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James H. Millonig, PhD
Resident Faculty Member
Center for Advanced Biotechnology and Medicine
Assistant Professor
Department of Neuroscience and Cell Biology
UMDNJ-Robert Wood Johnson Medical School
679 Hoes Lane
Piscataway, NJ 08854-5638
Tel: 732 235 3391
FAX: 732 235 4850
Email: Millonig@CABM.rutgers.edu

Grant Title: The development of spinal cord therapies through a genetic analysis of mouse spinal cord development

Grant Number: 04-30033-SCR-E-0

Grant Period: 7/01/04-6/30/07

Date: 3/10/08

Summary of Research:
The research funded by the New Jersey Commission on Spinal Cord Research is focused on a mouse mutant called *vacuolated lens* (*vl*). *Vacuolated lens* mutants display spina bifida, which is the failure of the developing neural plate to fold and fuse to generate the spinal cord. Spina bifida is a common human disorder that has a multi-factorial basis (genetic predisposition plus environment) and often results in leg weakness or paralysis.

Our research goals were 2-fold: one, to identify the gene disrupted by the *vl* mutation so that new pathways important for neural tube closure and spinal cord development could be discovered, and two, to develop a mouse model that reflects the multi-genic basis of human spina bifida.

Our genetic cross demonstrated that the *vl* spina bifida/spinal cord phenotype was due to a mutation in the orphan G protein coupled receptor (GPCR) called *Gpr161*. GPCRs constitute a large superfamily of proteins that are commonly used by cells to sense and respond to their environment. There are over 360 non-sensory GPCRs in the human genome. The ligand for ~200 of these receptors have been identified while the remaining 160 receptors are orphan GPCRs because their endogenous ligands are not known. It is well established the environmental signals are important for folding and fusion of the neural plate as well as later aspects of spinal cord development (Moury and Jacobson, 1989). The identity of these environmental factors is largely unknown. Thus, our cloning of the *vl* locus has identified a previously unrecognized ligand-receptor system necessary for neural tube closure and spinal cord development.

Our genetic cross also demonstrated that the *vl* spina bifida phenotype could be rescued by unlinked genetic loci. Five modifying loci (Modifiers of *vacuolated lens*-Modvl) were mapped (*Modvl1-5*) and established *vl* as one of the first multi-genic mouse models for studying the basis of human spina bifida (see attached, Matteson et al., 2008; Korstanje et al., in preparation).

These findings led to the aims of our proposal:
Aim 1:
Define the expression pattern of *Gpr161* during development
Determine the effect of the *vl* mutation on Gpr161 function
Characterize the *vl* embryonic spinal cord phenotype

Aim 2:
Determine whether two of the modifiers (*Modvl4* and *5*) were sufficient to rescue *vl* spina bifida phenotype

Aim 1 update
Define the expression pattern of Gpr161 during development
RT-PCR experiments determined that *Gpr161* was expressed in developing mouse embryos (E8.5-11.5). We then determined the spatial expression pattern of *Gpr161* by performing *in situ* hybridizations. During spinal cord development, we demonstrated that *Gpr161* transcripts were restricted to the lateral neural folds of the neural plate (E8.0-E9.5) and then later in dividing neuronal progenitors (see attached, Matteson et al., 2008). These experiments demonstrated that *Gpr161* is expressed in the developing CNS, identifying a novel receptor necessary for spinal cord formation and development.

The *vl* mutation also causes congenital cataracts. Consistent with this mutant phenotype *Gpr161* is expressed at all stages of lens development: lens pit (E10.5), lens vesicle (E11.5), primary lens fiber cells (E12.5) and differentiating secondary lens fiber cells (E14.5)(Matteson et al., 2008).

Gpr161 is also expressed in a number of other structures from E9.5-E12.5 including the fore and hindlimbs (E12.5) and the retina (E10.5-E14.5), suggesting a role for *Gpr161* in their development (Matteson et al., 2008).

Determine the effect of the vl mutation on Gpr161 function

The *vl* mutation (8 bp deletion) is located in exon 4 of the *Gpr161* and is expected to cause a frameshift and premature stop codon 50 nucleotides 3' of the deletion. This results in the incorporation of 16 novel amino acids (GAHGRRTVPGTQQQHR) and truncation of the GPCR at residue 386, deleting 143 (of 203) amino acids of the C terminal tail (Fig 1d, Matteson et al., 2008). The mutant protein is predicted to be ~15 kDa smaller than wild type (wt) Gpr161 (ca.expasy.org). Western analysis performed on lysates isolated from cells transfected with N-terminal myc-tagged wt and mutant (*vl*) Gpr161 identified two major isoforms, with the smallest band migrating at the predicted size (~58 kDa) and a larger band at ~70 kDa (Fig 1c, Matteson et al., 2008). This larger band likely represents a post-translationally modified version of Gpr161 (ca.expasy.org, McVector v9.0). The mutation reduces both protein products by ~15 kDa, consistent with the mutation truncating the C terminal tail. Reduced steady state levels of the mutant isoforms were also observed (Fig 1c).

Receptor-mediated endocytosis is a common mechanism by which GPCR signaling is attenuated and is regulated by C terminal tail phosphorylation. To investigate the effects of the mutation on Gpr161 plasma membrane targeting and intracellular localization, wt and *vl*-myc-Gpr161 constructs were transfected into HEK293T cells. To distinguish cell surface versus intracellular receptors, these studies were performed under non-permeabilized and permeabilized conditions. In non-permeabilized cells, both wt and *vl*Gpr161 were targeted to the plasma membrane. In permeabilized cells, a different staining pattern was detected with wtGpr161 displaying an intracellular punctate pattern and *vl*Gpr161 localized to the cell surface (Supplemental Fig 3, Matteson et al., 2008). To characterize this difference further, wt and *vl*Gpr161 plasmids were either co-transfected with expression constructs that target GFP to different subcellular compartments (plasma membrane, ER, nucleus) or the transfected cells were incubated with FITC-labeled Transferrin, an endosome marker. For wtGpr161 significant co-localization with Transferrin was observed, indicating that Gpr161 is present in endosomes. Minimal co-localization was observed with GFP targeted to the plasma membrane (Fig 3; Supplemental Fig 4, Matteson et al., 2008) while no overlap with ER or nuclear-GFP was observed (data not shown). For *vl*Gpr161, co-localization was detected for plasma membrane targeted GFP but not with FITC-Transferrin (Fig 3; Supplemental Fig 4, Matteson et al., 2008), ER-GFP or nuclear-GFP (data not shown). These results demonstrate that *vl* affects Gpr161 intracellular localization and disrupts Gpr161 internalization from the plasma membrane into the endosome compartment. Since endocytosis of GPCRs attenuates receptor signaling, these finding are consistent with the *vl* mutation affecting Gpr161 signaling during development.

Characterize the vl spinal cord phenotype

Previous published reports indicate that the dorsal midline of the developing spinal cord is attenuated in *vl* mutant embryos. This published phenotype raised the hypothesis that the roof plate, a signaling center located on the dorsal midline and necessary for spinal cord development, might be perturbed by the *vl* mutation. We have now performed *in situ* hybridizations for a series of roof plate markers (*Bmp6*, *Bmp7*, *Gdf7*, *Wnt1*, *Lmx1a*) and have discovered that their domain of expression is wider in *vl* mutants than control embryos. This was further confirmed by counting the number of roof plate cells using immunohistochemical markers. These data are consistent with the *vl* mutation resulting in an increase in the number of roof plate cells. Thus, Gpr161 function is needed for roof plate development.

Because roof plate signaling is required for subsequent spinal cord development, Gpr161 and its ligand may be used in the future as possible therapeutic to direct stem cells towards a spinal cord neuronal fate.

Aim 2 update

Determine whether two of the modifiers (Modvl4 and 5) were sufficient to rescue vl spina bifida phenotype

Our *vl* genetic cross that identified the mutation in *Gpr161* also mapped the genetic positions of the *vl* modifiers. This analysis mapped 5 modifiers and we discovered that MOLF alleles for chromosome 15 (*Modvl4*) and chromosome 18 (*Modvl5*) segregated with the absence of any obvious *vl* phenotype (see attached, Korstanje et al., in preparation). This suggested that MOLF alleles on these chromosomes were sufficient to rescue all the *vl* associated phenotypes including spina bifida.

However, because these initial mapping experiments were performed on mice that had a mixture of C3H and MOLF alleles throughout the genome, it was unknown whether the MOLF alleles for *Modvl4* and *5* were sufficient to rescue the *vl* phenotypes. To test this possibility, we generated *Modvl4* and *Modvl5* congenic mice where the entire genome was C3H expect for the important regions of chromosome 15 or 18, which retained the MOLF alleles.

To generate these congenics, C3H and MOLF wild type mice were mated to each other. The F1 progeny (C3H/MOLF) were then crossed back to C3H mice. At the next generation we selected mice that were MOLF for chromosome 15 or 18 but had the greatest proportion of C3H alleles for the rest of the genome. We continued this mating scheme and analysis for 6-8 generations until the entire genome was C3H except for the desired regions of chromosome 15 or 18 that were MOLF. We are in the final stages of generating the *Modvl4* congenic but have successfully generated the *Modvl5* congenic.

The *Modvl5* congenic mice were then mated to the *vl* mutant that are on an isogenic C3H background. This produced F1 *+/vl*, *+/Modvl5* $^{C3H/MOLF}$ mice, which were then mated back to *+/vl* mice. If the MOLF alleles on chromosome 15 were sufficient to rescue the *vl* phenotypes, we would expect *vl/vl Modvl5* $^{C3H/MOLF}$ mice to be phenotypically normal while littermate *vl/vl Modvl5* $^{C3H/C3H}$ mice would display defects. This is exactly what we observed (number of mice=229, P=.0005), indicating that one copy of chromosome 15 MOLF alleles is sufficient to rescue the *vl* phenotypes including spina bifida. These results further establish *vl* as one of the first multi-genic mouse models of spina bifida.

Foxe3 and Modvl3

We also mapped a modifier to chromosome 4 that specifically affected the *vl* cataract phenotype, *Modvl3*. To identify candidate genes, this region of chromosome 4 was scanned for biologically relevant candidates based upon expression and disease phenotypes. This analysis identified *Foxe3*, which is a winged helix forkhead transcription factor expressed in the developing lens and when mutated causes cataract and other lens associated diseases in humans and mice. Knock-down of *Foxe3* in zebrafish also leads to a lens phenotype (Medina-Martinez and Jamrich, 2007). *Foxe3*^{C3H} and ^{MOLF} were sequenced and two novel SNPs (mouse dbSNP build 127) were identified: a T to C transition at bp 68 and an A to C transversion at bp 499. The transversion does not result in an amino acid change while the T^{C3H} to C^{MOLF} transition replaces a leucine (L^{C3H}) with a proline (P^{MOLF}) at amino acid 23 in the N terminus of the protein (Fig 4a, data not shown, Matteson et al., 2008).

This region of *Foxe3* was then sequenced in 22 other mouse strains. 18 of the strains had the A allele (L^{23}) at bp 68 while only four had a C allele (P^{23})(Supplemental Fig 8a, Matteson et al., 2008). Although P^{23} is not commonly observed in different mouse strains it is evolutionarily conserved in rat, cow, rhesus, chimp and human (Fig 4b, Matteson et al., 2008). Given that proline commonly disrupts protein secondary structure, bioinformatics for Foxe3^{C3H} and ^{MOLF} were performed. For Foxe3^{C3H} a b-sheet is predicted to extend from amino acids 22-28 followed by an a-helix from 28-34. The P^{23} substitution in Foxe3^{MOLF} shortens the b-sheet and inserts a turn at amino acid 28 preventing the formation of the a-helix (Supplemental Fig 8b, Matteson et al., 2008). These bioinformatic data along with the evolutionary conservation of the proline suggests that the L^{23} to P^{23} alteration could functionally alter Foxe3.

If this amino acid change were responsible for the cataract modifying effect, we would predict that genetic backgrounds with the L^{23} allele would not affect the penetrance of the *vl* cataract phenotype. To investigate this possibility, a *vl* Balb/c intercross was then performed. 109 F_2 C3H/Balb progeny were generated (32 +/+, 63 +/*vl*, 13 *vl*/*vl*) with 100% of the *vl*/*vl* mice displaying an obvious cataract. These findings indicate that the Balb/c background does not modify the *vl* cataract phenotype, consistent with the P^{23} allele in *Foxe3* contributing to the *Modvl3* modifying effect.

We then investigated whether the L^{23} to P^{23} alteration affects the activity of Foxe3. Because the N terminus of other forkhead transcription factors function as trans-activators, $Foxe3^{C3H}$ and ^{MOLF} were co-transfected with a luciferase (luc) construct driven by a consensus Foxe3 binding site (26). To test the effect of the L^{23} to P^{23} alteration, these constructs were transfected into HEK293T cells, which do not express endogenous *Foxe3* (data not shown, cgap.nci.nih.gov/SAGE). Both $Foxe3^{C3H}$ and ^{MOLF} increased luc activity over the binding site alone. Moreover, $Foxe3^{MOLF}$ resulted in significantly lower luc activity than $Foxe3^{C3H}$, indicating that the P^{23} allele functionally alters the transcriptional activity of Foxe3 (Fig 4c, Matteson et al., 2008). The lower activity of $Foxe3^{MOLF}$ is also consistent with the MOLF background enhancing the cataract phenotype. Thus, we provide sequence, protein modeling, genetic and functional data supporting *Foxe3* as a gene responsible for the cataract modifying effect of *Modvl3*.

Our goal now is to use a similar strategy to identify the gene(s) that rescue the *vl* phenotypes for *Modvl5*. Examining the genome for this region of chromosome 18 identified 3 GPCRs as biologically relevant candidates. It is known that GPCRs form oligomeric complexes with each other (Breitwieser, 2004, Milligan, 2004) so if these GPCRs interact with Gpr161 they may be able to bypass the effect of the *vl* mutation. We are investigating this possibility and since the ligand for these candidate GPCRs is known, if our hypothesis is correct this would suggest that the same ligand binds to Gpr161.

Project challenges: Nothing out of the ordinary

Implications for future research: Our Aim 1 analysis has uncovered a new pathway important for neural tube closure, roof plate and subsequent spinal cord development. The identification of the environmental factor that acts as a ligand for Gpr161 could be used to decrease the incidence of spina bifida in a manner similar to folic acid. Since signaling from the roof plate is required for proper spinal cord development, Gpr161 and its ligand could be used in the future by stem cell biologists to generate roof plate and spinal cord neurons in individuals affected by spinal cord disease and injury.

Our Aim 2 analysis has identified modifiers that can rescue *vl* Gpr161 phenotypes, indicating that these modifier genes likely function in the same pathway as Gpr161. Our goal is to clone the genes responsible for *Modvl4* and *5* as we have for *Modvl3*. This will provide additional molecular insight into the causes of spina bifida and the pathways that regulate spinal cord development. It will also potentially identify additional molecular reagents to treat spinal cord disease and injury.

Future plans: Our current plans are to publish two more papers concerning the modifiers and then apply for federal money to continue our research for *Modvl5*.

Publications:

Matteson PG, Desai J, Korstanje R, Lazar G, Borsuk TE, Rollins J, Kadambi S, Joseph J, Rahman T, Wink J, Benayed R, Paigen B, Millonig JH. (2008) The orphan G protein coupled receptor, *Gpr161*, encodes the *vacuolated lens* locus and controls neurulation and lens development *Proc Natl Acad Sci USA* 105: 2088-2093

Korstanje R, Desai J, Rollins J, Lazar G, Spurr M, Joseph J, Kadambi S, Cherry A, Paigen B, Millonig JH. Quantitative trait loci affecting phenotypic variation in the *vacuolated lens* mutant mouse (in preparation *Physiol Genomics*)