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TITLE: Animal Models of Hereditary Peripheral Neuropathies.

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The following is a progress report for a Moran Foundation award to Dr. G. J. Snipes in the Department of Pathology, Baylor College of Medicine. Our long-term goal is to understand the molecular basis of myelination, from axon-Schwann cell contact to the expression of myelin genes. Achieving this goal has been facilitated by the finding that mutations in a small number of genes encoding for myelin structural proteins (Peripheral Myelin Protein-22, *PMP22*; Myelin Protein zero, *MPZ/P0*; Connexin 32, *GJβ1*), molecules putatively regulating axon-Schwann cell interactions (e.g. *MTMR2*, *GDAP*), and transcription factors that may directly regulate myelin gene expression (*SOX10* and *EGR2*), give rise to heritable neuropathies known as the Charcot-Marie-Tooth (CMT) related disorders that are characterized by myelin loss. Understanding the pathogenesis of these mutations will provide important insights into the molecular mechanisms important for myelination and Schwann cell biology.

The specific aims of the original proposal are explicitly stated followed by a narrative where progress towards the aims is provided. Overall, our goals were twofold: 1) to understand the pathogenesis of Pmp22 associated neuropathies; and, 2) to understand the regulation of myelin gene expression. As the original application was rather ambitious relative to the size of our laboratory, many of our proposed studies are ongoing.

Aim 1. Determine whether sequestration of CNX is a mechanism for the *Tr-J* neuropathy
Aim 2. Determine whether prolonged association between PMP22 and CNX (or other components of the CNX cycle) is a mechanism for the *Tr-J* neuropathy
In order to investigate the role of ER association degradation (ERAD) pathways in the *Tr-J* neuropathy, we have obtained cDNAs for CNX (EST consortium) and ER mannosidase I (in collaboration with R. Sifers , Baylor and K. Moremen U. Ga) and have cloned them, individually, bicistronic with the hygromycin resistance gene so that we can monitor the effects of co-transfections (e.g. PMP22-GFP and ER mannosidase I) in pools and at the single cell level. Activity of ER Mannosidase I has been postulated to regulate ER egress of abnormal glycoproteins via degradation pathways. As both aggresome and intracellular myelin figure formation are believed to be ERAD pathways, ER mannosidase I may play an important role in either the pathogenesis of CMT or its treatment. To test whether limiting CNX provided a basis for the intracellular accumulation of PMP22-*Tr-J*, we produced stably transfected 293 cells over-expressing CNX and transiently transfected the various PMP22-GFP test constructs (PMP22wt, PMP22N41A, PMP22*Tr-J*, and PMP22N41A/*Tr-J*) into the CNX or wild-type 293 cells. Over-expression of CNX was confirmed by Western blot. Quantitation of the intracellular aggregates formed by the various PMP22-GFP constructs in each of the transfected cell lines did not reveal any difference due to the presence of increased CNX. We have been unable to create 293 cells stably over-expressing ER Man I. This is probably due to toxicity associated with increased degradation of newly synthesized proteins that is promoted by over-expression of ER Man I. We plan to make inducible ER Man I over-expressing cell lines to test for the ability of ER Man I to enhance the degradation of mutant PMP22 in a pulse-chase paradigm in short term experiments.

As an extension of aim 1, we have investigated whether ER retention is a common mechanism for PMP22 and myelin protein zero (*MPZ*/P0) mutations. We screened a variety of PMP22- and P0-GFP fusion constructs carrying representative disease causing mutations and found that PMP22 is commonly associated with ER retention whereas only a few MPZ/P0 mutations are intracellular retained. We also found distinguishable ER retention mechanisms between PMP22 and MPZ/P0 based on colocalizations with different ER chaparones. Additionally, we eliminated the sole N-glycosylation site in PMP22 by site-directed mutagenesis (PMP22N41A) and created an N-terminal GFP fusion protein. By transient transfection, the PMP22N41A-GFP fusion protein is expressed on the plasma membrane indicating that the glycosylation site, (and CNX binding), is not necessary for PMP22 assembly and transport to the plasma membrane. We produced a double mutant PMP22 protein, PMP22*Tr-J*-N41A-GFP, to test whether the absence of the glycosylation site would allow the *Tr-J* mutant PMP22 protein to escape ER quality control. When transiently transfected into 293 cells, the compound mutant PMP22 protein was retained in the ER compartment and formed intracellular inclusions. Double-label immunofluorescence confirmed that the doubly mutant PMP22 was no longer associated with CNX in the abnormal intracellular inclusions. Immunofluorescence studies, however, revealed that the doubly mutant PMP22 protein colocalized in intracellular inclusions with another ER chaperone protein, Bip. Thus, there appears to be functional redundancy for ER retention of mutant proteins, at least for PMP22. These experiments are ongoing.

Aim 3. Identify signal transduction pathways involved in the down-regulation of myelin gene expression in the *Tr-J* neuropathy

We believe that PMP22 mutations cause significant stress to the ER as a basis of the "gain of function" of this class of mutations. We hypothesized that the ER stress is transduced to the nucleus where, among other things, myelin gene expression is down-regulated to explain low levels of myelin protein mRNA production observed in Tr mice despite the paucity of myelin. As previously reported using semi-quantitative PCR to interrogate the activation of the UPR as assess by upregulation of BiP and CHOP, we have demonstrated that the UPR is not significantly activated in *Tr-J* sciatic nerves. We have also screened for whether the ER overload response is activated in *Tr-J* sciatic nerves. The ER overload response is associated with activation of nuclear factor-kB. In collaboration with Dr. P. Barker, McGill University who has engineered a transgenic mouse expressing *lacZ* under the control of an NF-κB responsive element (NF-kB reporter mice), we have crossed the NF-κB reporter mice with *Tr-J* mice and assayed for altered β-galactosidase activity in peripheral nerves. These studies also yielded negative results in that there was no difference in the *lacZ* activity in the peripheral nerves of normal compared to *Tr-J* mice. Our plan it two-fold: We have generated transgenic mice in which the PMP22 gene controls *lacZ* expression. We will breed the PMP22/*lacZ* mice with the *Tr-J* mice to determine the time course of PMP22 inactivation (as evidenced by changes in β-galactosidase expression) during the course of the *Tr-J* neuropathy. Then, as we proposed as a contingency, we will screen cDNA microarrays with cDNAs prepared from normal and *Tr-J* sciatic nerves taken at appropriate time points based on the results of the breeding studies to determine which ER quality control pathways are perturbed.

Aim 4. Develop a mouse model for studying the pathogenesis of Dejerine-Sottas syndrome.
Aim 5. Develop a mouse model for studying the effects of the SOX10 mutation on myelination.
During the last year we have developed a multipurpose plasmid based cloning vector for the production of transgenes targeting cDNAs to myelinating Schwann cells *in vivo*. We created a novel 8-base cutter rich polylinker in a modified pBluescript vector and have subcloned the Schwann cell enhancer (SCE) from the myelin basic protein gene fused to a minimal heat shock protein (HSP) 68 promoter driving the expression of a bicistronic expression cassette composed of a multicloning site for cDNA expression followed by the internal ribosome entry site (IRES) derived from poliovirus for translation of a 3' enhanced green fluorescent protein (EGFP) reporter cassette. We are now subcloning several cDNAs into this vector. These cDNAs include CNX (see aim 1), EDEM (aim 2), dominant negative EGR2 (aim 4) and dominant-negative SOX10 (aim 5).

Mpz/P0 is an excellent model for the study of myelin gene regulation by SOX10. The *Mpz*/P0 gene is relatively small (7 kb) [Feltri, 1999 #1012]; postnatally, it is only expressed in Schwann cells; it encodes for a single mRNA species; and, appropriate *Mpz*/P0 /*lacZ* constructs, which require both 3' and 5' elements relative to the first coding exon, can efficiently model endogenous *Mpz*/P0 gene regulation [Feltri, 1999 #1012]. Accordingly, we have isolated bacterial artificial chromosomes containing the murine *Mpz*/P0 gene and contiguous sequences known to be required for efficient *Mpz*/P0 expression. 1) We have engineered a series of *Mpz*/P0/*lacZ* gene reporter constructs by cloning *lacZ* in frame with the start codon for *Mpz*/P0 and deleting 3' and 5' elements to define the minimal portion of the *Mpz*/P0 gene required for efficient reporter gene expression in myelinating Schwann cells. Our preliminary results indicate that a –6, +7 kb (1/1 lines) and –5, +3 kb (2/2 lines) *Mpz*/P0/*lacZ* construct in transgenic mice targets β-galactosidase to myelinating Schwann cells. 2) Though preliminary, we have successfully targeted the –6, +7 *Mpz*/P0/*lacZ* construct to the *Hprt* locus in ES cells and derived chimeric mice (in collaboration with Dr. Martin Matzuk, Baylor College of Medicine) that express high levels of β-galactosidase in the peripheral nerves that are present in a small tail biopsy stained with Bluo-gal. As one of our goals is to study the role of SOX10 in myelin gene expression, we have mutated potential SOX10 binding sites in the proximal *Mpz*/P0/*lacZ* transgene in order to inactivate their function. We will then study the effects of these mutations on *lacZ* expression *in vivo* following targeting to the *Hprt* locus in ES cells and generating transgenic mice. If eliminating the SOX10 binding sites affects *Mpz*/P0 expression, this would be the first in vivo evidence for the function of any transcription factor in the regulation of compact myelin proteins. In addition, since SOX10 mutations cause demyelinating neuropathies, it would be strong evidence that SOX10 mutations could directly affect myelin gene regulation as a basis for hereditary neuropathies.

In summary, we have made good progress towards understanding the effects of the *Tr-J* mutation in *Pmp22* in the biology of Schwann cells and in developing tools for the study of the regulation of myelination. We appreciate the support of the Moran Foundation.