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Structures and functions of cilia during vertebrate embryo development

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Abstract

Cilia are hair-like structures that project from the surface of cells. In vertebrates, most cells have an immotile primary cilium that mediates cell signaling, and some specialized cells assemble one or multiple cilia that are motile and beat synchronously to move fluids in one direction. Gene mutations that alter cilia structure or function cause a broad spectrum of disorders termed ciliopathies that impact virtually every system in the body. A wide range of birth defects associated with ciliopathies underscores critical functions for cilia during embryonic development. In many cases, the mechanisms underlying cilia functions during development and disease remain poorly understood. This review describes different types of cilia in vertebrate embryos and discusses recent research results from diverse model systems that provide novel insights into how cilia form and function during embryo development. The work discussed here not only expands our understanding of in vivo cilia biology, but also opens new questions about cilia and their roles in establishing healthy embryos.

KEYWORDS
birth defects, ciliopathies, embryonic development, multiciliated cells, primary cilia

1 | INTRODUCTION

Cilia are microtubule-based organelles that perform critical functions during embryonic development and throughout adult life. There are two basic types of cilia: immotile cilia and motile cilia. Cilia that are motile work together to move fluids over epithelial surfaces, whereas immotile cilia serve as signaling hubs that sense and process cues that guide cell behaviors such as migration, proliferation and differentiation (Gerdes et al., 2009; Mitchison & Valente, 2017; Reiter & Leroux, 2017). Work over the last two decades in the fields of genetics, cell and molecular biology, and embryology have linked defects in the formation and/or function of cilia with several congenital multisystem disorders that are referred to as ciliopathies. Ciliopathies present with a broad spectrum of severity, partially overlapping phenotypes, and genetic heterogeneity (Focșa et al., 2021; Hildebrandt et al., 2011; Horani & Ferkol, 2021; Hyland & Brody, 2021; Waters & Beales, 2011). While several gene mutations that alter cilia are embryonic lethal, a window into the wide-ranging functions for cilia during vertebrate embryonic development is provided by the breadth of multisystem birth defects associated with ciliopathies. Examples include neural tube defects, kidney cysts, polydactyly, organ laterality defects, respiratory problems, and congenital heart defects. The mechanisms underlying cilia functions during development and disease are under active investigation. Significant insights into cilia form and function have come from powerful studies using single-celled organisms, including ciliates (Soares et al., 2019, 2020; Valentine & Van Houten, 2021) and the green alga

Chlamydomonas reinhardtii (Pan, 2008; Snell et al., 2004), or invertebrate animal models such as planaria (Basquin et al., 2015) and the roundworm *Caenorhabditis elegans* (Bae & Barr, 2008; Blacque & Sanders, 2014). However, this review focuses specifically on cilia in vertebrate embryos. Here, I give a brief overview of cilia architecture and dynamics and reference recent review articles that provide in-depth coverage of these topics. I then discuss different types of embryonic cilia and review selected examples of recent work that has provided new insights into mechanisms underlying cilia functions during embryo development. Finally, I consider open questions and remaining gaps in our understanding of embryonic cilia and their impacts on human health.

2 | CILIA ARCHITECTURE

Cilia are ancient organelles that have a highly conserved core architecture, which includes a basal body, a transition zone, and an axoneme (Figure 1). The first of these structures, the basal body, serves as the base of the cilium and is derived from the oldest or "mother" centriole in the cell (Breslow & Holland, 2019; Hall & Hehnly, 2021; Kumar & Reiter, 2021). The mother centriole is a barrel shaped structure made up of nine microtubule triplets decorated with distal and subdistal protein appendages. During ciliogenesis, the mother centriole—tethered to its daughter centriole—docks at the plasma membrane and becomes the basal body (Figure 1). Distal appendages of the mother centriole function as transition fibers to link the basal body to the ciliary membrane. The basal body then functions as the microtubule organizing center that forms the microtubule-based axoneme of the cilium. Second, the transition zone is located between the basal body and the axoneme, and functions as a "ciliary gate" that controls which proteins enter and exit the cilium (Figure 1) (Gonçalves & Pelletier, 2017; Jensen & Leroux, 2017; Reiter et al., 2012). The transition zone contains Y-links that connect the axoneme to the overlying membrane and multi-protein complexes that regulate cilia protein trafficking. Finally, the axoneme is a cylindrical structure composed of nine microtubule doublets that protrudes from the cell surface and forms a specialized ciliary membrane (T. Ishikawa, 2017; Zhao et al., 2022) (Figure 1).

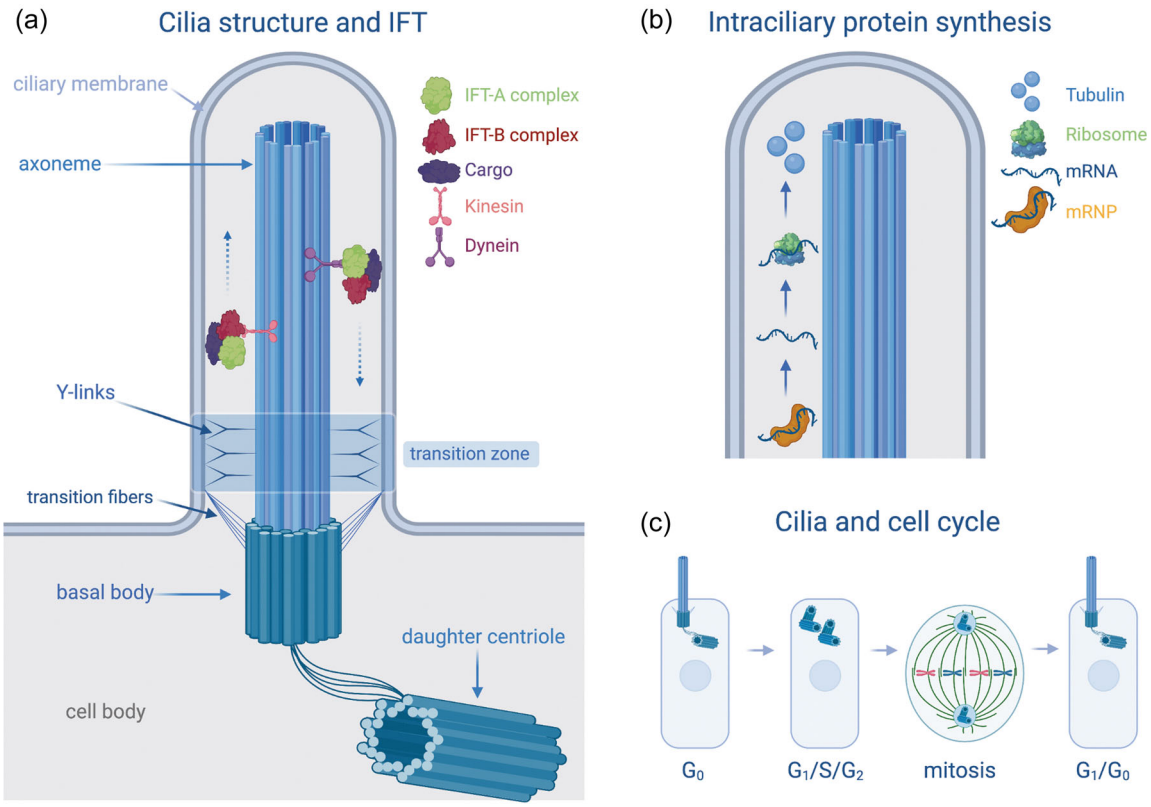

FIGURE 1 Core structure and dynamics of cilia. (a) The mother centriole in the cell becomes the basal body of the cilium, which is connected to the plasma membrane by transition fibers. Y-links and multi-protein complexes form a transition zone adjacent to the basal body that selects proteins to enter and exit the cilium. The microtubule-based axoneme projects from the surface of the cell and creates a specialized ciliary membrane. The intraflagellar transport (IFT) system moves protein cargos inside cilia. The multi-subunit IFT-B complex interacts with Kinesin-2 to move up the axoneme, and the IFT-A complex interacts with Dynien-2 to move down. (b) In addition to IFT, recent work has uncovered protein translation inside motile cilia. Messenger ribonucleoprotein (mRNP) complexes are proposed to deliver mRNAs to cilia that are translated by ribosomes to generate cilia structural proteins such as Tubulin. (c) Cilia typically assemble when cells exit the cell cycle (G_0). Upon re-entry of the cell cycle, cilia disassemble and centrioles duplicate during $G_1/S/G_2$ phases in preparation for division. Following mitosis, cilia reassemble in G_1/G_0 phases.

Although contiguous with the plasma membrane, the membrane covering the axoneme has a distinct lipid and protein composition. The enzyme inositol polyphosphate-5-phosphatase E (INPP5E) localizes to the cilium where it converts the phospholipids PI(4,5)P3 and PI(4,5)P2 to PI(4)P (Bielas et al., 2009; Jacoby et al., 2009), which favors localization of specific cell signaling proteins (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). Building upon these conserved components, the precise architecture and composition of a cilium is cell type-specific and depends on the interplay of multiple regulatory mechanisms.

3 | DYNAMIC REGULATION OF CILIA

Cilia are dynamic structures during embryonic development. Assembly, maintenance, and disassembly of cilia is a tightly regulated process that depends, in part, on intraflagellar transport (IFT). IFT—first described in the cilia-like flagella of *Chlamydomonas reinhardtii* (Kozminski et al., 1993) and subsequently found to be well conserved in eukaryotes—is a molecular transportation system that moves ciliary proteins along the axoneme (Jordan & Pigino, 2021; Prevo et al., 2017; Webb et al., 2020). The IFT machinery consists of multiple protein subunits that form two complexes known as IFT-A and IFT-B. These complexes form polymers called "trains" that move building materials and signaling molecules up (anterograde transport) and down (retrograde transport) the microtubule-based axoneme (Figure 1a). IFT-B interacts with the plus-end directed microtubule motor Kinesin-2 at the cilium base to drive anterograde transport to the cilium tip, and IFT-A interacts with the minus-end motor Dynein-2 to facilitate retrograde transport of proteins down and out of the cilium. IFT machinery interacts with a multi-protein complex called the BBSome (named for the ciliopathy Bardet-Biedl Syndrome) that links IFT to signaling proteins (Garcia et al., 2018; Nachury & Mick, 2019; Wingfield et al., 2018). Initially implicated in delivering G-protein-coupled receptors (GPCRs) to cilia (Berbari et al., 2008; Jin et al., 2010), it is now recognized that the BBSome largely regulates the exit of activated signaling receptors and other cargo from cilia (Lechtreck et al., 2009, 2013; Liu & Lechtreck, 2018; Nager et al., 2017; Ye et al., 2018). The balance of anterograde to retrograde transport—which controls ciliary composition and mediates axonemal elongation, maintenance, and retraction—is regulated by signaling pathways and, in some cases, the external environment. Based on these dynamics, several models to explain the precise control of cilia length have been proposed (Avasthi & Marshall, 2012; H. Ishikawa & Marshall, 2017; W. Wang et al., 2021).

In addition to axonemal dynamics, the molecular contents of cilia are also dynamic and highly regulated. Proteins made in the cell body that are destined to enter cilia are largely transported by trafficking pathways to the cilium and then vetted by the ciliary transition zone. Membrane proteins can be sent to cilia in vesicles from the Golgi, via recycling endosomes, or by diffusing through the plasma membrane (Morthorst et al., 2018; Mukhopadhyay et al., 2017). Components of trafficking pathways that deliver proteins to cilia include Rab GTPases, the exocyst, and the IFT protein IFT20 (Blacque et al., 2018; Mukherjee et al., 2014; Nachury & Mick, 2019; Zhao et al., 2022). The IFT-A complex, which meditates retrograde IFT, is also involved in delivering cargo to cilia. In mammalian cells, the tubby family proteins Tub and Tulp3 interact with IFT-A and PI(4,5)P2 to traffic structurally diverse membrane proteins to cilia (Badgandi et al., 2017; Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Hilgendorf et al., 2019; Hirano et al., 2017; Mukhopadhyay et al., 2010, 2013; Sun et al., 2012). Proteins that arrive at the base of the cilium next encounter the transition zone, which is thought to function both as a size-exclusion gate for soluble proteins and a diffusion barrier for membrane proteins (Garcia-Gonzalo & Reiter, 2017; Jensen & Leroux, 2017; Takao & Verhey, 2016). Mutations that disrupt the transition zone can alter the protein composition of cilia by allowing proteins to leak in or out. The exact molecular mechanisms used to "select" proteins for entry and exit of cilia remain unknown. For membrane-bound proteins it is proposed that Y-links organize transition zone protein complexes at the ciliary membrane to create a barrier that requires an approved transport system, such as IFT or LIFT (lipidated intraflagellar transport), for successful entry or exit from the cilium (Jensen & Leroux, 2017). In recent years, the molecular composition of the transition zone has been extensively analyzed. Genetic and biochemical studies have characterized multi-protein complexes that function at the transition zone to regulate the contents of cilia (Garcia-Gonzalo et al., 2011; Sang et al., 2011; Williams et al., 2011). Two major complexes identified—the NPHP complex and the MKS complex—are named after the ciliopathies Nephronophthisis (NPHP) and Meckel Gruber syndrome (MKS). Cell biology experiments and super-resolution microscopy studies have provided insights into the regulation of transition zone assembly and disruptions of transition zone complexes in ciliopathy patients (Garcia-Gonzalo & Reiter, 2017). Interestingly, recent work indicates proteins can be synthesized inside motile cilia (Hao et al., 2021). Using proteomics, transcriptomics, and super-resolution imaging, the authors identified ribosome components, translation factors, mRNA, and nascent peptides in cilia (Figure 1b). In addition, this study implicated the RNA-binding protein FMRP in delivering mRNA-protein (mRNP) complexes to cilia. These findings change how we think about protein dynamics in cilia, and suggest intraciliary translation may be used to complement IFT delivery of proteins needed to maintain cilia structure. Further work is needed to investigate molecular details of this mechanism, test whether proteins are synthesized in immotile cilia, and determine if/how mRNPs are selected at the transition zone.

The assembly and disassembly of cilia is dynamic during developmental processes, which include cell proliferation and cell differentiation (Patel & Tsiokas, 2021). Several cilia assembly and disassembly factors and pathways have been described (Hossain & Tsang, 2019; Lee, 2020; Sánchez & Dynlacht, 2016). In many cases, the appearance and disappearance of cilia is linked to the cell cycle (Kasahara & Inagaki, 2021). Since the basal body is derived from the mother centriole, cilia assemble when cells are quiescent and disassemble when cells enter the cell cycle as the centrioles are

needed for mitosis (Figure 1c). Several pathways, including Wnt signaling, Ca^{2+} signaling, and phosphoinositide signaling, have been found to activate the kinase Aurora A that regulates mitotic entry (Goto et al., 2017). Aurora A can phosphorylate/activate the histone deacetylase HDAC6, which deacetylates and destabilizes axonemal tubulin to promote cilia disassembly. Other factors implicated in cilia disassembly include HDAC2, Kinesin motor proteins, and polo-like kinase (L. Wang & Dynlacht, 2018). In stem cells, the cilia assembly/disassembly decision is linked to self-renewal versus differentiation (Bodle & Loboa, 2016; Lyu & Zhou, 2017; Yanardag & Pugacheva, 2021). One example is that cilia disassembly in neural progenitor cells has been implicated in neurogenesis. Mutations in patients with the birth defect microcephaly have been found to delay cilium disassembly in neural progenitors (Gabriel et al., 2016; W. Zhang et al., 2019). This delay leads to decreased proliferation and premature differentiation of neural progenitor cells, which is linked to a reduced pool of progenitors for neural development, and thus provides an explanation for microcephaly. In addition to developmental defects, dysregulation of cilia assembly/disassembly has been implicated in cancer (Cao et al., 2021; L. Wang & Dynlacht, 2018). While many studies of cilia dynamics have been done using tractable in vitro systems, tools developed for in vivo imaging studies (examples include [Ford et al., 2018; H. Zhang, Huang, et al., 2022]) provide opportunities to better understand the molecular cues and mechanisms that regulate the dynamic building and deconstruction of cilia in vertebrate embryos.

4 | TYPES OF EMBRYONIC CILIA

In vertebrate embryos, transcriptionally regulated variations in ciliary architecture and composition give rise to distinct types of cilia in different types of cells (Gerdes et al., 2009; Hyland & Brody, 2021; Mitchison & Valente, 2017). Most cells assemble an immotile cilium—referred to as a "primary cilium" because there is one cilium per cell—that contains membrane proteins that can sense external chemical and/or mechanical cues, and thereby serves as a signaling hub for the cell. Specialized cell types with motile cilia—referred to as multiciliated cells—build many cilia that beat in concert to move fluids. This section gives an overview of these types of embryonic cilia and highlights exceptions to these general rules.

4.1 | Immotile cilia

Immotile primary cilia typically have what is called a "9 + 0" ultrastructure that refers to the 9 microtubule doublets of the ciliary axoneme (Figure 2a-a'). In contrast, motile cilia generally have a "9 + 2" structure that includes a central pair of microtubule doublets (Figure 2b-b'). Primary cilia are often referred to as sensory cilia because, in part, receptors for ligands of several cell-cell signaling pathways can localize to the ciliary membrane. These include hedgehog (HH), G-protein-coupled receptor (GPCR), and transforming growth factor-beta (TGFβ) pathways. Other pathways modulated by primary cilia are WNT and receptor tyrosine kinase (e.g., FGF, PDGFRα, and IGF) signaling. Known details about the roles for primary cilia in biochemical signaling pathways have been described in recent reviews (Anvarian et al., 2019; Elliott & Brugmann, 2019; Sreekumar & Norris, 2019). One of the most widely studied examples of cilia-mediated signaling is the HH pathway. Several cellular and molecular mechanisms by which defects in primary cilia and HH signaling cause birth defects have been reviewed (Andreu-Cervera et al., 2021; Ingham, 2022; Murdoch & Copp, 2010). Examples include mutations in IFT proteins that alter ciliary transport (Huangfu et al., 2003; Keady et al., 2012) and *INPP5E* mutations that alter ciliary phosphoinositide content (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Jacoby et al., 2009), which disrupts HH signaling and result in polydactyly and neural tube defects. The involvement of primary cilia in critical signaling pathways during development is consistent with the broad spectrum of birth defects associated with ciliopathies.

In addition to sensing biochemical signals, primary cilia have been implicated as mechanosensors. Prominent models suggest stretch-activated ion channels that localize to the ciliary membrane allow primary cilia to sense being bent/deformed by mechanical stimuli, such as fluid flow, by permitting ions to enter the cilium to trigger signaling cascades (Prasad et al., 2014; Spasic & Jacobs, 2017). This paradigm stems largely from work investigating the cation channel Polycystin 2 (PC2; encoded by the *PKD2* gene) that can interact with the Polycystin 1 protein (PC1; *PKD1*) to form a mechanosensitive complex that can localize to the primary ciliary membrane to regulate calcium ion (Ca^{2+}) entry (Gargalionis et al., 2019; Piperi & Basdra, 2015). Mutations in *PKD2* or *PKD1* are associated with autosomal dominant polycystic kidney disease, but exactly how loss of PC2 or PC1 causes kidney disease remains unclear. In kidney, primary cilia on epithelial cells have been proposed to detect urine flow through the nephrons and collecting ducts (Praetorius & Spring, 2003). Imaging experiments using fluorescent Ca^{2+} sensors in several different contexts indicate fluid flow can trigger an increase of intracellular Ca^{2+}, and that this response depends on primary cilia and the PC1-PC2 complex. Examples include work in kidney epithelial cells (Nauli et al., 2003), vascular endothelial cells (Nauli et al., 2008), bile duct cholangiocytes (Masyuk et al., 2006), and zebrafish (Yuan et al., 2015) and mouse (Mizuno et al., 2020) embryos. Results from these studies support a model that mechanoactivation of PC1-PC2 increases the intraciliary Ca^{2+} concentration that subsequently stimulates the release of Ca^{2+} from intracellular stores to raise cytoplasmic Ca^{2+} levels that influence downstream signaling. However, a study of multiple cell types from transgenic mice that express a cilia-targeted Ca^{2+} sensor concluded primary cilia are not Ca^{2+}-responsive mechanosensors (Delling et al., 2016). Differences in experimental techniques, including mouse embryo culture conditions, have been proposed to explain these disparate results (Mizuno et al., 2020). Additional work is needed to further clarify ciliary mechanisms during cellular mechanosensation.

Specific structural modifications of immotile cilia facilitate additional sensory functions. First, during development of photoreceptor cells (rods and cones) in the vertebrate retina, a highly

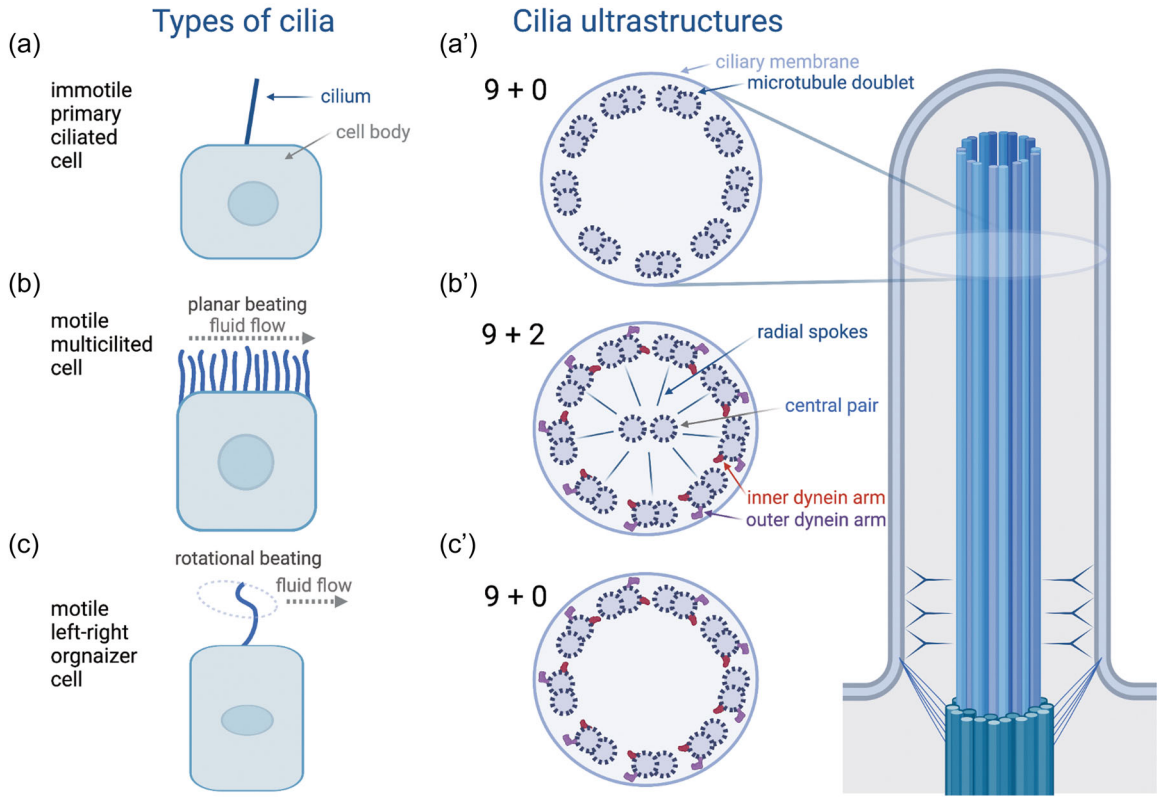

FIGURE 2 Types of cilia in vertebrate embryos. (a–c) Diagrams of cells with different types of embryonic cilia. (a) Immotile primary cilia mediate cell signaling. (b) Motile multi-cilia beat synchronously to generate directional fluid flows. (c) Motile left-right organizer (LRO) cilia beat with a helical pattern to drive fluid flows. (a'–c') Diagrams of cross sections through the different types of cilia. (a') Immotile primary cilia have a 9 + 0 arrangement of microtubule doublets. (b') Motile multi-cilia have a 9 + 2 arrangement that includes a central pair of microtubules and radial spokes. These cilia also have inner and outer dynein arms that generate movement. (c') Motile LRO cilia have a 9 + 0 arrangement with dynein arms.

modified 9 + 0 primary cilium forms the outer segments that are packed with membranous structures containing phototransduction proteins, including the GPCR protein Rhodopsin, that detect photons and mediate vision (Barnes et al., 2021; Bujakowska et al., 2017; Chen et al., 2021). Like other primary cilia, photoreceptor cilia consist of a basal body, transition zone (called the connecting cilium), and axoneme, but have a highly specialized ciliary membrane that has evolved for efficient photon capture and conversion of light into electrical signals. Importantly, mutations in cilia genes that alter photoreceptor structure and/or function cause retinal degeneration (Bachmann-Gagescu & Neuhauss, 2019). Second, in contrast to most cell types that form a single primary/sensory cilium, olfactory sensory neurons develop multiple sensory cilia (McClintock et al., 2020). These cilia express distinct cilia membrane-localized G protein-coupled odorant receptors. Odorant binding to these receptors activates intracellular signaling that leads to membrane depolarization and action potentials that transmit odor information to the brain (Su et al., 2009). Some ciliopathy mutations can disrupt olfactory cilia and cause anosmia (Kulaga et al., 2004; McEwen et al., 2007). A third example of a structural modification of sensory cilia is the 9 + 2 kinocilium found on vertebrate hair cells, which is an exception to the rule that immotile cilia have a 9 + 0 architecture. Hair cells in the inner ear convert sound vibrations into electrical signals—a process called mechanoelectrical transduction (MET)—to provide the basis for hearing. During embryonic development, hair cells form several actin-based stereocilia and one true microtubule-based kinocilium to form a highly organized hair bundle (Nayak et al., 2007). When sound deflects the hair bundle, MET channels on stereocilia open to depolarize the hair cell (Caprara & Peng, 2022; Cunningham & Müller, 2019; Zheng & Holt, 2021). The kinocilium is not directly involved in MET, but rather is required for planar cell polarity during hair cell development that mediates the arrangement of stereocilia necessary for hearing (D. Wang & Zhou, 2021).

4.2 | Motile cilia

Motile cilia have a 9 + 2 ultrastructure, and also contain radial spokes and dynein motor complexes that enable motility (F. Zhou & Roy, 2015) (Figure 2b'). Radial spoke complexes connect the 9 outer microtubule doublets to the central pair, and inner dynein arm (IDA) and outer dynein arm (ODA) complexes, which are regularly spaced along the axoneme, hydrolyze ATP to drive planar cilia beating (T. Ishikawa, 2017). Motile cilia are typically found on specialized

epithelial multiciliated cells (MCCs) that project ~50–300 cilia from their apical surface that beat in sync to move fluids in one direction (Brooks & Wallingford, 2014) (Figure 2b). In human embryos, MCCs develop in the respiratory tracts, brain ventricles, and reproductive tracts (Hoque et al., 2022; Hyland & Brody, 2021; Ringers et al., 2020). MCCs in the respiratory tract move mucus to remove trapped dirt and pathogens (called mucociliary clearance) to protect against infections. Multiciliated ependymal cells in brain ventricles move cerebrospinal fluid, which has both protective and signaling functions in the brain. Finally, during adult life, MCCs help move oocytes through the fallopian tubes and prevent sperm agglutination in efferent ducts in the female and male reproductive tracts. In contrast to uniform planar beating seen in fallopian tubes, efferent duct cilia use a whip-like beating that changes direction to create turbulent fluid flows that keep sperm from sticking together (Yuan et al., 2019). Regulatory factors that control development of MCCs—which include signaling pathways, transcription factor networks, and centriole amplification mechanisms—have been identified, but the molecular mechanisms used to generate these cells in developing embryos are still under investigation (Boutin & Kodjabachian, 2019; Lewis & Stracker, 2021; Spassky & Meunier, 2017).

An exception to the rule that motile cilia have a 9 + 2 structure and assemble on multiciliated cells is the motile 9 + 0 cilium that forms as one cilium per cell in an embryonic fluid-filled structure called the "organ of asymmetry" or more recently the "left-right organizer" (LRO) (Figure 2c-c'). In several vertebrate embryos, these monocilia in the LRO play a critical role in establishing the vertebrate left-right body axis and subsequent development of essential left-right asymmetries in visceral organs such as the heart and gastrointestinal tract (Amack, 2014; Blum & Ott, 2019; Hamada, 2020; Little & Norris, 2021). LRO cilia have dynein arms but lack radial spokes and the central pair (Figure 2c'), such that these cilia beat with a helical pattern rather than the typical planar beating of 9 + 2 cilia (Shinohara et al., 2015). In mouse, fish, and frog embryos, monocilia beat synchronously in the LRO to generate a right-to-left fluid flow that directs asymmetric expression of signaling cascades that break bilateral symmetry in the embryo (Essner et al., 2005; Nonaka et al., 1998; Okada et al., 2005; Schweickert et al., 2007). This includes upregulation of the TGFβ family member *NODAL* in the left lateral plate mesoderm where it regulates downstream factors, such as the transcription factor *PITX2*, associated with asymmetric morphogenesis of visceral organs (Franco et al., 2017; Grimes & Burdine, 2017; Shiratori & Hamada, 2014). This asymmetric *NODAL-PITX2* signaling is achieved by specific degradation of mRNA encoding the NODAL inhibitor DAND5 on the left side of the LRO, which depends on cilia-driven fluid flow (Lopes et al., 2010; Marques et al., 2004; Schweickert et al., 2010). However, exactly how flow is sensed by LRO cells is not fully understood (Cartwright et al., 2020; Dasgupta & Amack, 2016; Norris, 2012; R et al., 2019; Smith et al., 2014).

Defects in the formation or function of motile cilia result in disorders that can be referred to as "motile ciliopathies" (Wallmeier et al., 2020). One of these disorders, called primary ciliary dyskinesia (PCD), results from mutations that paralyze motile cilia (Horani & Ferkol, 2021; Lucas et al., 2020; Gravesande & Omran, 2005). Most newborns with PCD suffer from respiratory stress within 24 h and then develop progressive defects associated with compromised mucociliary clearance, which include chronic airway infections and progressive airway damage (bronchiectasis). In addition, otitis media and hearing loss are common in PCD children. In adulthood, male PCD patients are often infertile due to defects in sperm flagella and, although cilia motility in fallopian tubes is not essential for oocyte transport, subfertility and ectopic pregnancy has been reported in female patients (Leigh et al., 2019). In very rare cases, PCD patients develop hydrocephalus that may be caused by disrupted CSF flows (Wallmeier et al., 2022). Approximately 50% of PCD patients have defects in organ left-right asymmetry. Most of these patients have a complete mirror-image reversal of the left-right axis, called *situs inversus totalis*, which has little or no clinical consequence. However, ~6%–10% of PCD individuals have isolated let-right axis defects characterized as *situs ambiguous*/heterotaxy syndrome that can be associated with a congenital heart defect (Best et al., 2019; Kennedy et al., 2007). The combination of left-right axis malformations and airway defects, known classically as Kartagener's syndrome, was first linked to defects in motile cilia in the 1970s (Afzelius, 1976). Distinct from PCD, a disorder referred to as reduced generation of multiple motile cilia (RGMC) is caused by mutations in factors that control development of MCCs (Boon et al., 2014; Wallmeier et al., 2014). Respiratory MCCs in RGCM patients have fewer motile cilia, which results in airway symptoms seen in PCD patients. Left-right axis defects are not present, consistent with unaffected motile monocilia in the LRO. Defective MCCs in efferent ducts cause sperm agglutination, duct obstruction, atrophy of testis, and ultimately male infertility in mouse models, and likely underlie infertility in male patients (Aprea et al., 2021; Hoque et al., 2021; Terré et al., 2019; Yuan et al., 2019). Interestingly, the incidence of hydrocephalus is higher in RGMC patients than in PCD (Wallmeier et al., 2022). The congenital malformations associated with these motile ciliopathies highlight functions for motile cilia during embryonic development.

5 | MECHANISMS UNDERLYING EMBRYONIC CILIA FORM AND FUNCTION

Several model embryos, including mouse, zebrafish, and frog, have been used effectively to analyze cilia proteins and provide insights into cellular and molecular mechanisms underlying cilia functions during vertebrate development. In addition, cultured human cell lines, and more recently, human organoids, have begun to shed light on human embryonic cilia. Functions for cilia in specific developmental contexts have been described in several previous review articles that I highly recommend (Anvarian et al., 2019; Drummond, 2012; Goetz & Anderson, 2010; Sreekumar & Norris, 2019). Here, I discuss selected examples of recent work that highlights model systems and provides new insights into mechanisms underlying (1) primary cilia functions during embryo implantation and placenta development, (2)

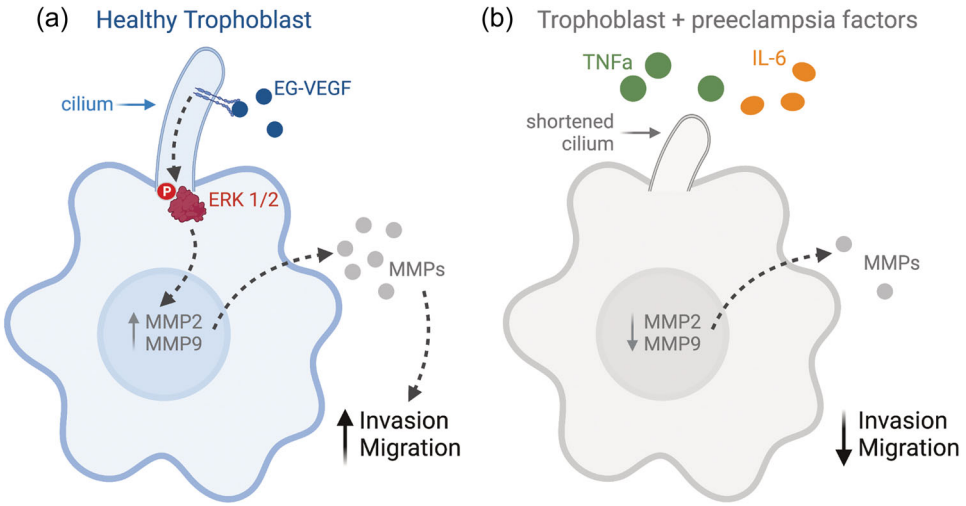

FIGURE 3 Primary cilia regulate human trophoblast cell migration and invasion. (a) In healthy trophoblasts, primary cilia mediate EG-VEGF signaling that upregulates MMP expression to enhance migration and invasion. (b) In trophoblasts treated with TNFa or IL-6, which are cytokines upregulated in pre-eclampsia patients, primary cilia are shortened, MMP expression is lowered, and migration is reduced. EG-VEGF, endocrine gland-derived vascular endothelial growth factor.

development of multiciliated cells that drive unidirectional fluid flows, and (3) cooperation between motile cilia and immotile cilia to break bilateral symmetry of embryos. These studies advance our mechanistic understanding of how cilia function during embryonic development and open new avenues for future research.

5.1 | Primary cilia-mediated signaling during embryo implantation and placenta development

Cilia are likely to have multiple functions during the earliest stages of mammalian reproduction. Known examples discussed above include motile cilia in efferent ducts that are critical for production of healthy sperm, and motile cilia in fallopian tubes that assist transport of ovulated oocytes and fertilized embryos. However, additional functions for cilia have been challenging to investigate and are still emerging. Recent work has identified links between immotile primary cilia and human embryo implantation and placenta development, and thereby implicate cilia dysfunction in associated health risks. During the process of implantation, trophoblast cells of the blastocyst bind to and invade the decidua of the uterine wall. Following complete implantation of the blastocyst, trophoblast cells differentiate and contribute to the placenta (Turco & Moffett, 2019). Anomalies in these processes can result in pregnancy complications, including pre-eclampsia (PE). PE is characterized by placental defects that can increase vascular resistance and subsequently raise maternal blood pressure (Rana et al., 2019). The causes of PE are not understood, but defects in trophoblast proliferation, invasion, and differentiation have been proposed to contribute to PE and underlie findings of shallow trophoblast invasion in PE patients (Fisher, 2015).

Recent studies suggest primary cilia on trophoblast cells mediate signaling cascades that regulate their migration and invasion (Ritter et al., 2020; C. Y. Wang et al., 2017, 2019). First, in a study by C. Y. Wang et al. (2017), analysis of cultured cell lines derived from human placenta (3A-subE cells) or human trophoblasts (HTR-8/SVneo cells) revealed these cells have primary cilia, and that the GPCR receptor PROKR1 (Prokineticin1 receptor 1) localizes to these cilia. Importantly, primary cilia were also detected in first trimester human placental tissue, and PROKR1 localized to these cilia. PROKR1 serves as a receptor for Prokineticin1, also known as endocrine gland-derived vascular endothelial growth factor (EG-VEGF), which is a signaling factor that regulates trophoblast invasion and placental development (Brouillet et al., 2013). Adding EG-VEGF to cell cultures was found to increase 2-dimensional migration of trophoblast cells and enhance transwell plate invasion through Matrigel. Mechanistically, EG-VEGF was found to increase phosphorylation/activation of the intracellular kinases ERK1/2 and upregulate expression of the matrix metalloproteinases MMP2 and MMP9 that are involved in matrix degradation during trophoblast invasion. Interestingly, phosphorylated ERK1/2 was enriched at the base of primary cilia. To test the function of the primary cilia in trophoblast cells, siRNA was used to knockdown expression of the IFT protein IFT88. IFT88 knockdown cell cultures had fewer and shorter cilia, and had reduced ERK1/2 activation and MMP 9 expression. In addition, IFT88 depletion inhibited EG-VEGF-induced trophoblast invasion. Together, these findings suggest primary cilia mediate an EG-VEGF- > ERK1/2-> MMP signaling cascade that promotes trophoblast invasion (Figure 3a). In follow-up work, the authors identified microRNAs miR-141 and miR-200a that can downregulate EG-VEGF expression, ERK1/2 activation, and MMP9 activity, and suppress EG-VEGF-induced migration and invasion of cultured trophoblasts (C. Y. Wang et al., 2019). Additionally, expression of miR-141 and miR-200a reduced the number and length of primary cilia in trophoblasts in vitro. In PE patients, expression of circulating miR-141 and miR-200a

were increased, and EG-VEGF levels were decreased, relative to healthy pregnant women. In normal human placenta, ~15% of cells are ciliated in the first trimester (this progressively decreases in the second and third trimesters), whereas only ~3% of cells have cilia in first trimester placenta from PE patients. These findings suggest defects in primary cilia and/or dysregulation of EG-VEGF in trophoblasts may contribute to PE.

In agreement with the work by C. Y. Wang et al. (2017), a study by Ritter and co-workers (Ritter et al., 2020), identified primary cilia in trophoblasts in healthy first trimester human placenta and in human trophoblast cell lines (HTR-8/SVneo, SGHPL-4, and HIPEC-65). In addition, cilia were detected in 3-dimensional trophoblast spheroids and in primary trophoblasts isolated from healthy placenta. However, primary trophoblasts isolated from PE patients had significantly shorter cilia. Previous work from this group indicates that the inflammatory cytokines IL-6 and TNF-α—which are increased in PE patients (C. C. Zhou et al., 2011)—reduce primary cilia in adipose-derived mesenchymal stem cells (Ritter et al., 2018). Consistent with these previous results, treating trophoblast cell lines or spheroids with IL-6 or TNF-α reduced cilium length. IL-6 or TNF-α treatments that shortened cilia reduced MMP2 and MMP9 expression and cell migration in trophoblast cultures (Figure 3b). It remains unclear whether IL-6 or TNF-α impact microRNA (e.g., miR-141 and miR-200a) levels in PE patients. Taken together, these studies (Ritter et al., 2020; C. Y. Wang et al., 2017, 2019) suggest primary cilia on human trophoblast cells regulate signaling pathways that mediate migration and invasion of these cells. Further work is needed to test whether these cilia functions are recapitulated in more physiologically relevant models and ultimately (if possible) in vivo. Interestingly, trophoblast cilia may be unique in humans since cilia were not detected in trophoblast lineages in transgenic reporter mice that fluorescently label all embryonic cilia (Bangs et al., 2015). In addition to new functions for trophoblast cilia, other emerging work suggests (1) primary cilia dysfunction in human placental mescenchymal stromal cells may contribute to PE (Romberg et al., 2022) and (2) mutation of the cilia gene DYNC2H1 is associated with recurrent miscarriage (Hassan et al., 2022). It will be interesting to follow future work to learn more about the mechanisms underlying how cilia function during early human development.

5.2 | Development of functional multiciliated cells to move fluids

How an epithelial cell becomes multiciliated is an intriguing cell biology question. Work in multiple model systems has identified signaling pathways, transcriptional networks, and developmental steps involved in generating multiciliated cells (MCCs), but the underlying mechanisms are not fully understood (Collins et al., 2021; Lewis & Stracker, 2021; Mahjoub et al., 2022; Spassky & Meunier, 2017). Cells specified to become MCCs upregulate transcription factors, including GEMC1 (encoded by *GMNC*; *Geminin Coiled-Coil Domain Containing* gene) and Multicilin (encoded by *MCIDAS*), that promote cell cycle exit and centriole expansion (Lalioti et al., 2019; Terré et al., 2016; F. Zhou et al., 2015). New centrioles dock at the apical surface of the cell as basal bodies, which form rootlets that are involved in anchoring the motile cilium. GEMC1 and Multicilin induce expression of additional transcription factors, such as FOXJ1 and TP73, that function as master regulators of motile cilia formation. Nascent beating cilia then become oriented in response to planar cell polarity cues and fluid movement (Mitchell et al., 2007, 2009), which ultimately allows MCCs to drive directional fluid flows. Human mutations that alter Multicilin, FOXJ1, or TP73 have been associated with reduced generation of multiple motile cilia (RGMC) phenotypes (Wallmeier et al., 2022), and the identification of additional regulatory factors of MCCs is expected to provide new candidate genes for RGMC. The recent work by D'Gama and colleagues (D'Gama et al., 2021) and Yasunaga and colleagues (Yasunaga et al., 2022) discussed below offers new insights into the developmental programs and molecular mechanisms that give rise to functional MCCs.

D'Gama and colleagues (D'Gama et al., 2021) used zebrafish to investigate the developmental origins of ependymal MCCs that line the brain ventricles and spinal canal and are thought to contribute to the flow of cerebrospinal fluid (CSF). Defects in MCC function in the nervous system has been associated with hydrocephalus (Wallmeier et al., 2022). In zebrafish, cilia-driven CSF flow has also been linked to the spine malformation idiopathic scoliosis (Grimes et al., 2016; X. Zhang et al., 2018). To better understand development of ependymal MCCs, D'Gama, et. al. used glutamylated Tubulin antibodies and video microscopy as readouts for motile cilia, and analyzed expression of *FOXJ1* homologs (*foxj1a* and *foxj1b* in zebrafish) and *GMNC* (*gmnc*) in brains of larval, juvenile, and adult zebrafish. These analyses revealed that MCCs develop from monociliated cells during juvenile stages, and that MCCs in different regions of the brain differentially express *foxj1a*, *foxj1b*, and *gmnc* (Figure 4a). These differences in gene expression suggested that distinct types of MCCs develop in the brain. Furthermore, analyses of $foxj1a^{+/-}$;$foxj1b^{-/-}$ or $gmnc^{-/-}$;$foxj1b^{-/-}$ double mutants uncovered defects in specific types of ciliated cells, suggesting the development of motile ciliated cell types depends on differential activation of Foxj1 and Gmnc transcriptional programs. Finally, single-cell RNA sequencing identified two distinct clusters of ependymal cells that likely correspond to different regions of the brain. These results provide new insight into the development and diversity of ependymal MCCs from embryonic to adult stages of life. Interestingly, $foxj1a^{+/-}$;$foxj1b^{-/-}$ mutants (which have reduced ependymal MCCs) and $gmnc^{-/-}$ mutants (which completely lack MCCs) develop enlarged brain ventricles reminiscent of hydrocephalus, but do not show scoliosis. However, scoliosis was observed in a subset of triple $foxj1a^{+/-}$;$foxj1b^{-/-}$;$gmnc^{+/-\ or\ -/-}$ mutants, suggesting the scoliosis phenotype does not result solely from loss of MCCs, but rather is associated with the combined loss of *foxj1a*, *foxj1b*, and *gmnc*. In light of these results, future work could aim to develop new tools to further clarify the relationship(s) between ependymal cell types, cilia, CSF flow, and scoliosis.

In addition to identifying transcriptional networks that control development of MCCs, recent work has elucidated physical mechanisms used to orient the multiple motile cilia in these cells. Yasunaga

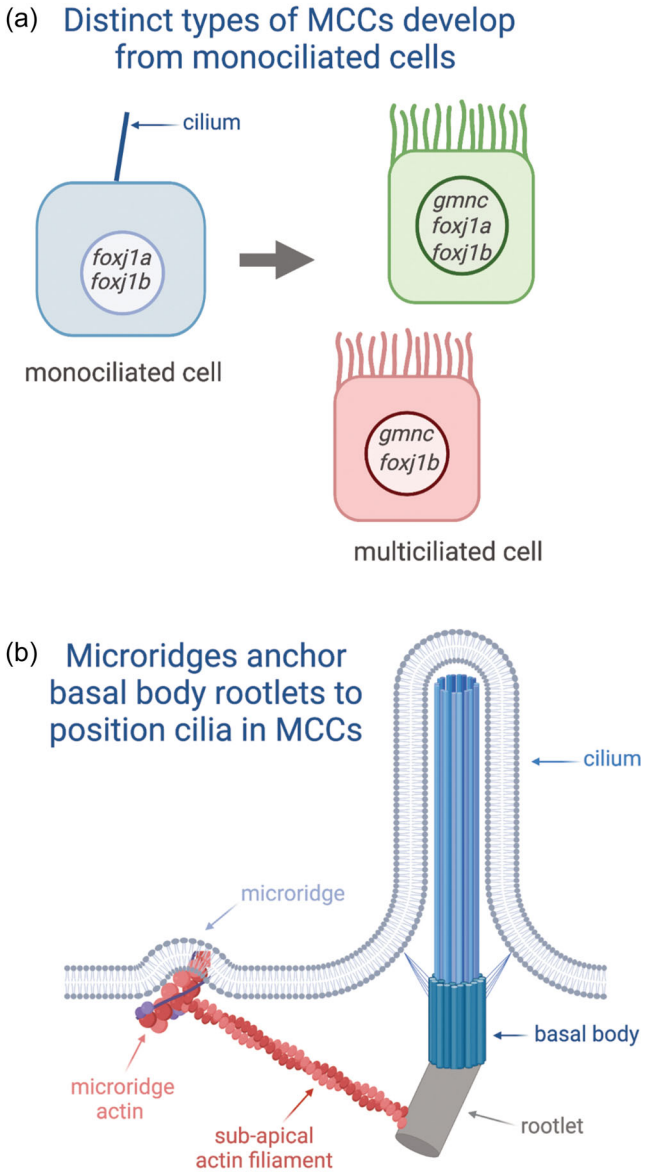

FIGURE 4 Development of transcriptional diversity and physical links in multiciliated cells. (a) Schematic representation of development of ependymal multicilited cells (MCCs) in the zebrafish brain. MCCs develop from monociliated cells during juvenile stages and show distinct cell types based on differential expression of FoxJ1 and Gmnc factors that mediate MCC transcription programs. (b) Diagram of a single motile cilium at the apical surface of a MCC. Sub-apical actin filaments connect ciliary rootlets to actin-based microridges to anchor and orient each cilium.

and colleagues (Yasunaga et al., 2022) used MCCs on the epidermis of the frog (*Xenopus leavis*) embryo as an in vivo model system to investigate how cilia are anchored to the apical membrane in the correct orientation. In previous work, actin filaments have been found to have several roles during cilia development in MCCs. Examples include an apical actin cytoskeleton involved in basal body docking (Pan et al., 2007), and a subapical actin network functionally linked to cilia polarization (Werner et al., 2011). However, exactly how the position of individual cilia becomes fixed in MCCs to establish synchronized beating and generation of directional fluid flow has remained unclear. To address this question, Yasunga, et. al. analyzed epidermal MCC development and identified sub-apical actin filaments associated with basal body rootlets that linked anterior rootlets with the apical plasma membrane. Basal bodies that lacked these filaments were randomly oriented, suggesting polarization of cilia in MCCs depends on the formation of rootlet associated actin filaments. Next, the Formin protein Fhod3, a known actin filament nucleator, was found to localize to basal body rootlets. Fhod3 depletion disrupted rootlet orientation, suggesting Fhod3 nucleates sub-apical actin filaments from ciliary rootlets necessary for proper cilia positioning. Imaging experiments indicated that these sub-apical actin filaments anchor ciliary rootlets at enigmatic actin-based structures called microridges that form on the apical surface of epithelial cells (Figure 4b). To test the requirement of microridges, the authors took two approaches using (1) an inhibitor of nonmuscle myosin II to block apical constriction that drives microridge formation and (2) optogenetic disruption of the protein Ezrin that is a component of microridges. In both experiments, disrupting microridge structure altered ciliary rootlet orientation. Together, these results identify a function for microridges as a physical anchor point for basal body rootlets that fix the position of individual cilia in MCCs. This study opens a new area of research to further understand the biophysical connections between basal bodies, microridges, and apical membrane in diverse types of ciliated cells.

5.3 | Cooperation between motile cilia and immotile cilia to establish the left-right body axis

The finding of altered left-right (LR) asymmetry of internal organs in patients with PCD/Kartagener's syndrome provides a clear link between motile cilia and the developmental process of establishing the LR body axis (Afzelius, 1976). Work in the 1990s identified motile monocilia that beat to generate a leftward flow of extraembryonic fluid in the cavity of the ventral node in the mouse embryo, which was found to be essential for determining the LR axis (Nonaka et al., 1998). Subsequent work identified analogous ciliated structures in several vertebrate embryos, which are now referred to as the embryo's left-right organizer (LRO) (Blum et al., 2009; Dasgupta & Amack, 2016; Grimes & Burdine, 2017; Hamada, 2020; Little & Norris, 2021). In mouse models, mutations that paralyze LRO cilia or manipulations that disrupt leftward flow were found to alter LR asymmetry (Nonaka et al., 2002; Okada et al., 1999; Supp et al., 1997, 1999). However, the mechanistic basis for how this mechanical flow is sensed by left-sided cells and translated into molecular signals has been an open question in developmental biology for decades. In the mouse LRO, two populations of cilia have been described: 1) motile cilia primarily found in the central pit of the LRO and 2) immotile cilia primarily on the larger crown cells at the periphery (McGrath et al., 2003). This arrangement led to a "two cilia" hypothesis that fluid flow generated by motile cilia is sensed by immotile cilia (McGrath et al., 2003; Tabin & Vogan, 2003;

Yost, 2003) (Figure 5a). Consistent with this model, ciliary localization of the mechanosensitive cation channel protein Pkd2 is specifically required in crown cells for normal LR patterning (Yoshiba et al., 2012). Additionally, mutations in the Pkd1 homolog Pkd1l1, which interacts with Pkd2, alters LR asymmetry in mouse (Field et al., 2011) and fish (Kamura et al., 2011). Furthermore, live imaging of Ca^{2+} indicators and treatments with Ca^{2+} antagonists have implicated asymmetric Ca^{2+} signaling in establishing left-sided Nodal activity and subsequent LR axis development. Together, these results support a mechanosensing mechanism in which bending of immotile LRO cilia by leftward fluid flow opens cilia-localized stretch-activated Pkd cation channels to allow Ca^{2+} entry into left-sided crown cells that induces degradation of *dand5* mRNA (encoding a Nodal inhibitor) to activate left-sided Nodal signaling (Figure 5b). Asymmetric Nodal activity at the LRO then triggers Nodal-Pitx2 signaling in left lateral plate mesoderm, which is thought to provide LR cues to developing organs such as the heart (Figure 5c). Alternative models propose chemosensing mechanisms that are mediated by leftward movement of secreted signaling molecules (Nonaka et al., 1998; Okada et al., 2005) or vesicles (Tanaka et al., 2005). Results from Yuan and colleagues (Yuan et al., 2015) using zebrafish and Mizuno and colleagues (Mizuno et al., 2020) using mouse have shed new light on the roles of cilia and Ca^{2+} in sensing flow, and provide evidence that supports the mechanosensory hypothesis.

Similar to mouse, the zebrafish LRO, called Kupffer's vesicle (Amack & Yost, 2004; Essner et al., 2005; Kramer-Zucker et al., 2005), has motile cilia that generate flow and immotile cilia that may sense the flow. In Kupffer's vesicle, which does not have crown cells, immotile cilia are randomly distributed throughout the LRO. Thus, a function for immotile cilia in zebrafish LR signaling has remained unclear (Ferreira et al., 2017; Sampaio et al., 2014; Tavares et al., 2017). To test the hypothesis that flow induces Ca^{2+} entry via mechanosensory immotile cilia, Yuan and colleagues (Yuan et al., 2015) developed a double reporter system in zebrafish that uses a green fluorescent Ca^{2+} sensor targeted to cilia and a red fluorescent Ca^{2+} sensor localized in the cytoplasm. Live imaging of Ca^{2+} signals revealed intraciliary Ca^{2+} oscillations (ICOs) in immotile LRO cilia. Although ICOs were observed throughout the LRO, ICOs were found to be biased to the left side. It was then determined that ICOs were frequently followed by an increase in cytoplasmic Ca^{2+} level in the same cell. Next, using gene knockdowns and mutant embryos, it was determined that left-biased ICOs depend on (1) motile LRO cilia and (2) the channel protein Pkd2. These results are consistent with a model in which flow triggers Pkd2-mediated Ca^{2+} entry into LRO cells via sensory cilia (Figure 5b). To block ICOs, the authors targeted cilia with the Ca^{2+} binding protein parvalbumin that can act as a Ca^{2+} sink. Suppressing ICOs disrupted LR asymmetry of Ca^{2+} signals, *dand5* mRNA, *southpaw* (Nodal homolog in zebrafish) mRNA, and heart laterality. Finally, expressing the Ca^{2+} sink specifically in LRO cilia was found to alter LR patterning, indicating a cell-autonomous function for intraciliary Ca^{2+}. Together, this study identified for the first time Pkd2-dependent ICOs in LRO cilia that function during LR axis determination. One surprising result of this work is that the frequency of asymmetric ICOs peaked at developmental stages (between the 1 and 4 somite stages) that are significantly earlier than the onset of robust cilia motility and asymmetric flow in the LRO (reported between the 4 and 6 somite stages [G. Wang et al., 2012]) and the degradation of *dand5* mRNA (reported at 8 to 10 somite stages [Lopes et al., 2010]). Future work could aim to develop new tools to precisely connect the dots between ICOs, Ca^{2+} signaling, and *dand5*.

Taking a similar approach as described above in zebrafish, Mizuno, et. al. (Mizuno et al., 2020) generated transgenic mouse strains that express cilia-localized or cytoplasm-localized fluorescent

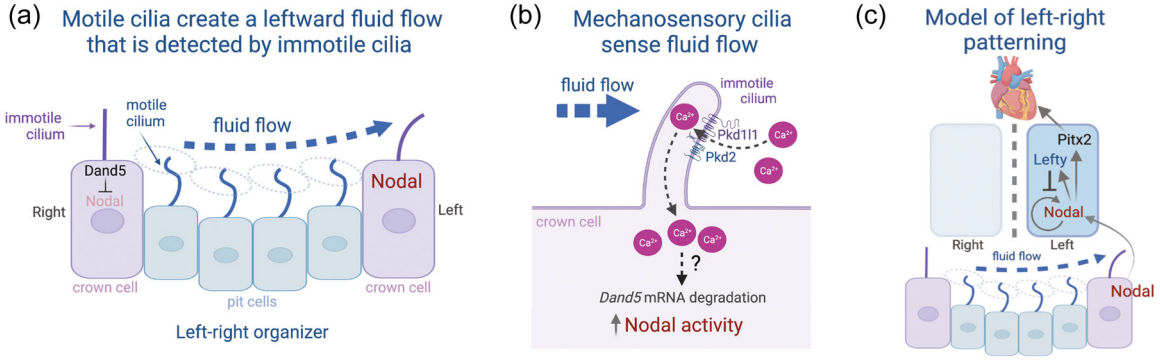

FIGURE 5 Motile cilia work with immotile sensory cilia to establish left-right asymmetry. (a) Diagram of the cellular architecture of the left-right organizer in the mouse embryo. Central "pit cells" have motile monocilia that beat in a vortical pattern to generate a leftward fluid flow, and peripheral "crown cells" have immotile cilia that sense the directional flow. Flow induces degradation of *dand5* mRNA and thereby activates Nodal signaling in left-sided crown cells. (b) A mechanosensory model for sensing flow. Bending of immotile cilia by fluid flow opens stretch-activated Pkd cation channels (made of Pkd1l1-Pkd2 complex) to allow Ca^{2+} entry into crown cells. Ca^{2+} from the cilium activates cytoplasmic Ca^{2+} release, likely from endoplasmic reticulum, that is proposed to mediate downstream signaling that ultimately results in degradation of *Dand5* mRNA and subsequent increased Nodal activity. (c) Model for vertebrate left-right patterning. Asymmetric Nodal activity generated at the left-right organizer induces Nodal signaling cascades in left lateral plate mesoderm. Nodal activates its own expression in neighboring cells, as well as the Nodal inhibitor Lefty and the transcription factor Pitx2. Left-sided Pitx2 expression is thought to instruct asymmetric morphogenesis of internal organs, including the heart.

Ca^{2+} sensors specifically in crown cells of the LRO. Live imaging of cultured mouse embryos captured Ca^{2+} transients in crown cell cilia. As in zebrafish, these ciliary transients were observed on both the left and right sides of the LRO, but were more frequent on the left side. Also consistent with findings in zebrafish, loss of cilia motility or Pkd2 abolished LR asymmetry of Ca^{2+} transients in crown cell cilia. Next, dual analysis of ciliary and cytoplasmic Ca^{2+} spikes in the same cells revealed these events often—but not always—occurred together in crown cells and were highly synchronized. Further analysis of mutants that lack cilia identified two types of cytoplasmic Ca^{2+} transients in crown cells: Type 1 transients are LR asymmetric and depend on cilia, and Type 2 transients are symmetric across the LRO and occur in the absence of cilia. The authors then used a pharmacological approach to further probe Ca^{2+} signals in crown cells. Results from these experiments implicated inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from endoplasmic reticulum (ER) is necessary for LR asymmetric cytoplasmic Ca^{2+} transients, whereas L-type channels and store-operated channels are dispensable. Importantly, treatments with thapsigargin, a small molecule that alters ER Ca^{2+} and reduced cytoplasmic Ca^{2+} transients in crown cells, identified a subset of ciliary Ca^{2+} spikes that occur in the presence of thapsigargin. This suggests thapsigargin-resistant Ca^{2+} signals originate in the cilia, rather than entering from the cytoplasm. This subset of ciliary transients was biased to the left side of the LRO. Again, in keeping with results from zebrafish, expression of cilia-localized parvalbumin in crown cells reduced ciliary and cytoplasmic Ca^{2+} transients and delayed asymmetry of a Dand5 reporter construct. The authors propose that Type 1 Ca^{2+} transients that depend on leftward flow and Pkd2 and are localized on preferentially localized on the left side of the LRO are the Ca^{2+} signals that translate flow into downstream asymmetric signaling—likely by inducing the degradation of *Dand5* mRNA (Figure 5b). Interesting future directions include how crown cells differentiate between Type 1 and Type 2 Ca^{2+} transients, and how left-biased Type 1 Ca^{2+} signals mediate *Dand5* degradation.

6 | FUTURE DIRECTIONS

Significant progress has been made towards understanding the structures and functions of cilia in vertebrate embryos. Yet, many open questions and gaps in our knowledge remain. Here I discuss challenges in two broad areas that the field is now poised to address. First, the state-of-the-art of the field has been steadily moving towards visualizing and quantifying in vivo cilia in real time. It is clear that cilia are highly dynamic structures that are best described in living tissues. Recent work also highlights findings that embryonic cilia structures and functions depend on developmental context and cell type. Already, many research groups have developed innovative imaging tools and approaches to capture tiny cilia in their native environments. The development of Ca^{2+} indicators described above in zebrafish (Yuan et al., 2015) and mouse (Mizuno et al., 2020) embryos provide good examples of quantifying dynamic cilia signals in real-time, which would not be possible using fixed samples. It will be important for the field to continue to develop novel methods to capture and quantify cilia dynamics in living systems. For example, as super-resolution microscopy and machine learning technologies continue to advance, there will be new opportunities to apply these methodologies to investigating, and ultimately understanding, cilia dynamics during embryo development.

The second challenge is to expand studies of human embryonic cilia. As suggested by trophoblast studies discussed above, it is likely that there are species-specific functions for cilia in human development that cannot be modeled in traditional animal systems. The rapidly advancing field of building stem cell-based synthetic human embryo-like structures (Simunovic & Brivanlou, 2017; Weatherbee et al., 2021; Zhai et al., 2022; M. Zhang, Reis, & Simunovic, et al., 2022) provides opportunities to move cilia studies from human cell cultures to self-organizing human organoids, embryoids, and gastruloids (Cruz et al., 2022). For example, recently developed human trophoblast organoids (Turco et al., 2018) may provide a more physiologically relevant model to further investigate primary cilia functions first identified in trophoblast cell cultures (Ritter et al., 2020; C. Y. Wang et al., 2017, 2019). It may also be possible to use embryoids or gastruloids to identify and study cilia in a human left-right organizer (LRO). So far, the human LRO has remained elusive. An additional feature of studying human organoids is the possibility of generating organoids from ciliopathy patients (Forbes et al., 2018; van der Vaart et al., 2021). This approach facilitates direct studies to pinpoint how cilia dysfunction impacts development and disease. Future work in these broad areas—innovative real time quantification of cilia dynamics and functional analysis of cilia in human development—is expected to advance our understanding of embryonic cilia and their impacts on human health.

AUTHOR CONTRIBUTIONS

Jeffrey D. Amack: Conceptualization; writing – original draft; funding acquisition; writing – review and editing.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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