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Zebrafish as a Model for Studying Ciliary Development and Disease

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Abstract Cilia play crucial roles in numerous biological processes, from cell signaling to tissue homeostasis, and their dysfunction can lead to a group of disorders known as ciliopathies. Zebrafish (*Danio rerio*), due to its genetic tractability and transparency during early development, has become an important model organism for studying ciliary development and related diseases. This study analyzes the stages of ciliary development in zebrafish, including tissue-specific processes and the role of key signaling pathways, and explores how zebrafish models contribute to understanding various ciliopathies. It emphasizes genetic manipulation to induce ciliary defects and phenotypic analysis, and describes key observational techniques in zebrafish ciliary research, including high-resolution imaging, genetic markers, and fluorescent reporters. Case studies demonstrate the application of zebrafish in studying human ciliopathies, such as Joubert syndrome, Bardet-Biedl syndrome, and nephronophthisis, as well as kidney and liver ciliopathies. It is expected that this study will provide reference value for future research on ciliary related diseases, promote the understanding of the pathological mechanisms of fibrotic disorders, and develop treatment strategies.

Keywords Ciliarydevelopment; Zebrafishmodel; Ciliopathies; Geneticmanipulation; Observationaltechniques

1 Introduction

Cilia are microtubule-based organelles that extend from the surface of eukaryotic cells and play crucial roles in cellular signaling, motility, and sensory functions. They are involved in various physiological processes, including signal transduction, organ development, and tissue homeostasis. Abnormalities in ciliary structure and function can lead to a group of human diseases known as ciliopathies, which affect multiple organs and systems (Blacque et al., 2017; Zhang et al., 2022). Cilia are essential for the proper functioning of sensory structures such as the eye, ear, and nose, and are also involved in developmental processes like left-right asymmetry formation and limb morphogenesis (Leventea et al., 2016).

Zebrafish (*Danio rerio*) have emerged as a powerful model organism for studying ciliary development and related diseases due to their genetic similarity to humans and the conservation of ciliary structure and function across vertebrates. Zebrafish offer several advantages for ciliary research, including their rapid development, transparency during embryonic stages, and the availability of sophisticated genetic and imaging techniques (Sedykh et al., 2016). These features make zebrafish an ideal model for investigating the roles of cilia in organogenesis and for modeling human ciliopathies (Pinto et al., 2021; Liu et al., 2023).

This study will discuss the various types of cilia present in zebrafish, their roles in organogenesis, and the advantages of using zebrafish for ciliary research. It will also highlight recent advances in the field, including the development of transgenic zebrafish lines for in vivo visualization of cilia and the identification of new roles for ciliary proteins. This study emphasizes the importance of zebrafish as a model organism in cilia research, promoting our understanding of fibrosis.

2 Ciliary Development in Zebrafish

2.1 Early stages of ciliary formation

Ciliary formation in zebrafish begins during early embryogenesis, where cilia play a crucial role in cellular signaling, tissue morphogenesis, and body patterning. The Nlz1 protein has been identified as a key player in this process. Knockdown of nlz1 in zebrafish results in abnormal cell specification in Kupffer's vesicle (KV) and a

significant reduction in the number of cilia in KV, the pronephros, and the neural floorplate, leading to phenotypes reminiscent of human ciliopathies (Dutta et al., 2015). The Hippo signaling pathway and its transcriptional co-activator Yap are essential for ciliogenesis during zebrafish kidney development. Knockdown of yap results in pronephric cysts and other ciliopathy-related abnormalities, which can be rescued by full-length yap mRNA (He et al., 2015).

2.2 Tissue-specific ciliary development

Ciliary development in zebrafish is tissue-specific, with different types of cilia present in various organs. For instance, endothelial cilia are crucial for maintaining vascular integrity during development. Mutations in intraflagellar transport genes, which are essential for cilia biogenesis, lead to increased risk of developmental intracranial hemorrhage, highlighting the importance of cilia in the vasculature (Kallakuri et al., 2015). In the pronephros, cilia are essential for maintaining duct integrity and proper morphogenesis. Disruption of ciliary function in this tissue leads to pronephric cysts and disorganization of the pronephric duct. Cilia in the retina are involved in sensory functions, and their dysfunction can lead to retinal dystrophy, as observed in zebrafish models of Joubert syndrome (Rusterholz et al., 2022).

2.3 Role of signaling pathways in ciliogenesis

Several signaling pathways are involved in ciliogenesis in zebrafish. The Wnt signaling pathway, both canonical and non-canonical, plays a significant role in ciliary formation. Nlz1 acts downstream of Foxj1a and Wnt8a in the canonical pathway and positively regulates Wnt11 in the non-canonical pathway to promote motile cilia formation. Prostaglandin signaling also regulates ciliogenesis by modulating intraflagellar transport. PGE2, synthesized by COX1 and COX2, binds to the EP4 receptor on the cilium, activating adenylate cyclase and cAMP signaling to promote anterograde intraflagellar transport (Jin et al., 2015). Cholesterol has been shown to be crucial for cilia biogenesis and function. Pharmacological inhibition of cholesterol synthesis in zebrafish leads to cilia dysfunction and organ malformations, suggesting that cholesterol governs critical steps of cilium extension (Maerz et al., 2019).

3 Zebrafish Models for Ciliopathies

3.1 Common ciliopathies studied in zebrafish

Zebrafish have been extensively used to model various human ciliopathies due to their genetic and physiological similarities to humans. Common ciliopathies studied in zebrafish include Joubert Syndrome (JBTS), nephronophthisis, and polycystic kidney disease (PKD). Joubert Syndrome is characterized by a distinctive cerebellar and brain stem malformation known as the "Molar Tooth Sign" and is associated with a variety of other ciliopathy phenotypes such as retinal dystrophy and fibrocystic renal disease (Rusterholz et al., 2022). Nephronophthisis, another ciliopathy, leads to cystic kidney disease and has been effectively modeled in zebrafish to study the function of related genes (Molinari et al., 2018). Polycystic kidney disease, characterized by the formation of fluid-filled cysts in the kidneys, has also been modeled in zebrafish, providing insights into the disease's progression and potential therapeutic strategies (Zhu et al., 2021).

3.2 Genetic manipulation to model ciliary defects

Genetic manipulation in zebrafish has been pivotal in modeling ciliary defects. Techniques such as morpholino oligonucleotide (MO) injections and CRISPR/Cas9 genome editing have been employed to knock down or mutate genes associated with ciliopathies. For instance, the IFT46 gene, essential for cilium formation and maintenance, has been manipulated in zebrafish to create transgenic lines that exhibit ciliopathy-like phenotypes, including cystic kidneys and pericardial edema (Lee et al., 2023; Wang et al., 2024). TALEN technology has been used to generate tmem67 mutants, which model Meckel syndrome type 3 (MKS3) and exhibit renal cysts and ciliary abnormalities. These genetic tools allow for precise control over gene expression and the study of specific ciliary defects in zebrafish.

3.3 Phenotypic analysis of ciliopathies in zebrafish

Phenotypic analysis of zebrafish models of ciliopathies involves detailed observation of ciliary structure and function, as well as the associated physiological abnormalities. For example, zebrafish models of Joubert

Syndrome have been used to study retinal dystrophy and other tissue-specific phenotypes, revealing distinct mechanisms depending on the affected gene (Figure 1) (Rusterholz et al., 2022). In nephronophthisis models, ciliary phenotypes are assessed in various developmental structures, such as Kupffer's vesicle, to understand the impact of gene knockdown on ciliary function. Advanced imaging techniques, including transmission electron microscopy (TEM) and electron tomography (ET), have been employed to characterize ciliary structures in zebrafish, providing high-resolution data on ciliary morphology and aiding in the validation of zebrafish as a model for primary ciliary dyskinesia (PCD) (Pinto et al., 2021). These phenotypic analyses are crucial for understanding the pathomechanisms of ciliopathies and developing potential therapeutic interventions.

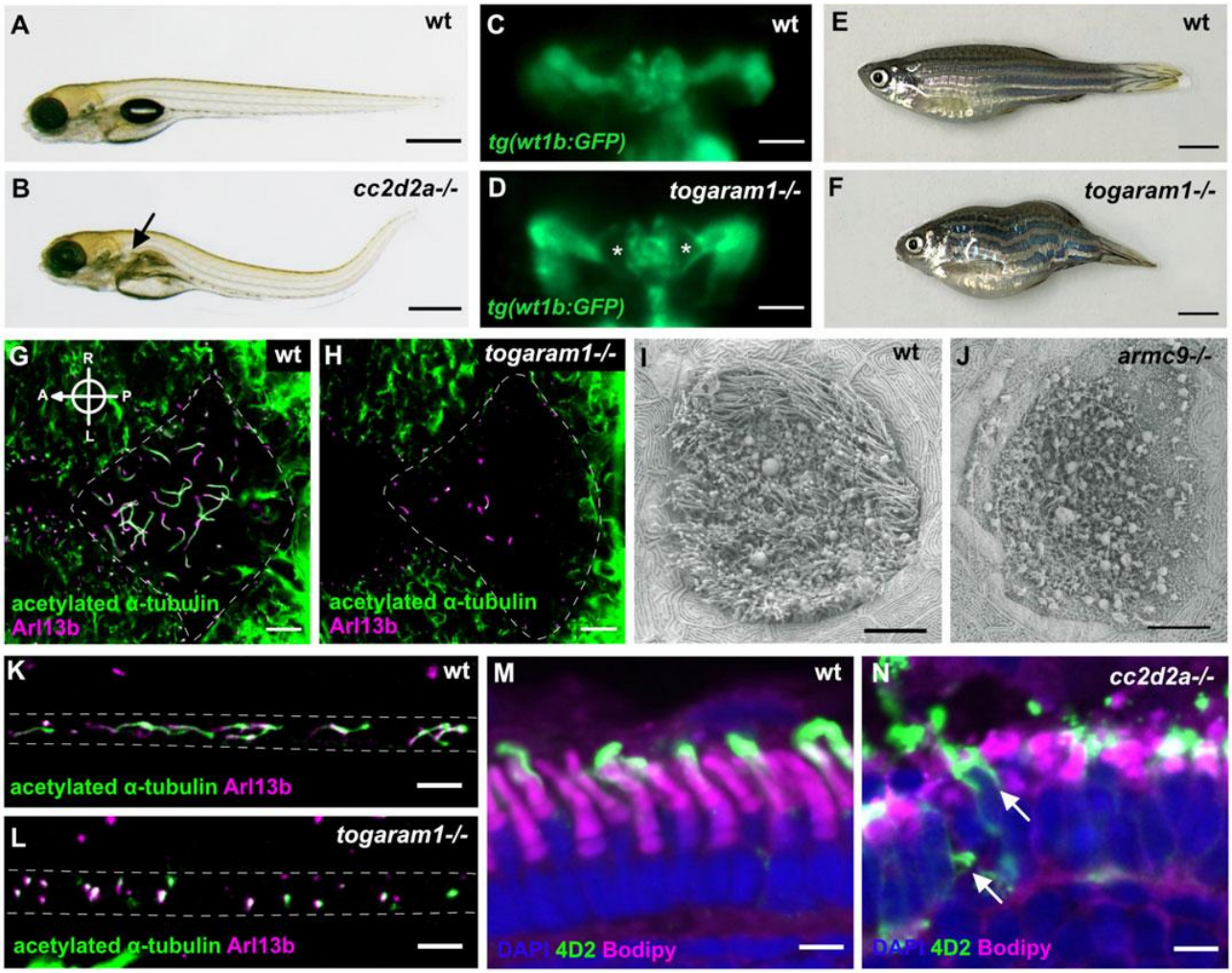

Figure 1 Various types of motile and immotile cilia in zebrafish show distinct acetylated tubulin or *Arl13b* signal patterns (Adopted from Rusterholz et al., 2022)

Image caption: A: Wild-type zebrafish larvae; B: *cc2d2a* gene knockout mutant larvae; C: Wild-type embryo; D: *togram1* gene knockout embryo; E: Wild-type adult zebrafish; F: *togram1* gene knockout adult zebrafish; G: Neural cilia of wild-type zebrafish shown with green label (*acetylated α-tubulin*) and magenta label (*Arl13b*); H: Significant reduction and structural defects of cilia after togram1 knockout; I: Cross-section of wild-type zebrafish retina; J: Comparison between wild-type and *armc9* gene knockout; K: *Acetylated α-tubulin* (green) and *Arl13b* (magenta) labeling in wild-type cilia; L: Corresponding labeling in *togram1* knockout cilia, showing significantly weakened dual labeling signals; M: Immunofluorescence labeling in wild-type photoreceptor cells; N: Ciliary structure of photoreceptors after *cc2d2a* gene knockout (Adapted from Rusterholz et al., 2022)

4 Molecular Mechanisms Underlying Ciliary Dysfunction

4.1 Defective ciliary structure and function

Ciliary defects can lead to a variety of human diseases known as ciliopathies, which include conditions such as polycystic kidney disease, primary ciliary dyskinesia, and retinal degeneration. In zebrafish, the knockdown of

genes essential for cilia formation, such as *IFT46*, results in multiple phenotypes associated with ciliopathies, including kidney cysts, pericardial edema, and ventral axis curvature. These defects are characterized by shortened and abnormal cilia in various tissues, such as the kidney and spinal canal (Lee et al., 2015; 2018). The overexpression of the ciliary membrane protein *Arl13b* in zebrafish leads to an increase in ciliary length, which disrupts motility in motile cilia but retains signaling capacity in immotile primary cilia. This suggests that *Arl13b* plays a crucial role in ciliary membrane extension and length regulation (Lu et al., 2015).

4.2 Disruption of intraflagellar transport (IFT)

Intraflagellar transport (IFT) is essential for the assembly and maintenance of cilia by transporting proteins along the axoneme. Disruption of IFT can lead to severe ciliopathies. For instance, mutations in IFT74 result in ciliogenesis defects and slow photoreceptor degeneration in zebrafish, highlighting the critical role of IFT proteins in maintaining ciliary structure and function (Luo et al., 2021). Similarly, IFT52 mutations impair the assembly of the IFT-B complex and its localization to cilia, leading to decreased cilia length and associated ciliopathies such as short-rib thoracic dysplasia and congenital anomalies of the kidney and urinary tract (Dupont et al., 2019). The deletion of WDR31, along with RP2 and ELMOD1, results in the accumulation of IFT complex B components and kinesin KIF17 in cilia, indicating their role in regulating IFT and BBSome trafficking (Cevik et al., 2023).

4.3 Protein networks regulating ciliary maintenance

The maintenance of ciliary structure and function is regulated by complex protein networks. IFT proteins, such as IFT46 and IFT74, are crucial for ciliary development and maintenance. In zebrafish, IFT46 is expressed in various ciliated tissues and is localized to the basal body. Knockdown of IFT46 results in multiple ciliopathy-associated phenotypes, demonstrating its essential role in ciliary development 18. Additionally, the Hippo pathway effector YAP1 is modulated by IFT complex B proteins (IFT88, IFT54, and IFT20) during cardiogenesis in zebrafish, highlighting a noncanonical role for IFT proteins in regulating ciliary-related pathways (Peralta et al., 2020). Moreover, endothelial cilia play a critical role in maintaining vascular integrity during zebrafish development, with Hedgehog signaling being a major mechanism involved in this process (Kallakuri et al., 2015).

5 Observational Techniques in Zebrafish Ciliary Research

5.1 High-resolution imaging of cilia

High-resolution imaging techniques are crucial for studying the intricate structures and functions of cilia in zebrafish. Transmission electron microscopy (TEM) and electron tomography (ET) have been employed to analyze the axonemal structure of cilia in zebrafish, providing detailed insights into their morphology. For instance, TEM and ET were used to characterize the cilia in the olfactory pit (OP) and the left-right organizer (LRO) of zebrafish embryos, revealing structural similarities to human respiratory cilia. Additionally, a new protocol involving glycol methacrylate (GMA) embedding has been developed to preserve fluorescent signals in zebrafish embryos, allowing for high-resolution imaging of internal structures without the need for antibodies. This method is particularly useful when advanced imaging technology is not readily available.

5.2 Genetic markers for ciliary studies

Genetic markers are essential tools for identifying and studying cilia in zebrafish. The identification of cilia-specific markers, such as Nephrocystin-3 (*NPH*p3), has facilitated the visualization of cilia dynamics in vivo. A transgenic zebrafish line expressing a fusion protein of *NPH*p3 and mCherry under the β-actin promoter has been developed, enabling efficient labeling of cilia in multiple cell types from embryonic stages to adulthood without causing developmental or physiological defects (Zhang et al., 2022). This transgenic line allows for live imaging of ciliary dynamics and the trafficking of cilia proteins, providing valuable insights into the roles of these organelles.

5.3 Use of fluorescent reporters in live zebrafish embryos

Fluorescent reporters are widely used in zebrafish research to study ciliary function and dynamics in live embryos. The transparency of zebrafish embryos and larvae allows for non-invasive imaging during their rapid development. Fluorescent reporter molecules, such as those used in transgenic lines expressing fluorescent proteins under specific promoters, enable in vivo imaging of cilia and other cellular structures (Tonelli et al., 2020). For example,

fluorescent vital dyes and transgenic cell type-specific fluorescent reporters have been used to identify different cell types and visualize subcellular structures in primary cell cultures of zebrafish embryos (Figure 2) (Sassen et al., 2017). These techniques facilitate the study of ciliary function and related phenotypes in live zebrafish embryos, contributing to our understanding of ciliopathies and other cilia-related disorders.

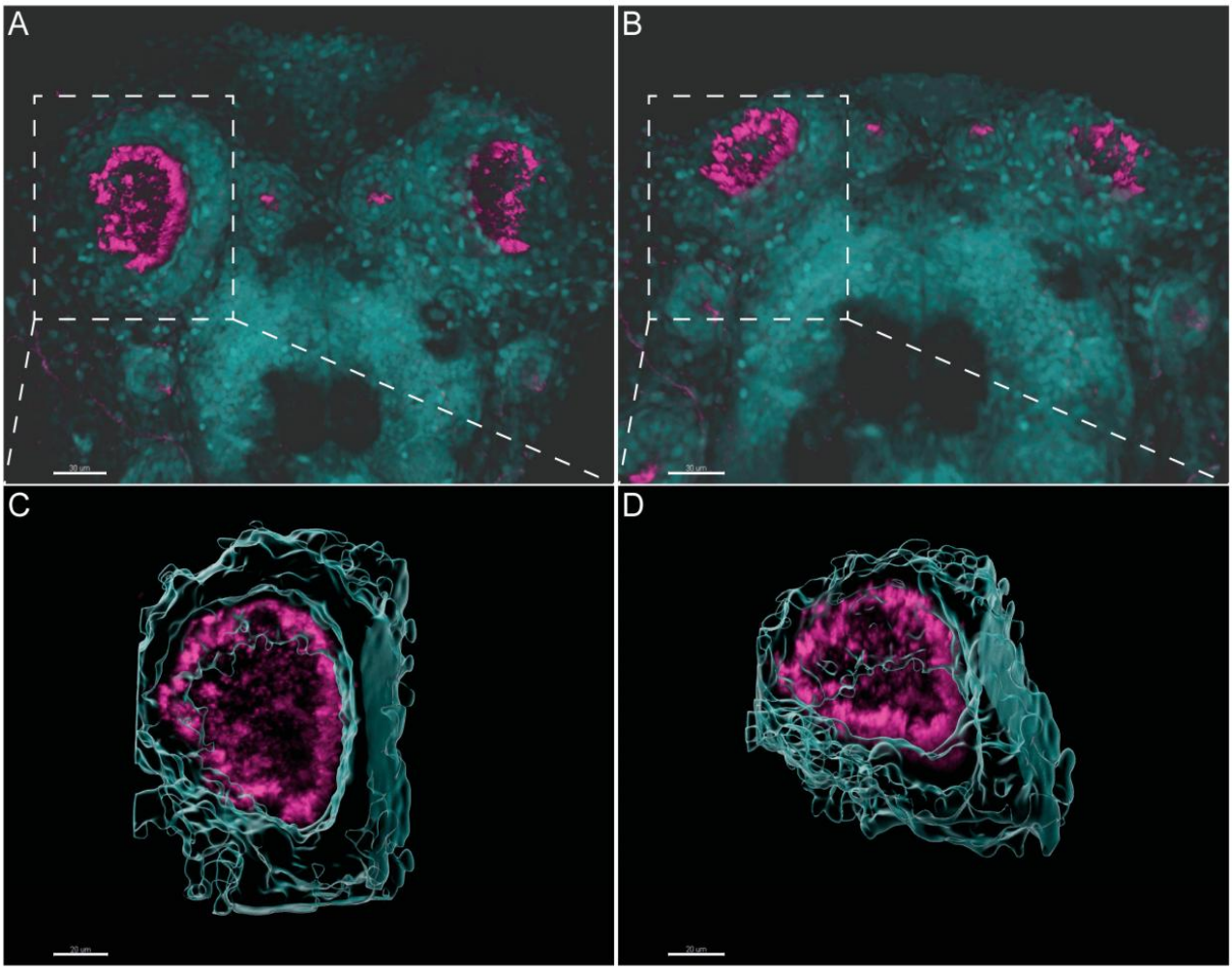

Figure 2 Three-dimensional imaging analysis helps to orient and localize the OP of zebrafish for TEM studies (Adopted from Pinto et al., 2021)

Image caption: (A, B) Immunofluorescent labelling with anti-acetylated α-tubulin shows the distribution of multiciliated cells in the OP of 4 dpf larvae. (C, D) 3D surface reconstructions from the respective OPs revealing the concave morphology of the organ when rotated. Anti-Acetylated α-tubulin immunofluorescence in magenta and DAPI in cyan. Scale bar 20 μm (Adopted from Pinto et al., 2021)

6 Case Study: Zebrafish in the Study of Human Ciliopathies

6.1 Application of zebrafish in understanding joubert syndrome

Joubert Syndrome (JS) is a neurodevelopmental disorder characterized by a distinctive brain malformation known as the "molar tooth sign" on axial MRI, along with symptoms such as hypotonia, ataxia, and abnormal eye movements. Zebrafish models have been instrumental in understanding the genetic and molecular mechanisms underlying JS. For instance, zebrafish models have been used to study the role of ARMC9, a basal body protein, in JS. Mutations in ARMC9 have been shown to cause typical ciliopathy phenotypes in zebrafish, such as curved body shape and retinal dystrophy, thereby supporting its role in JS (Weghe et al., 2017). Zebrafish models have helped elucidate the tissue-specific roles of proteins implicated in JS, revealing that different genes can cause distinct phenotypes in various organs (Rusterholz et al., 2022). These models are invaluable for exploring the pathomechanisms of JS and developing potential therapeutic strategies.

6.2 Investigating bardet-biedl syndrome with zebrafish models

Bardet-Biedl Syndrome (BBS) is another ciliopathy that has been extensively studied using zebrafish models. BBS is characterized by retinal degeneration, obesity, polydactyly, and renal abnormalities. Zebrafish have been used to model the retinal degeneration observed in BBS, which is caused by dysfunction in photoreceptor ciliary-related proteins. These models have helped clarify the role of BBS proteins in the primary cilium and their interactions with other ciliary modules, such as the intraflagellar transport (IFT) module (Delvallée and Dollfus, 2023). The use of zebrafish has also enabled the study of the nephronophthisis (*NPH*P) module, which is involved in the transition zone of primary cilia, furthering our understanding of the molecular mechanisms leading to BBS (Wang et al., 2022). These insights are crucial for developing targeted therapies for BBS and related ciliopathies.

6.3 Insights into nephronophthisis from zebrafish studies

Nephronophthisis (*NPH*) is a ciliopathy that primarily affects the kidneys, leading to cystic kidney disease. Zebrafish models have proven to be valuable in studying the genes associated with *NPH*. The transparency of zebrafish embryos and larvae allows for non-invasive imaging of ciliary phenotypes, facilitating the study of gene-specific knockdowns and their effects on ciliary function (Molinari et al., 2018). For example, imaging cilia within Kupffer's vesicle in zebrafish has been used to assess *NPH*-related phenotypes, providing detailed insights into the developmental structures affected by *NPH* genes. Zebrafish models have been used to investigate the variable phenotypes and penetrance of different ciliary transition zone mutants, which include *NPH*-associated genes. This research has highlighted the tissue-specific functions of these genes and the complex dynamics of ciliary phenotypes. These studies are essential for understanding the pathogenesis of *NPH* and identifying potential therapeutic targets.

7 Case Study: Zebrafish in the Study of Kidney and Liver Ciliopathies

7.1 Modeling autosomal recessive polycystic kidney disease (ARPKD) in zebrafish

Autosomal recessive polycystic kidney disease (ARPKD) is a severe genetic disorder characterized by the formation of cysts in the kidneys and liver, primarily caused by mutations in the *PKHD1* gene, which encodes the Fibrocystin/Polyductin (FPC) protein. Zebrafish have emerged as a valuable model for studying ARPKD due to their genetic and physiological similarities to humans. Research has shown that mutations in the *DZIP1L* gene, which encodes a ciliary-transition-zone protein, also contribute to ARPKD. Studies using zebrafish have demonstrated that DZIP1L localizes to centrioles and the distal ends of basal bodies, playing a crucial role in maintaining the periciliary diffusion barrier (Olson et al., 2019). This barrier is essential for the proper ciliary-membrane translocation of polycystin-1 and polycystin-2, proteins implicated in ARPKD pathogenesis (Song et al., 2016).

7.2 Zebrafish as a model for studying hepatic fibrosis

Hepatic fibrosis, often associated with ciliopathies, is another area where zebrafish models have provided significant insights. The liver, like the kidney, is affected in ARPKD, leading to fibrosis and other complications. The FPC protein, encoded by PKHD1, is localized not only in the cilium but also in the basal body and other cellular compartments. This localization is crucial for understanding the unique tissue patterning events controlled by FPC, which are not influenced by polycystin-1 (PC1). Zebrafish models have been instrumental in elucidating these mechanisms, offering a clearer picture of how ciliary defects contribute to hepatic fibrosis and providing a platform for testing potential therapeutic interventions (Ma, 2020; McGrew, 2024).

7.3 Investigating the role of ciliary defects in hepatorenal ciliopathies

Ciliary defects are central to the pathogenesis of hepatorenal ciliopathies, including ARPKD. The primary cilium, a cellular organelle, plays a pivotal role in maintaining renal tubule morphology and preventing cyst formation. In ARPKD, mutations in PKHD1 and DZIP1L disrupt the function and biogenesis of ciliary proteins such as FPC and polycystin-1/2. Zebrafish models have been crucial in studying these disruptions. For instance, while DZIP1L mutations compromise the ciliary expression of polycystin-1/2, FPC deficiency does not affect their biogenesis and localization, indicating divergent mechanisms leading to cyst formation. These findings underscore the complexity of ciliary functions and their impact on kidney and liver diseases, highlighting the importance of zebrafish as a model organism in this research area (Lu et al., 2017; Ma, 2020).

8 Concluding Remarks

Zebrafish have proven to be an invaluable model for studying ciliary development and related diseases. The transparency of zebrafish embryos and their rapid development allow for detailed observation of ciliary structures and functions. Studies have shown that zebrafish possess both primary and motile cilia, which are essential for various physiological processes. For instance, research has demonstrated the presence of motile cilia in the olfactory pit and the left-right organizer, with structural similarities to human respiratory cilia. These findings underscore the utility of zebrafish in understanding the genetic and molecular underpinnings of ciliary development and dysfunction.

The potential for future research using zebrafish models in ciliary-related diseases is vast. Given the genetic and physiological similarities between zebrafish and humans, zebrafish can be used to explore the pathogenesis of diseases such as Primary Ciliary Dyskinesia (PCD) and other ciliopathies. The ability to perform high-resolution imaging and genetic manipulation in zebrafish makes it possible to identify novel genes and pathways involved in ciliary function and disease. Additionally, zebrafish models can be employed in high-throughput drug screening to discover new therapeutic compounds for treating ciliary-related disorders.

Zebrafish models are increasingly recognized for their importance in translational medicine. Their genetic tractability, coupled with the conservation of disease-related genes and pathways, makes them an excellent system for studying human diseases and developing new treatments. Zebrafish have been used to model a wide range of conditions, from cardiovascular and metabolic diseases to neurodegenerative disorders and liver diseases. The insights gained from zebrafish research have led to the identification of new drug targets and the development of therapeutic strategies that are now being tested in clinical trials. The continued use of zebrafish in biomedical research promises to accelerate the discovery of effective treatments for a variety of human diseases, highlighting their critical role in the future of precision medicine.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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