Damien Islek

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Prof Jeff

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Research Proposal Draft:

Introduction:

Zebrafish are a model organism, which has been the subject of scientific inquiry for some time. The development of neurons has always been an area of interest when trying to understand the human brain and zebrafish are an excellent media to learn about the human nervous system. In zebrafish, neurons are of special interest because their mode of axonogenesis and axon pathfinding in the brain of zebrafish embryo involves a lot of the same pathways which exist in humans. These neurons, during vertebrate development, project axons from their cell bodies and use signaling molecules to guide their growth towards their designated target cell.

In zebrafish, primary commissural ascending neurons (CoPA), are interneurons which, during development, project an axon ventrally which crosses the midline and grows dorsally; where it then turns and ascends the spinal cord towards the CNS (Hjorth & Key 2002; Chitnis & Kuwada 1990). Each CoPA neuron has a target cell in the CNS, and use signaling molecule gradients to mediate theses axons pathfinding and growth. CoPA neurons axon guidance is mediated by Planar Cell Polarity (PCP) and ligand concentration. Wnt is the ligand which regulates PCP in copa neurons of zebrafish; the concentration gradient of Wnt directs axon migration towards the proper target (Hayes et al 2013).

Planar Cell Polarity (PCP) coordinates the uniform orientation of cell structures and cell movements within the plane of tissue (Zallen, 2007). A group of evolutionarily conserved genes affecting PCP in all tissues are often referred to as the “core” PCP genes (Vladar et al 2009); these genes encode Frizzled (Fz), Flamingo (Fmi), Van Gogh (Vangl), Dishevelled (Dsh), Prickle (Pk) and Diego (Dgo) (Vladar et al 2009).

The main action in this pathway, which mediates cell process and cytoskeletal structure, is Frizzled binding to a Wnt ligand (Kohn and Moon, 2005; Jenny and Mlodzik, 2006). Wnt will bind to Fz extracellularly, which will change Fz’s affinity to DsH and cause it to interact with the intracellular protein. DsH then goes on to activate JNK (Boutros et al 1998); JNK is a mediator of cell movement via actin filament structure, so once it is activated by Dsh, it goes on to affect the actin cytoskeleton and change shape/movement (Yamanaka et al 2002). PCP requires Fz activation and subsequent membrane localization of Dsh; Fz activation occurs through the binding of Wnt ligand.

When studying the loss of function when mutant versions of these core proteins are inserted into a vertebrate genome, you see very similar effect. In vangl mutant zebrafish, embryos demonstrated neural tube morphogenesis defects (Hayes et al 2013). PTK7 was found to be a novel regulator in the planar cell polarity pathway (Lu et al 2004; Hayes et al 2013); in the experiment described in Lu et al 2004 it was demonstrated that mice with mutant ptk7 genes displayed a severe form of neural tube defect (NTD). Hayes et al in 2013 reported that ptk7 mutant zebrafish embryos demonstrated neural tube morphogenesis defects similar to those observed with Vangl mutant zebrafish; which is a known core PCP signaling mutant (Hayes et al 2013). So the same defects were seen in ptk7 mutant zebrafish and in vangl mutant zebrafish, suggesting the two are involved in the same signaling pathway, PCP (Lu et al 2004).

PTK-7 is a single polypeptide chain with one transmembrane domain. The extracellular domain is composed of several immunoglobulin like domains. The transmembrane domain is a combination of hydrophobic amino acids which come together to form an alpha-helix in the membrane. The intracellular domain of this protein contains a conserved tyrosine motif (Hayes et al 2013). It is not known which of these domains, if not all, are involved in regulating the PCP pathway in vertebrates. In order to determine which domains of the ptk7 protein are required in this pathway, Hayes et al 2013 tested the ability of deletion and substitution mutants to rescue wnt8 overexpression defects. It was seen that ptk7ΔICD (deleted intracellular domain) and ptk7EgfrTM (exchanged transmembrane domain) were able to rescue the wnt8 induced defects to the same extent as full length ptk7. It was also observed that Ptk7ΔECD (deleted extracellular domain) was not able to rescue wnt8 overexpression defects. This suggested that the extracellular domain most likely is what is regulating the PCP pathway in zebrafish (Hayes et al 2013). This domain could be involved in co-receptor activity with Frizzled and Wnt ligand.

Experiment:

PTK7 Rescue Experiment:

You first start off with a mutant-PTK-7 zebrafish. This mutation will cause this zebrafish to not have a function ptk-7 protein in the membrane to regulate the PCP pathway to help copa neuronal migration. These mutants are able to survive with this mutations, but not without defects; these defects include curvature of the spine and malformation in morphology. Generation of these zebrafish was done by an outside lab and are bread to be ptk7 deficient.

Next you will need to construct plasmids which contains modified versions of ptk7. This first starts with isolating and amplifying coding DNA (cDNA) for ptk7 protein in zebrafish using PCR. Specially designed primers will be used to start and stop the replication of the protein at certain sites to allow for substitution and deletion of the protein. Once these modified isolate and amplified cDNA fragments are complete for experimental set-up, they will be inserted into an entry vector through enzymatic recombination (Gateway Cloning). The plasmid containing the ptk7 cDNA of interest will have the DNA in between two sequences, attB1 and attB2. The DNA in between these sequences will be transferred over to the target vector by BP clonase crossing over attB1/attB2 with attP1/attP2. The entry vector plasmid contains a kanacillin resistance gene, which when grown on a kanacillin agar plate, will ensure that only bacteria containing this plasmid would survive

Once the DNA of interest is in the entry vector it can then be transferred over to a destination vector. This destination vector will have the tol2 transposase sites, the proper heat shock promotor, and poly A tail. The destination vector will be carrying a gene for ampicillin resistance. This will ensure that only bacteria with this plasmid will survive in an ampicillin/agar growth plate. This destination vector is modified to have different things on the tail and front end of the R, sites. On the front side you need a Tol2 site, HSP70, and on the tail end you have a Poly-A section, and Tol2 site.

BP Clonase splices the B and P sequences which forms a new sequence in the plasmid which contains the cDNA of interest, L sequence.

In this experiment, the mutant ptk7 zebrafish will be microinjected with a plasmid and mRNA of a transposon (Tol2) at the one cell stage of development. The plasmid will contain a modified version of ptk7, to either delete the intracellular/extracellular domains or substitute the transmembrane domain. The plasmid will also encode a heat shock promotor, a poly-A tail and GFP and M-Cherry tags; all of this will be in between two Tol2 transposase sites. The heat shock promotor will allow for the controlled activation of transcription of the gene.

Once mRNA and plasmid are microinjected into one cell stage embryo of ptk7 mutant zebrafish, the mRNA takes some time to be translated and folded into fully formed transposase protein, Tol2. Once it is formed, it will randomly insert the genetic information in between the two Tol2 transposase sites into the genome of the mutant zebrafish. By the time the mRNA is folded and plasmid genes are able to be incorporated, the organism will be at around 1064 cells, so not all of the cells will express the rescued version of the ptk7 protein.

Discussion:

Copa neurons are second order sensory neurons in zebrafish which are part of a touch evoked retraction movement. In the transgenic fish generated for this experiment, GFP is expressed in copa neurons; it lights them all up green. These neurons during deveolment in zebrafish without PCP gene defects, will send their axons up the anterolateral track up to the brain. This means the axon must first cross over to the opposite side of the spinal cord than change direction to go up the spinal cord.In fish which express mutant or non-functional versions of PCP core genes, including ptk7, these Copa neurons will tend randomly choose to go up (anterior) or down (posterior) the spinal cord after sending their axons to the opposite side of the spinal cord. If you look at the copa neurons of a matured ptk7 mutant zebrafish that was not affected, you will see close to a 50/50 distribution of axons going anterior and posterior.

In this experiment, the inserted genomic information has M-Cherry, so any cell which expresses the outside genetic code will fluoresce red. Copa neurons which fluoresce red and green are those which express M-Cherry and GFP, so they have the cDNA of the modified protein inserted into their genome. When analyzing the copa neurons of zebrafish in the rescue experiment, the neurons which express GFP and M-Cherry are the only ones you consider when comparing how many axons went anterior vs posterior. In the rescue experiment group whos plasmid had full length ptk7, you should theoretically see 100% of neurons go anterior who express both GFP and M-Cherry.