitochondrial changes to be used to predict clinical outcomes in HIV positive patients.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

We have developed a novel approach to the analysis of changes in the mitochondria which offers potentially novel insights into the effect of antiretroviral therapy (ART) on mitochondrial function and may provide a rapid screen to identify patients most at risk for neurocognitive decline. Our object was to identify an approach which will allow us to quantify the relative susceptibility of an individual to ART. We have identified mitochondrial mutations associated with ART and we predict that this increased mutation rate represents an increased intrinsic susceptibility to therapy. Further, we predict that patients exhibiting this phenotype are more likely to suffer neurocognitive decline with age and continued antiretroviral therapy.

Given this background, we propose to examine the mitochondrial mutation rate in patients being cared for in the Drexel University College of Medicine HIV/AIDS clinic, located in Philadelphia, Pennsylvania, USA and correlate the mutation rate with neurocognitive function in these patients using a battery of neurocognitive analyses that specifically test prefrontal dementia.

Specific Aims:

1A. Perform a retrospective analysis of mitochondrial mutations in two defined regions of the mitochondrial genome using DNA samples derived from patients enrolled in the Drexel University College of Medicine HIV/AIDS Genetic Analysis Cohort.

1B. Perform a longitudinal analysis of mitochondrial mutations in patients enrolled in the Drexel University College of Medicine HIV/AIDS Genetic Analysis Cohort.

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Expected Research Outcomes and Benefits

The project will provide the ability to tailor individual therapy for antiretroviral drugs based upon the patient's susceptibility to these drugs. This will help to limit the long-term impact of antiretroviral therapy on patient health.

Summary of Research Completed

Progress

A total of 434 patients have been enrolled into the DREXELMED HIV/AIDS Genetic Analysis Cohort to assess the role that genetic variation within the HIV-1 sequence plays in disease progression and potentially in the development of neurological complications. We collected blood from these 434 patients for drug testing and viral genetic assessment and determined clinical parameters for each patient at each visit. A total of 869 visits have been completed since the start of the project. Samples have been obtained from patients that are both naive to ART and are currently receiving ART and the level of compliance has been evaluated. Peripheral blood mononuclear cell DNA samples were obtained from the DrexelMed/HIV Genetic Analysis cohort. Due to the fact that the cohort has been followed longitudinally for several years, it was possible to obtain matched samples obtained at first visit when patients were naive to therapy and following 1.5-2 years of ART. This CURE grant funds an ongoing longitudinal evaluation of mitochondrial genetics in the DREXELMED HIV/AIDS Genetic Analysis Cohort. The work is performed in collaboration with an ongoing cognitive assessment that is being performed on these patients. To date 118 patients have undergone a full cognitive assessment provided a unique resource for the identification of mitochondrial markers and genetic polymorphisms correlated with poor cognitive status. The CURE funds have supported the mitochondrial sequence analysis which is ongoing. A total of 38 patients have undergone complete mitochondrial sequence analysis and mitochondrial sequencing is underway for an additional 80 patients. This will provide a cohort of 118 patients for whom both full cognitive assessment and full mitochondrial sequence data is available.

Mitochondrial Haplogroup Analysis

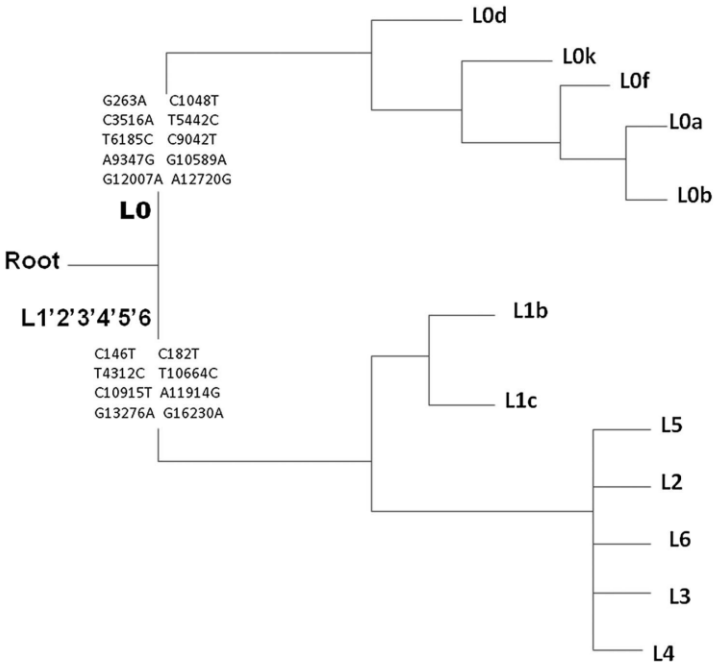
Recent studies have uncovered a relationship between the individual's mitochondrial haplogroup and HIV progression, the risk for Alzheimer's disease. In addition an analysis of patients in the CHARTER study (a large cohort longitudinal study of HIV positive individuals) revealed a decreased incidence of peripheral neuropathy in patients with the European mitochondrial haplogroup J and the African subgroup L1c receiving HAART. We are determining the mitochondrial haplogroup for the118 patients who have undergone the neurocognitive evaluation procedure developed by Dr. Libon. Additional patients who undergo the neurocognitive evaluation are also screened for mitochondrial haplogroup, circulating mitochondrial DNA, and are evaluated for mitochondrial mutation rate. This effort provides a growing database that includes neurocognitive evaluation and a variety of mitochondrial markers. This database is used both to identify correlations within the DrexelMed HIV/AIDS Genetic Analysis Cohort but also as a resource for comparisons with other HIV/AIDS cohorts such as the CHARTER study.

A total of 118 patients have undergone a comprehensive neurocognitive evaluations established by Dr. Libon and colleagues. This ongoing effort allows us to evaluate mitochondrial damage, mitochondrial DNA, and mitochondrial haplogroups as correlates to neurocognitive status.

During the current reporting period we have obtained complete mitochondrial genome sequences for 38 patients who have undergone full neurocongitive assessments. DNA samples from a further 80 patients are currently being processed for sequencing. The current set of sequences provides insight into the composition of the genetic profile of the cohort. The mitochondrial haplogroup of the majority of patients sequenced to date belongs to the African subgroup (Figure 1 A and B). Interestingly, a differential susceptibility to peripheral neuropathy has been described in HIV positive individuals receiving stavudine therapy. This association will be examined in the expanded cohort.

An additional analysis of point mutations associated with specific diseases revealed numerous mutations that may predispose to schizophrenia, encepalopathy, cardiomyopathy, and chronic migraine syndrome (Figure 2). Among these mutations are mutations that show greater disease association in specific haplogroups such as the encepalopathy mutation, which is associated with the L2b subgroup. These associations will be examined in greater detail once the sequence analysis of all 118 patients is completed and will be reevaluated as the study continues.

A



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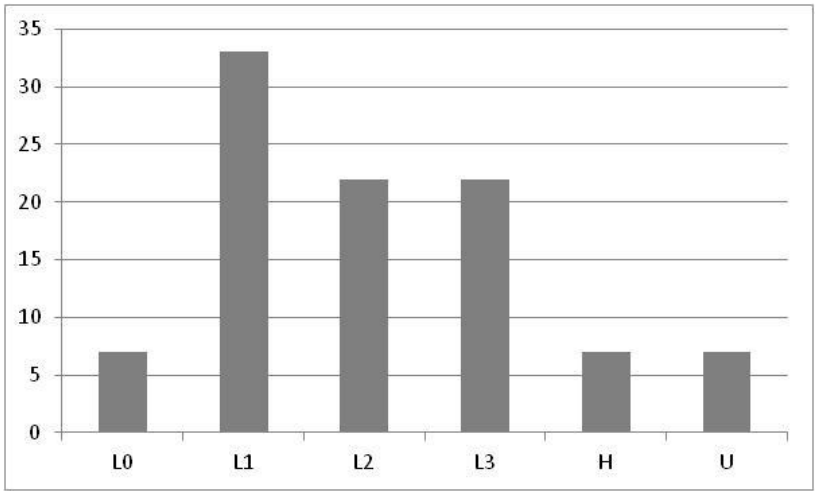

Figure 1. Haplogroup distribution in the DrexelMed HIV/AIDS Genetic Analysis Cohort
Complete mitochondrial sequence was obtained on 38 patients who have undergone complete neurocognitive analysis. Haplogroup assignment was performed using the Mitotoolbox program.
A) Schematic of African mitochondrial haplogroups.
B) percent distribution of mitochondrial haplogroups within the DREXELMED HIV/AIDS Genetic Cohort.

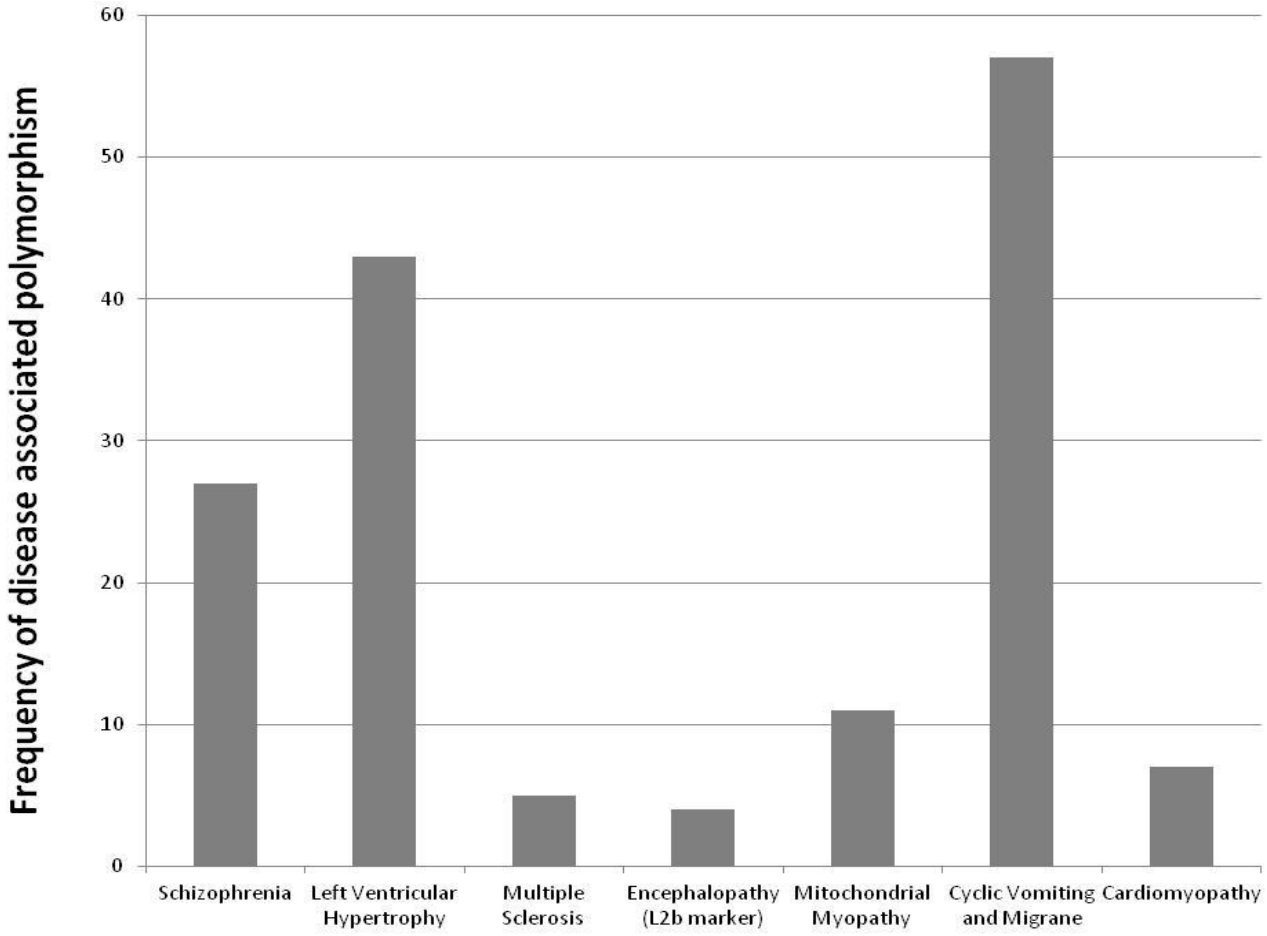

Figure 2. Mitochondrial disease associated mutations in the DrexelMed HIV/AIDS Genetic Analysis Cohort. Disease specific point mutations were identified using the Mitotoolbox program and are presented as the number of mutations within the 38 patients analyzed to date.