Zebrafish as a Key to Unlocking Human Genetic Diseases of Hearing

Erin Jimenez and Ashwin A. Bhandiwad

Introduction

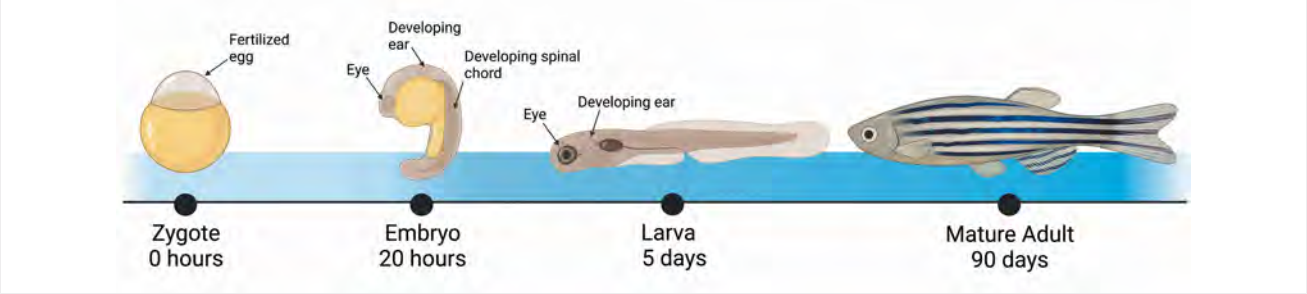
Hearing loss is a major cause of a disability affecting approximately 5% of the world's population and significantly decreases the quality of life (Leek and Molis, 2012). Although most hearing loss cases are caused by aging or noise exposure, congenital deafness affects 1 in 1,000 children (Toffler et al., 2015). Thus, a major motivation in hearing research is to understand how genes and the environment interact to affect auditory system development and function in hearing individuals and how the system fails in congenital and age-related diseases of hearing. If the system fails, are there any tools to reverse the process and restore hearing?

One of the most important tools for understanding the genetic causes of disease and potential avenues for treatment is genome sequencing. When the human genome was first sequenced in 2001, it cost an estimated $300 million and required coordination between 20 institutions across 6 countries. Today, the same task can be accomplished by a single research group for approximately $1,000 (Wetterstrand, 2013). Importantly, advances in computational power and analytical tools have generated large datasets of patient populations that have identified more than 110 deafness-related genes (Toffler et al., 2015). However, to bridge the gap between gene discovery and understanding how those genes influence hearing, we need a biological system with a similar genome to that of humans but that allows rapid experimentation with as little invasive surgery as possible.

Introducing Zebrafish as a Genetic Model

The study of zebrafish (*Danio rerio*), a freshwater minnow from south and southeast Asia, fills that gap and provides a biological system that allows us to model the genetics of human deafness and understand the mechanisms governing hearing. Zebrafish have become ideal organisms for studying genetics, development, and regeneration. This is because zebrafish share more than 70% of their DNA with humans. Thus, genetic discoveries in zebrafish are readily translatable to help study and understand human disorders such as hearing loss.

Figure 1. *Schematic of zebrafish life stages. Zebrafish exhibit rapid development with optical clarity and a functional auditory system within 5 days postfertilization. Stages (**left to right**): zygote (fertilized egg) contains a single cell; embryo (20 hours postfertilization) is transparent and has developing eyes, ears, and spinal cord; larva (5 days postfertilization) is free swimming with fully functional sensory systems; and adult (90 days postfertilization) is sexually mature but has pigment in characteristic stripes and is no longer transparent. **Arrows** point to relevant developing organs. Distance between points is not to scale. Image created with BioRender.com.*



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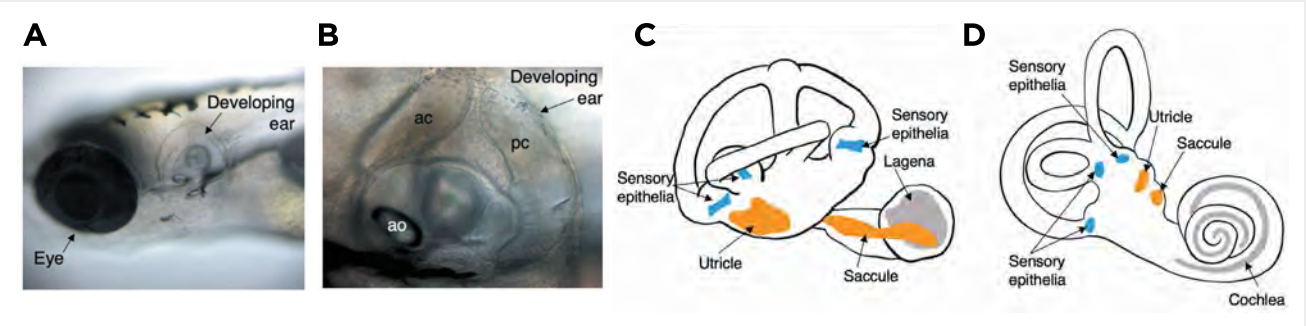


Figure 2. *The zebrafish hearing organ.* ***A:*** *lateral view of the head of a 10-day-old zebrafish with the inner ear. Arrows point to the eye and inner ear.* ***B:*** *close-up of zebrafish ear. Major structures of the developing inner ear are the anterior canal (ac), posterior canal (pc), and the anterior otolith (ao).* ***C:*** *schematic representation of the adult zebrafish inner ear showing three looped canals and associated sensory epithelia (**blue**) and the utricle (**orange**), saccule (**orange**), and lagena (**gray**) that have overlapping hearing and balance functions. Sensory epithelia are associated with the anterior and posterior canals and are involved in balance.* ***D:*** *schematic representation of the human inner ear showing the canals and associated epithelia and the major organs of hearing and balance: utricle (**orange**), saccule (**orange**), and cochlea (**gray**). The cochlea is the main structure for hearing and is closely related to the zebrafish lagena.*

Zebrafish, besides sharing much of the human genome, have other unique characteristics that make them ideal model systems. For example, they produce hundreds of transparent eggs at a time, and these develop into an adult within 90 days, allowing studies of heritability across multiple generations within very short time periods (**Figure 1**). Moreover, zebrafish larvae are nearly transparent in the first week of development, providing visual access to the entire organism without the need for invasive surgery (**Figure 2**). Together, these properties allow the study of how genes influence physiology and how genetic dysfunction causes disease in an intact animal.

Zebrafish are also important models for understanding many early symptoms of hearing loss, including perceptual issues such as the loss of sound identification and localization in noisy environments. In particular, because auditory neural pathways of zebrafish are like those in humans, zebrafish studies can help understand the genetic basis of hearing diseases and identify the neural pathways that are perturbed during hearing loss.

In this article, we discuss how studies of zebrafish, coupled with the recent advances in molecular biology and genetics, have given insights and developed tools for understanding the mechanisms of hearing. We discuss how genetic manipulations in zebrafish can allow us to turn genes on and off to understand their mechanisms of action in the auditory system. We show how zebrafish mutants with nonfunctioning genes have been used to model human hearing diseases. Finally, we discuss how the remarkable regenerative ability of zebrafish is revealing new molecular pathways to restore hearing function after damage to the hearing organ.

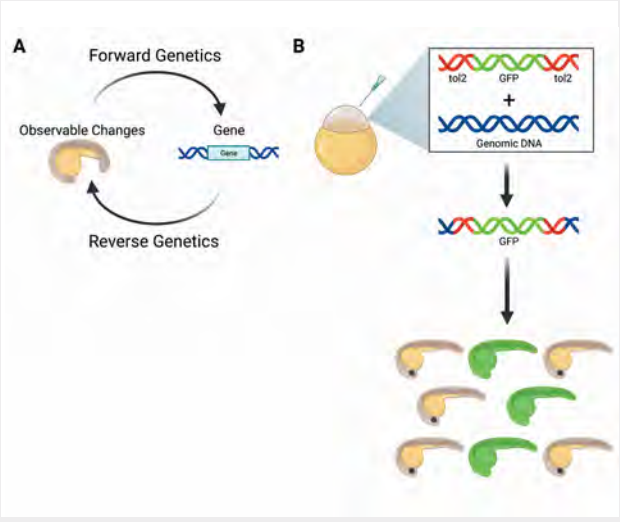
The Genetic Toolkit in Zebrafish

Early genetic experiments in zebrafish were conducted in the form of genetic screens, which generated large numbers of mutants (i.e., animals with characteristic biological changes caused by DNA alteration) by making random changes to the genome. The largest group of mutants was identified by a multi-institute consortium using a strategy called forward genetic screening (Nicolson et al., 1998). Forward genetics is a search strategy that starts with observed differences in behavior or physiology in mutant animals, followed by genome sequencing to find the mutation that gave rise to the observed effect (**Figure 3A**). Early genetic screens identified mutants with defects in hearing and balance and disrupted behaviors like the acoustic startle response, where the animal engages in a distinctive rapid swim away from the direction of a loud sound. Subsequent work isolated the genes that were disrupted in these mutants to understand the molecular machinery of hearing.

In contrast to forward genetics, reverse genetic approaches disrupt the function of specific genes that are suspected to have a role in hearing, followed by observing behavioral and physiological changes linked with that disruption. The most powerful reverse genetic approach to date is CRISPR, a genome editing tool that enables precise genome editing by removing, adding, or changing sections of the DNA sequence. Completion of the zebrafish genome (Howe et al., 2013) in combination with CRISPR technology has democratized reverse genetics and has made it possible to edit the genome easily and efficiently. Researchers can now test any "candidate" gene implicated in hearing and look for disruptions in auditory system development and function in zebrafish. These recent advances present an opportunity to explore the molecular mechanisms involved in hearing and position the zebrafish as an advantageous model system for studying hearing disorders.

In addition to forward and reverse genetic approaches, researchers have also developed methods to insert whole genes into zebrafish, a process called transgenesis (**Figure 3B**). A major technological breakthrough occurred in 1999 when researchers in Japan discovered a gene called *tol2*, which allows viruslike parasites to insert their own genes into the fish genome. Researchers co-opted this gene to insert custom engineered genes into the zebrafish genome (**Figure 3B**) (Kawakami and Shima, 1999). This was a transformational finding that allowed researchers to manipulate and observe a gene's effects on biological function. For example, *tol2* has been used in mutant fish that lack a gene called *clarin1* that is implicated in human hereditary hearing loss and renders the fish deaf. Using *tol2*, it is possible to insert a functional copy of the human *clarin1* gene into the zebrafish genome and restore hearing (Gopal et al., 2019). Together with forward and reverse genetics, *tol2*-based transgenesis has become a powerful tool to interrogate the development and function of the auditory system in zebrafish.

Figure 3. *Schematic of genetic tools used in zebrafish.* ***A:*** *forward genetics starts with an observed change in the zebrafish and identifies the gene mutation that causes it. Reverse genetics approaches generate mutations in the DNA sequence of a gene of unknown function and observe the effect on development and function of the zebrafish.* ***B:*** *schematic of transgenesis* tol2. *Green fluorescent protein (GFP) DNA, flanked by* tol2, *is injected into a single cell zebrafish zygote. GFP is randomly integrated into the zebrafish genome. If integration is successful, the embryo fluoresces green. Image created with BioRender.com.*



How Do Fishes Detect Sounds?

Zebrafish larvae provide a unique window into understanding how the inner ear structures detect and process sounds. When they are five days old, zebrafish larvae are almost completely transparent and have a fully functional auditory system (**Figure 2**).

At the cellular level, zebrafish and human auditory systems are almost identical; both use specialized sensory cells called hair cells to convert sound into electrochemical signals. Hair cells are identified by flexible hairlike structures on their surface called stereocilia, which are linked with one another at their tips (**Figure 4A**). Sound exerts a mechanical force on stereocilia and causes them to bend, resulting in a strain on the tip links. Strain on the tip links opens molecular pores called mechanoelectrical transduction (MET) channels and causes an influx of electrical current into the hair cell.

The current influx into the top of the hair cell stereocilia tip links propagates to the bottom of the cell to a specialized structure called the "ribbon synapse," named for its ribbonlike shape in cross section. Each cell contains three to five synaptic ribbons (Obholzer et al., 2008), which form the interface between hair cells and neurons that send information to the brain. Ribbon synapses act as a storage unit for large amounts of chemical signaling molecules and as a "conveyor belt" that releases stores of signaling molecules to activate neurons following cur-

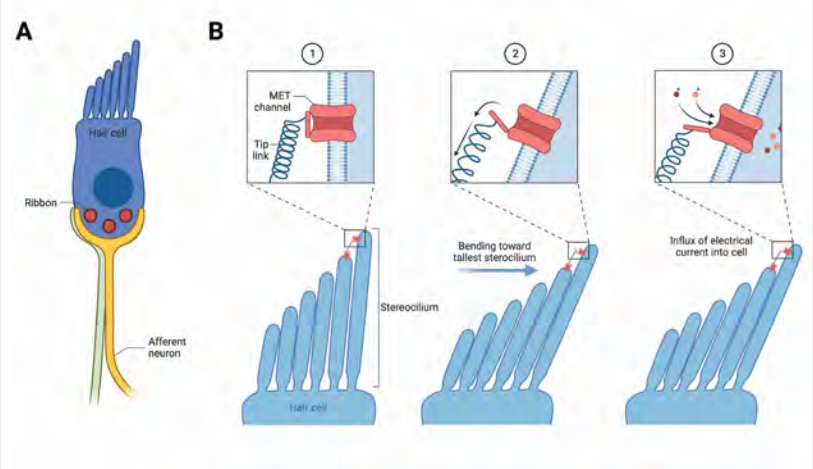


Figure 4. *Schematic of the hair cell.* ***A:*** *schematic of hair cell (**purple**) and auditory neuron (**yellow**) connected via ribbon synapses (**red**).* ***B:*** *schematic of mechanotransduction through hair cell tip links. Stereocilia are connected via tip links: (**1**) mechanotransduction channel (MET; **orange**) attached to a tip link (**blue**); (**2**) sound waves cause the cell bundle to "bend." When the bundle bends toward the tallest stereocilia, it creates a strain on the tip links and opens the MET channel; and (**3**) an open MET channel causes an influx of positively charged ions, resulting in an inward current. Image created with BioRender.com.*

rent influx. The mutation of genes associated with the ribbon synapse or chronic exposure to loud noise causes disruption and, in the extreme, detachment of ribbons from the hair cell-neuron interface. This process leads to auditory synaptopathy, a form of sensorineural hearing loss (Kindt and Sheets, 2018).

How Do Genes Regulate Mechanotransduction in Hair Cells?

Hair cells must transduce sounds with speed and precision, and they accomplish this through the interplay between the ribbon synapse and the MET channel **(Figure 4B)**. Because many of the genes that are implicated in hearing loss are associated with the tip links and the MET channel, this region within the hair cells has been a focus of study. Substantial evidence implicates two proteins, transmembrane-like channels (TMCs) 1 and 2 (TMC1 and TMC2, respectively), as crucial components of the MET channel (Pan et al., 2018). These proteins can be conceptualized as gates, with parts on the inside and outside of the cell. When multiple gates come together, they form a ring that allows positively charged ions to pass through. In zebrafish and humans, TMC mutations cause deafness and recent estimates suggest that TMC mutations are responsible for 3-8% of human hereditary hearing loss cases (Nist-Lund et al., 2019).

TMC1 and TMC2 are made in the cell body, far away from the stereocilia tips. How are they transported to the stereocilia tips correctly to form the MET channel? The answer starts from a serendipitous observation in a group of zebrafish larvae that failed to perform reflexive auditory escape behaviors. Genetic analysis of these fish showed a mutation in the gene *tmie,* which is also implicated in heritable congenital deafness in humans (Gleason et al., 2009). When hair cell stereocilia are bent manually with a small glass probe in zebrafish *tmie* mutants, there is no resulting current. However, if a fully functional *tmie* gene is inserted in hair cells using the *tol2* system, auditory function is restored (Pacentine and Nicolson, 2019).

Functional *tmie* can also be fused to a green fluorescent protein (GFP) that allows visualization of *tmie* proteins within the hair cell and its movement. Similarly, TMC1 and TMC2 can be fused with GFP to show their movement from the cell body to the stereocilia. A combination of transgenic expression techniques and live imaging experiments show that TMC1 and TMC2 fail to move into the hair bundles in *tmie* mutants. Conversely, if there is too much *tmie*, there is an overabundance of TMC1 and TMC2 in the stereocilia tips. Therefore, *tmie* seems to be a critical player in targeting the pore-forming units of the MET channel to hair cell bundles.

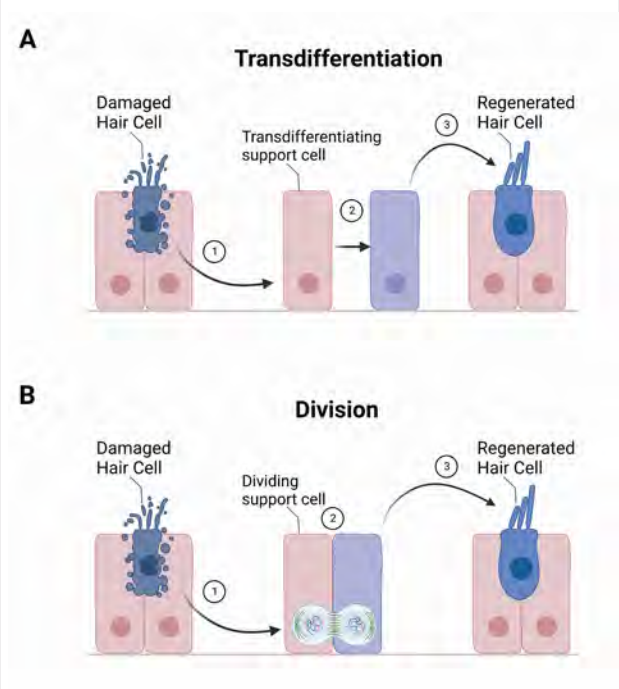
The transparency of the zebrafish inner ear has enabled the study of the ways mutations disrupt ribbons. The genes responsible for coding ribbon-specific proteins in zebrafish are *ribeye* and *ribeye b*, which make up two-thirds of the ribbon volume in hair cells (Chen et al., 2018). Nonfunctional *ribeye* genes coding ribbon proteins result in the reduction in size or loss of ribbons (Sheets et al., 2011) and failure of neurons to connect to and receive information from hair cells that subsequently cause hearing loss.

Hearing Regeneration and Zebrafish

Sensorineural hearing loss is the most common type of environmental exposure-related hearing loss and is caused by hair cell damage and death (Müller and Barr-Gillespie, 2015). In humans and other mammals, sensorineural hearing loss is permanent. However, zebrafish, like all fish species, continually produce hair cells throughout their lifetime or regenerate them in response to damage (Popper and Hoxter, 1984). New hair cells are born from neighboring cells called supporting cells. After damage and hair cell death, nearby supporting cells undergo molecular changes to either give birth to a new hair cell or directly transform from a supporting cell to a hair cell (**Figure 5**).

In contrast, supporting cells in mammals are thought to have lost this ability. One intriguing hypothesis for this loss of regeneration in mammals is that because hearing loss generally occurs after the reproductive age, there is a lack of evolutionary pressure on regenerative processes (Groves, 2010). However, mammalian support cells can be converted into hair cells in limited cases in the balance organ or in early development, suggesting that the molecular pathways are still present (White et al., 2006). Understanding how and why hair cells regenerate in zebrafish could therefore provide genetic and therapeutic targets to potentially initiate regeneration and reverse permanent hearing loss in humans.

Figure 5. *Hair cell regeneration in zebrafish.* ***A:*** *hair cell regeneration through direct transdifferentiation: (**1**) hair cell damage initiates transdifferentiation in supporting cells; (**2**) support cell is converted into a precursor hair cell (**purple**); and (**3**) precursor cells generate structures to become a new hair cell (**blue**).* ***B:*** *hair cell regeneration through division: (**1**) hair cell damage causes the support cell to divide into two; (**2**) newly formed support cell becomes a hair cell precursor (**purple**); and (**3**) it matures into a regenerated hair cell (**blue**). Image created with BioRender.com.*



Lateral Line Hair Cell Regeneration Is Experimentally Induced by Toxic Drugs

To find genes that stimulate hair cell regeneration, researchers have extensively studied the zebrafish lateral line, a distributed mechanosensory organ on the skin surface that detects water flow around the animal. The lateral line consists of morphological units called neuromasts, which are flower-shaped arrangements of the same sensory hair cells as in the ear, surrounded by supporting cells (Chagnaud and Coombs, 2013). Because lateral line hair cells are located on the skin surface, they are easy to visualize using fluorescent chemical dyes that enter hair cells only through a functional MET channel (Owens et al., 2008). This feature allows easy access with drugs and rapid testing of candidate genetic and therapeutic targets to assess regeneration.

Although hair cells are damaged by loud noise, it is more common and easier to induce damage using drugs such as neomycin and copper sulfate (Mackenzie and Raible, 2012). Like vital dyes, drugs can enter hair cells through MET channels. New genetic tools in zebrafish permit damaging adult zebrafish hair cells at will or through conditional targeted ablation. Conditional ablation allows precise control to damage hair cells without injuring the surrounding tissue throughout the adult inner ear and in the lateral line (Jimenez et al., 2021). Hair cell regeneration in both the lateral line and adult inner ear of zebrafish is rapid; hair cells grow back within 48 hours after initial damage. Rapid regeneration therefore enables capture of molecular responses that occur during regeneration; functional recovery is observed either behaviorally or through vital dye labeling.

Using Zebrafish to "Screen" for Hearing Regeneration Genes

Zebrafish studies make it possible to identify and catalog which genes control hair cell regeneration. Researchers

identified three sequential waves of genetic responses on the regenerating zebrafish lateral line. First, an inflammatory response is initiated after hair cell damage; second, a regeneration-specific program is activated; and finally, the developmental program is reactivated. Collectively, the dynamic molecular responses that occur in the zebrafish lateral line enable supporting cells to reenter the cell cycle and replace lost hair cells (Varshney et al., 2016).

Zebrafish lateral line regeneration studies have resulted in the identification of many hearing regeneration candidates with overlapping functions in regeneration and normal hair cell development. Because mutations in these genes are unhelpful to study regeneration because they also disrupt the overall development of other organ systems in zebrafish, researchers are focused on identifying hearing regeneration-specific genes that may be used as targets for therapeutics in humans. To date, only a few zebrafish regeneration genes have been identified that impact hair cell regeneration without disrupting normal development of other organs, including the ear. Combining the zebrafish model with the genome editing tool CRISPR, researchers can now test every candidate gene that is likely important for hearing regeneration, inactivate them in zebrafish, and look for disruptions or improvements in zebrafish hearing regeneration.

A mechanistic understanding of how many genes interact with each other to control hair cell regeneration has been accomplished in the adult zebrafish inner ear. By surveying active genes in the animal and using machine learning to analyze interactions of those genes with each other and the environment, we have found that zebrafish hair cell regeneration relies on a network of genes called transcription factors that work together to turn genes on and off (Jimenez et al., 2022). During early hair cell regeneration, one class of transcription factors initiates the regeneration response in the nearby supporting cells by turning on genes that convert support cells into stem cell-like immature hair cells. Another set of transcription factors then activates genes that give rise to the proteins and cellular structures that fully transform support cells into functional hair cells (**Figure 5**). Using genomics on regenerating zebrafish inner ears, the field is beginning to understand the molecular switches that activate hair cell regeneration from the surrounding cells in the inner ear. This technique suggests that the same transcription factors can be switched on in the mammalian inner ear to stimulate a regenerative response in humans.

Functional Hearing Regeneration in Zebrafish

In addition, sensorineural hearing loss can occur due to damage to the neurons that carry signals to the brain. It turns out that zebrafish can also regenerate neurons after damage and show functional recovery, again distinguishing them from mammals. Recent experiments have revealed that neurons also promote hair cell regeneration because when lateral line neurons are damaged, hair cell regeneration is disrupted (Hardy et al., 2021). Moreover, experiments in transgenic zebrafish with fluorescent dye-labeled hair cells and neurons have shown that regenerating neurons find and bind hair cells to reform the same connection pattern (Faucherre et al., 2010). The study of cellular and molecular mechanisms of hair cell and neuron regeneration in zebrafish is still nascent but already shows promise in co-opting these mechanisms to repair and reverse sensorineural hearing loss in humans.

How Do Sound Signals Travel from the Ear to the Brain?

Simply detecting sound by hair cells is not enough; inner ear neurons are critical for conveying sound information to the brain, which integrates information to generate the perception of hearing. Auditory neuron function and development studies in zebrafish have revealed remarkable specificity and selectivity of which signals get transmitted to the brain. Because of the abundance of high-resolution images, transgenic manipulations, and computational image analyses in zebrafish, it is possible to label individual neurons in a zebrafish and aggregate across multiple zebrafish to generate a map of all neurons. Aggregation reveals biological principles of how neurons connect with the inner ear and with each other to convert sound into hearing.

One common biological principle is preferential neuron wiring between hair cells and primary auditory-processing brain regions. For example, preferential neuron wiring is used for transmitting information selectively to brain regions that control behavioral responses. In early development, zebrafish use their auditory system primarily for threat detection. A loud and unexpected noise signals a predator's attack, and zebrafish respond with an escape response away from the direction of the sound with a reaction time of 5 milliseconds, about 30 times faster than in humans (Burgess and Granato, 2007). Given the importance of this behavior for survival, sensory neurons that connect the auditory system to initiate

this startle response develop earlier than sensory neurons used for other sensory modality pathways (Liu et al., 2022).

However, not all sounds are threats; performing escape behaviors to every sound is inefficient and energetically costly. Therefore, sensory signals need to be robust to the environmental context and filtered before reaching the escape-activating neurons. Sensory filtering is an important role of the brain; failure to filter irrelevant signals is a symptom of disparate neurological conditions from tinnitus to schizophrenia (Swerdlow et al., 2001). Because zebrafish and humans use the same signaling molecules for sensory signal filtering, zebrafish have also become an important animal model for understanding the genetics of schizophrenia and for developing new antipsychotic drugs (Langova et al., 2020).

How does the inner ear filter auditory information to prevent unnecessary escape responses? Simple and selective neuronal wiring also plays an important role in filtering out irrelevant sounds. In the escape system, auditory neurons that initiate the escape response also send information to regulatory neurons within the escape system. When the animal hears a moderately loud noise, regulatory neurons are preferentially activated and prevent the initiation of subsequent escape responses. The effect of these neurons can be seen in zebrafish by expressing a protein called iGluSnFr (pronounced "I glue sniffer"), which increases brightness in the presence of activating signaling molecules. When iGluSnFr is used to label escape neurons, sudden loud sounds cause an increase in iGluSnFr brightness and indicate a strong activation of the escape-response neurons. However, if the animal hears a moderate sound preceding the loud sound, iGluSnFr brightness does not increase. This phenomenon, also known as prepulse inhibition in studies of other animals, is specific to signaling between the auditory and escape neurons, suggesting that regulatory neurons block auditory neuron signals to suppress escape neurons (Tabor et al., 2018). Neurons can not only relay directional and contextual information (e.g., predator alert) but can also signal to self-regulation systems. Zebrafish, therefore, allow us to precisely understand how neurons communicate with each other to accomplish complex processes and ultimately to understand diseases where those processes fail, such as tinnitus and schizophrenia.

Conclusions

The zebrafish is a powerful model organism for the study of human disease. This is particularly the case for the study of human hearing because zebrafish and humans share common features such as the auditory and vestibular organs that play roles in hearing and balance. Zebrafish as a model system has many advantages over mice, including the ability to generate many offspring, trace living cell types, observe mutants that mimic genetic mutations found in human hereditary hearing loss, and learn the regenerative instructions involved in hearing regeneration.

Advances in molecular biology and genome-sequencing technology combined with the power of the zebrafish have provided an opportunity to understand auditory function and explore human deafness. With zebrafish, it is now possible to validate and interrogate nearly every human hearing loss gene to understand their mechanisms in a vertebrate. Moreover, the available genetic tools and transparency of the zebrafish embryo make it possible to visualize the hair cells, auditory neurons, and brain regions that convert sound signals into the sense of hearing.

Death of hair cells in the human inner ear results in permanent impairments to hearing and deafness. However, the zebrafish can regenerate their hair cells, offering unprecedented opportunities to identify new strategies to stimulate hair cell regeneration in mammals. By using the highly regenerative zebrafish combined with revolutionary approaches in biology, we are beginning to understand the molecular mechanisms involved in hearing regeneration.

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Contact Information

Erin Jimenez
erin.jimenez@nih.gov
National Human Genome Research Institute
National Institutes of Health
50 South Drive
Bethesda, Maryland 20984, USA

Ashwin A. Bhandiwad
ashwin.bhandiwad@alleninstitute.org
Allen Institute for Brain Science
615 Westlake Ave N
Seattle, Washington 98109, USA

For author bios, please go to
acousticstoday.org/bios-19-1-2