Ciliary protein localization in Caenorhabditis elegans: a review exploring gar-3 as an additional localization factor

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Abstract

gar-3, a gene that encodes a G protein-coupled acetylcholine receptor, plays a role in both neurotransmission and protein localization to synapses. We have begun to ask whether *gar-3* affects the localization of PKD-2 protein to primary cilia in *C. elegans*, a free-living nematode. Dysregulated PKD-2 localization has been linked to polycystic kidney disease in humans. Thus, understanding the factors that mediate PKD-2 protein localization to cilia might help us better understand the molecular basis of polycystic kidney disease. We chose to utilize *C. elegans* as our model organism as it is transparent and has cilia structure similar to that of mammalian kidney cells. In order to address our question, we use both genetic and reverse genetic techniques to reduce *gar-3* gene expression in *C. elegans* transgenic for PKD-2::GFP. These techniques allowed us to utilize fluorescent microscopy to assess PKD-2 localization to primary cilia in a living animal. In this paper, we describe our research in the context of cilia biology, polycystic kidney disease and what is known about *gar-3.*

Understanding ADPKD by Studying Model Organisms

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by fluid filled sacks (cysts) within the kidneys. These cysts crowd out the healthy tissue and ultimately contribute to kidney failure. Mutations in the genes, PC1/PKD-1 and PC2/PKD-2, which encode polycystin-1 and polycystin-2 respectively, cause most cases of ADPKD. The polycystins act as co-receptors and localize to primary cilia of human kidney epithelial cells where they are thought to sense fluid flow. In addition to the changes in polycystin localization and expression, ADPKD involves changes in gene expression of many other genes. Identification of these changes in gene expression may lead to a better understanding of the molecular pathways underlying ADPKD.

ADPKD belongs to a category of genetic diseases classified as ciliopathies; the underlying cellular defect in these diseases is dysfunctional, malformed, or absent cilia. Cilia are projections from the cell membrane and aid in both movement of the cell or fluids and transmission of signals to the cell. Motile cilia are able to move on their own while primary cilia lack the mechanism for intrinsic movement. Cilia are found on nearly all cells in the human body and play different roles depending on their location. For example, cilia lining the renal epithelia in the kidneys may act as flow sensors for urine while cilia in the developing heart play a vital role in the establishment of left-right asymmetry (Dasgupta and Amack 2016). In the Lyman Gingerich lab, we study the relationship between polycystin-2 localization, gene expression, cilia

function, and PKD using 2 model organisms: *C. elegans* and zebrafish.

Although the excretory system of *C. elegans* is not comparable to that of humans, aspects of ADPKD can be studied using *C. elegans* as a model. Primary cilia are found on cell surfaces of many different eukaryotic cells, including human kidney epithelial cells. By contrast, the only ciliated cells in *C. elegans* are a subset of neurons; thus, *C. elegans* can survive without functional cilia while vertebrates cannot. *C. elegans* are used as a model organism for studying ADPKD because the PKD-2 protein localizes to the primary cilia of some of their sensory neurons; this is similar to PKD-2 protein localization to cilia of human kidney epithelial cells. Thus, ciliary protein localization can be easily assessed in *C. elegans.* By tagging PKD-2 with the green fluorescent protein, GFP, the localization of PKD-2 to specific cilia in *C. elegans* can be easily assessed using a fluorescent microscope (Scholey et al. 2004 and Bae et al. 2008).

In order to explore cystic kidney disease in an organism with a kidney more similar to that of humans, we look to the zebrafish. Larval zebrafish have a pronephros (developing kidney) that is similar in structure and patterns of gene expression to the nephron of the adult human kidney. In addition, the pronephros is functional as it develops, and a number of cystic kidney mutants have been identified (Poureetezadi and Wingert 2016). This means that the zebrafish is a viable organism to study polycystic kidney disease and changes in gene expression as cyst development progresses. Our lab has found a number of genes with altered expression in a zebrafish model of cystic kidney disease (our own unpublished data). Because *C. elegans* provide a simpler system for assessing some aspects of cilia form and function, we turn to *C. elegans* for analysis of individual genes. This review will focus on methods of altering gene expression and analyzing PKD-2::GFP localization in *C. elegans* to provide background for analysis of *chrm5a/gar-3.*

chrm5a gene expression has been observed in fish at synaptic junctions and in the retinal pigmented epithelium, a ciliated layer of cells in the eyes. *chrm5a* encodes a muscarinic receptor that has G-protein-coupled acetylcholine receptor activity. In zebrafish larva with cystic kidneys, we observed a decrease in *chrm5a* expression, compared to wild-type larva (our own unpublished data). There could possibly be a link between the decreased expression of the *chrm5a* gene in mutant zebrafish and cystic kidneys. Our lab is currently investigating the role of *gar-3,* the *C. elegans* ortholog of *chrm5a*, in PKD-2 ciliary localization using genetic and reverse genetic approaches. We are using PKD-2::GFP localization as an indicator for PKD-related cilia defects.

The pattern of PKD-2::GFP mislocalization can indicate different types of abnormalities of the cilia. For example, the *cil* genes in *C. elegans* were identified and categorized based on their effects on PKD-2::GFP localization (Bae et. al, 2008). Six mutants were placed in together because they had abnormal distribution of PKD-2 in the dendrites; these genes influence how PKD-2::GFP is targeted to specific subcellular locations. Additionally, these mutants could possibly affect vesicular transport, PKD-2 localization in the endoplasmic reticulum, and PKD-2::GFP in the cilium (Bae et al., 2008). Five additional mutants were placed in a separate group, because the PKD-2::GFP localization within the cilium proper was abnormal compared to wild type. Thus, genes in this second group may specifically regulate PKD-2 localization and maintenance within the cilium itself (Bae et. al., 2008).

These mislocalization patterns in both groups were concurrent with dysfunctional cilia as evidenced by male mating behavior and olfaction assays. Thus, not only the localization of PKD-2 to the cilia but also the subciliary localization and amount of protein localized each has an important effect on cilia form and function. In addition, PKD-2::GFP mislocalization may be indicative of 1)specific defects in the trafficking of the protein, 2) defects in the structure of specific primary cilia, or 3) a more generalized defect in all primary cilia. To determine which is occurring, male mating behavior and olfaction assays are frequently combined with assessments of cilia structure to broaden the scope of cilia assessed.

Male mating behavior in *C. elegans,* is regulated by the male-specific ciliated neurons to which PKD-2::GFP localizes. The mating analysis techniques used in Bae et. al, 2008 assessed response behavior efficiency and mating efficiency because PKD-2 affects the sexual attraction to females, the ability to locate the vulva, and the active response to a potential mate. The results of these analyses demonstrated that mating behavior may not be solely dependent on PKD-2::GFP localization patterns as four mutants, *cil-2(my2), my9, cil-3(my11), and my14*, had normal male mating behavior. Additionally, two mutants, *cil-1(my15)* and *cil-5(my13)*, have normal male mating behavior but cannot reproduce successfully. Therefore, a mutation in one of the *cil* genes can result in defects in fertility, copulation, and/or ciliogenesis in addition to PKD-2::GFP mislocalization.

PKD-2::GFP mislocalization can be due to 1) specific defects in the trafficking of the protein, or 2) the structure of specific primary cilia, or 3) a more generalized defect in all primary cilia. To assess whether other primary cilia are also affected, behaviors dependent on other ciliated neurons can be analyzed. For example, *bbs-7(my13)* mutants have both abnormal mating behavior responses and abnormal chemotaxis to isoamyl alcohol but not diacetyl or benzaldehyde (Braunreiter et al. 2014). Perception of each of these chemicals involves a different set of ciliated neurons.

Dye-filling assays can be used to assess the structural integrity of neurons exposed to the external environment through ciliated endings. For example, in *cil-5(my13)* animals, amphid neurons fill weakly while phasmids are never fill with DiI. On the other hand, *xbx-1*(*my17*) animals are completely dye-filling defective, indicative of more global cilia defects (Bae et al., 2008).

When working in vertebrate kidney cells, a number of additional techniques can be employed. For example, immunolocalization of PC1 and PC2, Ca^{2+} microfluorimetry and generation of affinity-purified antibodies were common methods used to analyze flow-induced Ca^{2+} signaling in embryonic kidney cell cultures (Nauli et. al., 2003). Primary cilium are needed to detect fluid flow in cells, which is required for flow-induced Ca^{2+} signaling. Using mutated embryonic kidney cell cultures, short or no cilia formed with decreased expression of PC1 after spending one day in a medium that promoted cell division. Therefore, a mutation in PC1 affects production of cilia and is required for flow-induced Ca^{2+} signaling. However, Nauli and colleagues also found that using thrombin could increase cytosolic calcium in both mutant and wild-type cells indicating that the defects in the mutant cells were not due to an inability to move Ca^{2+}, but a specific defect in sensing fluid flow (2003).

chrm5a/gar-3 function

chrm5a/gar-3 encodes a muscarinic receptor in zebrafish and *C. elegans*, respectively. Initial characterization of this family of receptors in zebrafish involved cloning, phylogenetic analysis, and immunohistochemistry methods to analyze protein localization. The six genes identified were *chrm1a*, *chrm1b*, *chrm3a*, *chrm5a*, and *chrm5b*, also known as the chrm-odd genes. Using RT-PCR, total RNA count was quantified for four different developmental stages revealing that all genes were expressed at the metamorphic and adult stages, whereas only *chrm1a*, *chrm3b*, and *chrm5b* were expressed during the larval stage. These differences in timing of expression may reveal that the different genes have acquired different roles in development (Nuckels et. al., 2011).

Although we are uncertain how loss of *chrm5a/gar-3* expression might result in cystic kidneys and whether the link might involve changes in cilia receptor localization or cilia structure, we are curious whether this gene might be related to cystic kidney disease. Our approach mirrors that taken with *bbs-7(my13)* and *xbx-1(my17):* to downregulate *gar-3* gene expression and assess both PKD-2::GFP localization specifically and cilia structure and function more globally in *C. elegans.*

Methods and Primary Results

Unlike the research involving *my13* and *my17,* we plan to use both forward and reverse genetic techniques. To produce *C. elegans* which are both homozygous for a mutation in the *gar-3* gene and express PKD-2::GFP, we will cross strain VC657 with PT443. This will also enable us to examine males homozygous for the mutation in *gar-3*. Our second approach involves reducing *gar-3* mRNA using RNAinterference.

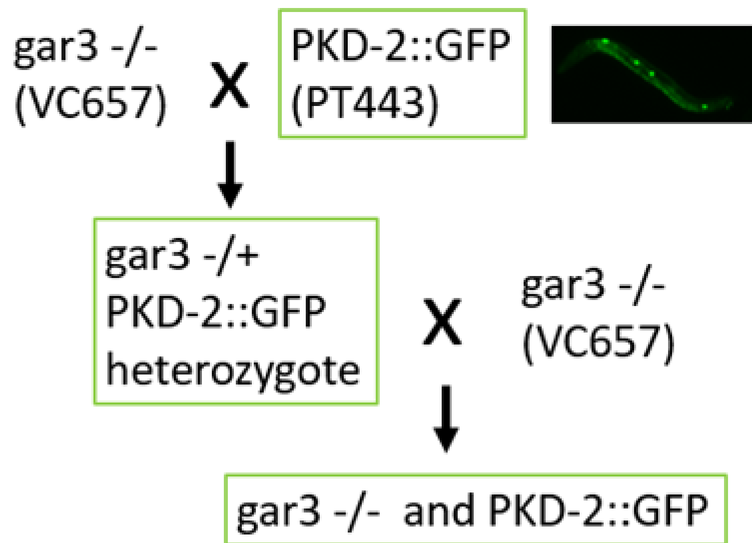

Figure 1. Genetic crossing scheme to analyze PKD-2::GFP localization in *gar3* mutant animals.

With both approaches, to analyze PKD-2::GFP localization to ciliated neurons, male progeny are mounted onto agar pads atop microscope slides with levamisole to prevent movement. *C. elegans* males are analyzed under the 100x oil objective with a GFP filter and UV light. Male-specific neurons in the head and tail normally contain PKD-2::GFP in the cell bodies and the cilia but not in the dendrites connecting the cell bodies to the cilia. In our initial analysis, five of six males homozygous for the mutation in *gar-3* lacked PKD-2::GFP expression in the male-specific tail neurons and exhibited exaggerated head movements. We have not yet done further analysis of the tail neurons to see if the mutation causes defects in the neurons themselves.

Conclusion

The zebrafish and *C. elegans* systems provide complementary advantages to efforts to understand the biology of cystic kidney diseases. Although the zebrafish pronephros is more similar to the human kidney, the optical transparency and accessibility of *C. elegans* cilia, combined with similarities in cilia structure and function, make both effective tools for understanding cilia and, potentially, the role of *gar-3*. Since our study was limited by our small sample size, we cannot draw conclusions about the effects decreased expression of the *gar-3* gene has on protein localization. Moving forward, experiments involving *gar-3* genetic variants including as strains VC670 and JD217 will be explored in addition to the VC657 variant.

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