Zebrfish: A Functional Refuge at the End of an Odyssey

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Editor's Note

For many of the readers of *Zebrfish*, the idea that zebrafish can inform the human disease community is not new. Yet, many of us can recall recent meetings, grant review committees, or other scientific forums when the validity of zebrafish as a model organism for human disease is called into question. “Tank to Bedside” is a quick highlight of the roles zebrafish are playing in the understanding of human disease, whether it be in the development of cancerous lesions, providing a model for drug efficacy in precision medicine studies, or as a functional validation of genomic sequencing variants identified in human patients with rare diseases. With seventy percent of human genes having at least one zebrafish orthologue, zebrafish are well established as a first “go-to” for novel disease gene functional studies. Each edition of “Tank to Bedside” will review some of the exciting ways that zebrafish are the best model for identifying mechanisms of human disease and provide talking points that you can use to talk with your nonzebrafish colleagues in politics, at parties, and even around the dinner table. Enjoy.

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FOR PEOPLE, BOTH ADULTS and children, suffering from undiagnosed and rare diseases, whole exome sequencing (WES) and sometimes whole genome sequencing technologies are adopted as the penultimate step in a diagnostic odyssey comprising many years of long journey marked by seemingly endless doctor visits and clinical tests. Despite the apparent clinical splendor of sequencing the whole exome or genome of a patient, getting to the final diagnosis and genetic answer is not so easy.

For some alleles a pathogenic sequence variant may be determined by *in silico* methods or by comparison with a well-curated set of previously published pathogenic alleles in sequence databases such as ClinVar (www.ncbi.nlm.nih.gov/clinvar/), the search for a causative gene may not end with sequencing. Identifying a novel human gene that has not been previously implicated in human disease often requires functional studies to determine significance, particularly where diseases are rare and only a few patients harbor pathogenic alleles in a given gene.

Functional studies can provide the needed piece of evidence in the effort to determine the pathogenicity of a given allele in a specific gene. These studies may answer the following questions: Is the gene expressed in the tissues damaged or not functioning in a given disease? Is the potential disease gene expressed during development or only in the

adult? Could loss or gain of function alleles of the candidate gene recapitulate the human condition in an animal model? It has become apparent that zebrafish are now widely accepted as an important model organism for functional studies of potentially pathogenic genes and may provide that final piece of evidence to end a diagnostic odyssey.

A search of Pubmed on March 21, 2016 with the terms “zebrafish and exome” brings up 161 references, with the oldest reference from 2010. Although these terms may not be all inclusive, it is still remarkable that for 161 novel human disease genes, zebrafish were the model organism of choice for functional studies to demonstrate clinical significance. The publications demonstrate the important role of zebrafish and the expertise of zebrafish investigators committed to their individual scientific interests and can be tapped by human geneticists to provide a critical piece of evidence.

But first, a brief explanation of nomenclature is in order. As geneticists, we are quite accustomed to the word “mutation.” For our nucleic acid-centered minds, this word merely means a variant in nucleic acid sequence. To the rest of the world, particularly the patient-centered world, “mutation” has more dire connotations and implies a negative value judgment to individuals who harbor a genetic variant. To communicate effectively with all communities and stakeholders, the human genetics community has moved to abandon the word

“mutation” and adopted more descriptive language. For a variant or allele that is associated with a disease or condition, the term “pathogenic variant” or “disease-causing variant” has become the more accepted term.¹

Here are some examples of recent reports over the past 6 months that describe human genetic discoveries where zebrafish provide functional evidence.

Cystic Renal Disease

Human ciliopathies are a group of conditions mediated by defective cilia function. These conditions can primarily affect a single organ such as the kidney resulting in cystic kidney disease or may be pleiotropic such as Bardet–Biedl syndrome where patients have cognitive, retina, kidney, and adiposity defects or Jobert syndrome where patients have kidney, cerebellar, retinal, and cognitive defects.^{2,3} Grampa *et al.*⁴ evaluated genomic material from 342 living patients and 200 fetal losses with cystic kidney disease using a targeted exome strategy where 1222 genes associated with cilia function were sequenced. One known cilia protein encoding gene, *NEK8/NPHP9*, encodes a serine/threonine kinase located at the inversin compartment of the cilial axoneme, distal to the transition zone, that was hypothesized to regulate the Hippo pathway. In four human families, biallelic novel non-sense and missense pathogenic variants of *NEK8* were identified. Functional studies from human fibroblasts were complemented by zebrafish studies to confirm the pathogenic nature of the alleles and to demonstrate the role of the Hippo pathway in the pathogenesis of disease. For zebrafish, morpholino (MO) knockdown of *NEK8* resulted in ciliopathy-related phenotypes that included body axis curvature, laterality defects, cyclopia, and renal cysts. Although wildtype human mRNA significantly reduced the ciliopathy phenotypes, mRNA harboring missense alleles from the human patients had no effect. Overexpression of human *NEK8* mRNA resulted in body axis curvature and pronephric abnormalities as did overexpression of *YAP*, a component of the Hippo pathway. *YAP* expression can be diminished by a drug, Verteporfin. Embryos injected with *NEK8* mRNA to induce overexpression were incubated in 20 μ M Verteporfin, and although the proportion of laterality defects remained unchanged, there was a significant reduction of kidney cystic defects supporting that *NEK8* may function through *YAP*.

Oligogenic Nature of Heterotaxy and Primary Ciliary Dyskinesia

Very few human genetic conditions have been caused by pathogenic alleles in more than one gene in the same individual. Yet, when patients present with a phenotype and two mutations within the same pathway or cell functional unit are discovered to harbor pathogenic variants, it is difficult to conclude that both variants are contributing to disease without functional studies. Primary ciliary dyskinesia is a rare human condition characterized by chronic lung and sinus infections and often randomization of the left/right body axis. Li *et al.*⁵ sequenced a total of 174 heterotaxy/primary cilia dyskinesia patients from four academic medical centers using either WES or a limited targeted exome. In that study they found that six patients who harbored pathogenic alleles in *DNAH6* also harbored a pathogenic allele in a second known ciliary gene.

To determine the role of trans-heterozygous affects of *DNAH6* and *DNAI1* or *DNAH5*, experiments were carried out in both zebrafish and mouse lung epithelial cells. In zebrafish, subthreshold dosages of MOs targeting *dnah6* as well as *dnai1* or *dnah5* were injected simultaneously. Although the subthreshold dosages caused little phenotypic difference, injection of both MOs together had a significant effect on heterotaxy. Similarly, subthreshold dosages of siRNA against *Dnah6* as well as *Dnai1* or *Dnah5* injected simultaneously resulted in cilia defects in mouse lung epithelial cells. Taken together, these experiments support the potential clinical significance of transheterozygous mutations in heterotaxy and primary ciliary dyskinesia.

MMP21 Pathogenic Variants Are a Novel and Common Cause of Human Heterotaxy

MMP21 is a secreted protein with a structural conformation consistent with a matrix metalloprotease. By WES, Guimier *et al.* identified autosomal recessive pathogenic variants of *MMP21* in a surviving twin with heterotaxy and resulting congenital heart disease whose twin did not survive because of severe structural cardiac defects consistent with heterotaxy.⁶ Furthermore, screening of 264 probands with heterotaxy identified *MMP21* pathogenic variants in nearly 6% of affected probands. Mouse embryos engineered to have mutations similar to the human patients exhibited similar defects, and knockdown of *mmp21* by both translation blocking and splice site MOs in zebrafish resulted in abnormal cardiac looping.

TRNT1, a Novel Gene for Nonsyndromic Retinopathy

Human retinopathies refer to a group of conditions where there is degeneration or dysfunction of photoreceptors, typically rods, cones, or both, resulting in the diminished light detection by the eye. Age of onset is variable with the most severe condition, Leber congenital amaurosis, results in near blindness at birth. Rod/cone dystrophies or cone/rod dystrophies result in a more gradual loss of photoreceptors, causing blindness anywhere from the first decade of life to middle age. Although nearly 200 genes have been associated with retinopathies, diagnostic challenges exist for patients who suffer from this group of blinding conditions and do not possess pathogenic variants in previously discovered genes using targeted exome approaches. To study patients undiagnosed for retinopathy, DeLuca *et al.*⁷ performed WES in an apparently healthy individual who was losing vision and identified biallelic variants in *TRNT1*, a gene not previously associated with retinitis pigmentosa. To find another affected case, the group analyzed genomic DNA from 1729 individuals having retinitis pigmentosa who had been previously studied by targeted sequencing of retinopathy genes. They identified two siblings with retinitis pigmentosa with *TRNT1* biallelic disease-causing variants. *TRNT1* had been previously associated with a condition characterized by congenital sideroblastic anemia, B-cell immune deficiency, recurrent fevers, and cognitive disability (SIFD) (OMIM 616084). *TRNT1* is a tRNA nucleotidyltransferase that catalyzes the addition of three nucleotides, CCA to the 3' end of a transfer RNA. Zebrafish *trnt1* is conserved with human with expression in

the retina, brain, lateral line, and tail blood-forming regions. Three separate splice blocking MOs generating hypomorphic knockdown of *trnt1* expression resulted in microphthalmia, diminished touch response, decreased CCA incorporation in tRNAs, and a reduction in light response. Based on the genetic findings, the human patients who were previously thought to be otherwise healthy, suffered from a mild sideroblastic anemia. Of note, the morphant zebrafish exhibited reduction of hemoglobin staining. Taken together, the zebrafish functional studies demonstrated that hypomorphic alleles of *TRNT1* cause retinopathy with mild sideroblastic anemia that is an allelic to a more severe condition, SIFD.

References

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