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Invited Mini Review

The primary cilium as a multiple cellular signaling scaffold in development and disease

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Primary cilia, single hair-like appendage on the surface of the most mammalian cells, were once considered to be vestigial cellular organelles for a past century because of their tiny structure and unknown function. Although they lack ancestral motility function of cilia or flagella, they share common ground with multiciliated motile cilia and flagella on internal structure such as microtubule based nine outer doublets nucleated from the base of mother centrioles called basal body. Making cilia, ciliogenesis, in cells depends on the cell cycle stage due to reuse of centrioles for cell division forming mitotic spindle pole (M phase) and assembling cilia from basal body (starting G1 phase and maintaining most of interphase). Ciliary assembly required two conflicting processes such as assembly and disassembly and balance between these two processes determines the length of cilia. Both process required highly conserved transport system to supply needed substance to grow tip of cilia and bring ciliary turnover product back to the base of cilia using motor protein, kinesin and dynein, and transport protein complex, IFT particles. Disruption of ciliary structure or function causes multiple human disorder called ciliopathies affecting disease of diverse ciliated tissues ranging from eye, kidney, respiratory tract and brain. Recent explosion of research on the primary cilia and their involvement on animal development and disease attracts scientific interest on how extensively the function of cilia related to specific cell physiology and signaling pathway. In this review, I introduce general features of primary cilia and recent progress in understanding of the ciliary length control and signaling pathways transduced through primary cilia in vertebrates. [BMB Reports 2012; 45(8): 427-432]

INTRODUCTION

Recent findings in cellular signaling during mammalian development highlight the importance of antenna-like organelles, primary cilia, which were once forgotten and considered to be vestigial structure for a past century. Single hair-like appendage protrudes from the surface of the most of mammalian cell types except hematopoietic cells (1). Primary cilia contain nine sets of microtubule doublet structure (9 + 0) ensheathed by the specialized membrane. Primary cilia are divergent version of cilia distinct from conventional motile cilia that have additional central microtubule pair (9 + 2) and found on multiciliated cells to play a role in generating force by beating for directional movement of fluid (2). In contrast to motile cilia, primary cilia is involved in sensory process and cellular signal transduction. Making cilia, ciliogenesis, in cells depends on the stage of cell cycle due to reuse of centrioles for cell division forming mitotic spindle pole (M phase) and assembling cilia from basal body (starting G1 phase and maintaining most of G0 phase) (3). To assemble the cilia, centrioles dock to the apical membrane of the cells first and posttranslationally modified tubulins start to polymerize from mother centrioles to grow away from the surface of the membrane. At the same time, disassembly process occurred during ciliogenesis and balance between these two processes determines the length of cilia (4). Both process required highly conserved transport system to supply needed substance to grow tip of cilia and bring ciliary turnover product back to the base of cilia using motor protein, kinesin and dynein, and transport protein complex, Intraflagellar Transport (IFT) particles (5, 6). Disruption of ciliary structure or function causes multiple disorders in human exemplified in congenital genetic disorders called ciliopathies (7, 8). They affect major body organ development and homeostasis. Although molecular basis of ciliopathies are largely unknown, more and more studies revealed that understanding of molecular mechanisms of ciliogenesis and signaling pathways routed through cilia are critical to reveal the pathogenesis of these human genetic diseases.

In this review, I introduce general features of primary cilia and recent progress in understanding of the ciliogenesis and role of primary cilia focusing on cellular signaling.

CILIARY STRUCTURE AND CILIOGENESIS

Cilium has a characteristic microtubule-based structure, axoneme, which is nine pairs of cylindrically arranged microtubules and proximal end of it emerges from centrosome-derived basal body as shown in Fig. 1A. Cilia are classified as motile and

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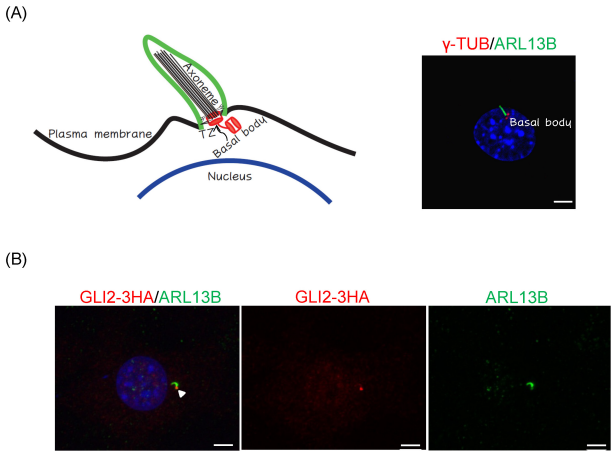

Fig. 1. Primary cilium in mammalian cell. (A) (Left) Schematic drawing of primary cilium in mammalian cell. It protrudes from plasma membrane and axoneme grows from modified mother centriol, basal body. The ciliary compartment was excluded from cytoplasm by transition zone (TZ). (Right) Example of immunofluorescent staining of primary cilium in NIH3T3 cell. Basal body was stained with anti-gamma tubulins Ab (red, γ-TUB) and primary cilium was detected with anti-Arl13b antibody (green). (B) Primary cilia played as a signaling center as shown in (B). SHH activate Gli2 transcription factors to turn on the Shh target genes. Gli2 transcription factors localize to the tip of cilia and post-translationally processed to transform as an active-form with unknown mechanism. Arrowhead indicates ciliary tip localized Gli2 transcription factor (red). Scale bar = 5 μm

nonmotile based on their motility. Motile cilia have conserved role of ancient organelle, flagella, responsible for movement of fluid and other substance. Ultrastructure of motile cilia has additional central pair of microtubule which is referred to as "9 + 2" structure such as flagella. They also have inner and outer dynein arms which endow motile cilia to beat one direction in a coordinated manner. Ensemble of ciliary motility generates directional power to set in a motion of fluid or exogenous substance in airway surface or ventricular system in the brain. Breakdown of this sophisticated system in respiratory tissue or brain results in airway disease or hydrocephalus. In contrast to motile cilia which reside in specific tissues, most of mammalian cells display single antenna-like structure which lacks dynein arms and central pair of microtubule resulting in immotility.

Building the cilia in cells begins with migration of centrioles to the cell surface and they dock onto the plasma membrane. After docking, mother centriole starts to grow to form ciliary skeleton, axoneme, by microtubule filament polymerization and concurrently ciliary membrane extended together (2). At the base of cilia triplet microtubule of mother centriole shift to doublet structure and this region is called 'transition zone' which contained Y-shaped structure to connect doublet microtubule to the ciliary membrane (9). Transition zone plays as a barrier to separate cilium from cytoplasm by gating import of ciliary proteins. Surprisingly, gating property of transition zone resembles and employs same machinery as nuclear pore complexes (NPCs) (10, 11). Nuclear transport system utilizes NPCs to mobilize proteins containing nuclear localization signals (NLSs) or export signals (NESs) in and out of nucleus. Ciliary proteins also known to contain nuclear localization signal (NLS)-like sequences, termed ciliary localization sequences (CLSs). Small molecules (less than 40 kDa) could enter the cilia but higher than that required CLS to import into the cilia with the aid of importin and Ran GTPase. It is a quite amazing to know how much ciliary entry system share commonality with nuclear transport system. It would be also interesting to see whether ciliary transport system needs ciliary export system or not. If so, to identify the NES-like sequences, ciliary export sequences (CESs) in ciliary proteins based on analogy of nuclear export system is worth pursuing coming days.

Cilia do not have a machinery to synthesize proteins and other substance to elongate. Therefore, they require a specialized transport system to deliver needed ciliary proteins to the tip and assemble axoneme. Intraflagellar transport (IFT) is a motor-driven protein complex that transports ciliary proteins in a bidirectional manner from base to the growing tip or vice versa. IFT is a multiprotein complex subdivided it into two subtypes, A and B. IFT B is a kinesin-II motor driven ~14 proteins to deliver cargo proteins toward plus end microtubules as an anterograde transport. The cargoes for IFT B mainly consist of axonemal proteins that run beneath the ciliary membrane to allow axonemal growth. Nevertheless, dynein 2 motor-assisted IFT A complex is responsible for retrograde transport moving cargoes to minus end direction to recycle them (5). Dynamic balance between anterograde and retrograde transport determines the flow rate of proteins into and out of cilia and ciliary length (4).

SIGNALING PATHWAYS IN CILIARY LENGTH CONTROL

Primary cilia maintain their length ranging from 1 to 10 μm in mammalian cells. Many factors are involved in controlling a steady state of cilia length and diverse intracellular signaling pathway could affect multiple layers of cilia length control steps. Consensus on common ciliary length control effector is cAMP signaling pathway. To identify the signaling pathways regulating ciliary length, Shah group screened chemical inhibitors of signaling pathway in immortalized kidney collecting duct line (IMCD) or primary kidney or bone cells and identified common signaling factors, cAMP and intracellular calcium affected the length of cilia (12). Increased cyclic AMP levels and PKA activity in cells stimulates the growth of cilia up to twice of normal length of cilia in 3 hours. They also identified decrease in intracellular calcium level also causes lengthening of cilia almost 2-fold. These processes did not require protein synthesis because it is not significantly affecting cilia length shown by cycloheximide treatment experiment. Furthermore, they found that siRNA-mediated adenylate cyclase (AC) knockdown blocked cilium length increase by lowering the intracellular Ca^{2+} level. This result indicates that crosstalk between Ca^{2+} and cAMP level exists in ciliogenesis of mammalian epithelial and mesenchymal cells.

Interestingly, increase of cAMP or decrease of Ca^{2+} by drug compromised the speed of anterograde transport but not retrograde transport (12).

In Chlamydomonas, lithium, an inhibitor of glycogen synthase kinase 3 β (GSK3β) causes flagellar elongation by decrease GSK3β activity (13). It implicates that GSK3β in Chlamydomonas restrict the growth of flagellar to maintain proper length of flagellar. Consistently, lithium modulates length of primary cilia in the nervous system and fibroblast-like synoviocytes (14). Interestingly, the cilia lengthening effect of lithium is rather decreasing in cAMP and calcium level than GSK3β activity inhibition. In their system, blocking of ACIII activity mimics the effect of lithium on cilia elongation. These inconsistency between green algae and mammalian system regarding roles of GSK3β and lithium on flagellar or cilia lengthening remains to be defined.

The NIMA related kinase (Nek) family kinases have recently been shown that they played a key role in regulating cilia length control and congenital mutations on them cause ciliopathies. Nek family kinases have 11 members in mammalian genome and Nek1 and 8 has a tie with ciliogenesis based on mouse genetic studies (15). Mice carrying truncated Nek1 mutation display polycystic kidney disease (PKD) (16). Overexpression of Nek1 in IMCD cells affect ciliogenesis by reducing number of ciliated cells and ciliary localization of Nek1 is required for ciliogenesis (17). NEK8 (G448V) missense mutation in the C-terminus also causes PKD and wildtype of NEK8 localizes to primary cilia (18). Cultured primary kidney epithelial cells from Nek8 mutant mice have longer cilia in length (19). Interestingly, Nek8 mutant mice show abnormal epidermal growth factor receptor (EGFR) expression and cAMP signaling. These results might explain that increased cAMP levels account for cilia lengthening in Nek8 mutant mice.

Moreover, there are well-known signal transduction pathways which also involved in cilia length control at the different steps of ciliogenesis. Downregulation of FGF signaling by FGF receptor 1 morpholino in zebrafish showed that it reduced the length of cilia. This event occurred through downregulation of ciliogenic transcription factors, foxj1 and rfx2, and of the IFT gene, IFT88 (20).

The Mammalian target-of-rapamycin (mTOR) pathways sensing the state of nutrients, growth factors and cellular energy level control metabolic states in cells. Recently mTOR signal transduction pathway reversibly modulates ciliary length in zebrafish model system (21, 22). Suppression of mTOR pathway by rapamycin in zebrafish resulted in short cilia and pathway activation increases the length of cilia. Interestingly the underlying mechanism of cilia length control of mTOR lies in downstream target of mTOR complex, S6 kinase 1(S6k1). Protein synthesis is required for cilia length regulation of mTOR complex through S6k1 suggesting that mTOR pathway might regulate the ciliary protein expression in vertebrate.

Other kinases and phosphatase also involved in cilia length control with unknown mechanisms. Cilia length control factors are well conserved throughout model system such as Chlamydomonas and vertebrate system (23). In Chlamydomonas, four different long flagellar mutants were cloned and two of them were kinases, cell cycle-related kinase (CCRK) and male germ cell kinase (MAK) (23). Loss of MAK in mouse model system result in abnormal long cilia in photoreceptor cells and human genetic studies revealed that mutation in MAK cause retinitis pigmentosa in human (24-27). CCRK has also been shown that it affects ciliogenesis in zebrafish model system (28). In contrast to Chlamydomonas studies, knockdown of CCRK expression in vertebrate compromises ciliary structure rather than length.

Cdc14 phosphatase is another example of factors regulating ciliary length. Cdc14 localize to centrosome and known to act on reversing Cyclin-dependent kinase 1 (CDK1)-dependent phosphorylation events. Using an antisense morpholino knock-down of CDC14 showed that shortening the cilia length in zebrafish embryo (29).

SIGNALING PATHWAYS WITHIN PRIMARY CILIA

Primary cilia deviate from ancient motility function to evolve playing diverse sensory perception and signal transduction center in mammals. As sensory roles, primary cilia involved in mechanosensation, olfaction and vision. In renal tubule cells fluid flow in tubule bends primary cilia to generate calcium signaling. Olfactory receptor neurons protrude dendritic knobs with odorant receptor containing 15-20 sensory cilia to detect odorant. Binding of odorant to G-protein coupled receptor in olfactory sensory cilia triggered cAMP signaling and led to depolarization of neurons by cAMP-gated ion channel opening to sense the smell. Finally photoreceptor sensory cilia in retina are modified form of primary cilia that connect the outer segment and cell body in photoreceptor cells. Outer segment of photoreceptor cell is specialized compartment to detect light stimulus and contain continuously turnover photoreceptors. Connecting cilia played a role to supply photoreceptors from cell body to outer segment.

Primary cilia are implicated in multiple cellular signaling pathways in animal development and homeostasis. They are involved in two main signaling pathways, Hedgehog and Wnt, during animal development and disease. Primary cilia in Hedgehog signaling are extensively studied and highly regarded as critical organelle in transmitting its signaling. Briefly, the secreted Sonic hedgehog (Shh) protein in mammals binds to the transmembrane receptor, Patched1 (Ptch1) to release its inhibition of Smoothened (Smo). Smo activation subsequently leads to Gli transcription factors activation with unknown mechanisms which turn on the Shh target gene expressions (30-32). Surprisingly, recent forward genetic screening studies in mouse enlighten us that primary cilia deeply linked to vertebrate Hedgehog signaling in animal development (33). In mouse development Shh specified ventral motor neurons and interneurons from neural tube precursor cells. Two IFT protein, IFT 88 or 172,

and kinesin motor protein, Kif3A, mutant mice embryo displayed the reduced Shh signaling and loss of ventral neurons in neural tube development. Currently, exploding interests in primary cilia-mediated signaling in vertebrate animal development were made by this stark finding. Molecular basis of primary cilia in Shh signaling was extensively scrutinized and revealed that Shh binding to Ptch1 displace cilia-residing Ptch1 resulting in translocation of activated Smo into ciliary membrane compartment (34, 35). Smo translocation affects the ciliary tip localized Gli transcription factor activation by unknown mechanisms (Fig. 1B). Without primary cilia, Gli proteins cannot be converted to active form of transcription factors and result in turning off Shh target gene expression. Several lines of evidences indicate that post-translational modification of Gli occurred in the primary cilia to sway Gli metabolism and transcriptional activity (36, 37).

Wnt signaling pathway has been linked to primary cilia, whereas there are controversial reports and they lack a firm ground compared with Hedgehog signaling. Wnt signaling realm consists of canonical and non-canonical segments. Canonical Wnt signaling converges on β-catenin and stability of β-catenin is posttranslationally regulated by casein kinase I (CKI), glycogen synthase kinase 3β (GSK3β) and ubiquitin-proteasome system (UPS). Changes in nuclear level of β-catenin reactivate canonical Wnt pathway and target gene expression. UPS machinery tightly decrease cytosolic β-catenin at the ciliary basal body. Downstream effector of canonical Wnt signaling, dishevelled (Dvl), stabilize β-catenin to activate signaling. In contrast to canonical pathway, noncanonical Wnt pathway is independent of β-catenin and grouped several subdivision based on downstream signaling effectors such as Dvl, cGMP/Ca^{2+}, small GTPase (RAP1), receptor tyrosine kinase-like orphan receptor 2 (ROR2), PKA, GSK3β, aPKC, receptor related to tyrosine kinase (RYK) and mammalian target of rapamycin (mTOR) (38). Dvl-dependent noncanonical Wnt pathway corresponds to planar cell polarity (PCP). PCP is important for polarizing cells within the apical/basal plane. Interestingly, evidences are recently accumulating that PCP pathways played a role in ciliogenesis. The basal body localized inversin (Inv) protein was activated by non-canonical PCP pathway and interacts with Dvl to exerts its effect on canonical Wnt signaling by Dvl degradation (39-41). It eventually results in β-catenin destruction and pathway off. In experiment with mouse embryo, primary fibroblasts and embryonic stem cells, cilia defect mutants, *kif3a*, *Ift88* and *Ofd1* increase canonical Wnt signaling cellular response to ligand (42). Based on these studies primary cilia could restrict the cellular response of Wnt signaling. However, recent studies from zebrafish and mouse discord a role of primary cilia in Wnt signaling. Maternal/zygotic ift88 zebrafish mutant showed disrupted Hedgehog signaling but normal Wnt signaling (43). Likewise, analysis of cilia mutant mouse embryo could not detect any significant difference in Wnt target gene expression as wildtype embryos (44). It still remains unresolved whether primary cilia are involved in canonical Wnt signaling.

CILIOPATHY

Cilia in our body exist broadly as motile or nonmotile form and share common microtubule doublet structures. Therefore, human congenital disruption of ciliary structure or function causes pleiotropic developmental disorder with variable degree of spectrum. More than dozen diseases are attributed to cilia formation or function defect and affect most of organ, for example, kidney, brain, limb, eye, ear, liver and bone. These rare ciliopathies include Joubert syndrome (JBTS), nephronophthisis (NPHP), autosomal dominant and recessive polycystic kidney disease (ADPKD and ARPKD), Meckel-Gruber syndrome (MKS), and Bardet-Biedl syndrome (BBS). Phenotypes related ciliopathies are classified two categories, motile cilia or immotile cilia-related disorders. Original finding in ciliopathy from Kartagener's Syndrome is improper establishment of our body's left-right asymmetry causing situs inversus and unable to sweep away exogenous substance by mucocilia in respiratory tract systems causing sinusitis and bronchiectasis (45). Later, left-right determination defect is attributed to the malfunction of motile cilia in embryonic nodal cilia (46). In addition to this, motile cilia disruption causes hydrocephalus in brain, and respiratory tract infections. Sensory and signaling defects in cilia cause retinal degeneration in the eye, anosmia in olfactory epithelium, kidney cyst formation and obesity. These phenotypes are inconsistent among ciliopathies and showed wide spectrum of symptoms.

Pathology of ciliopathy patient showed very diverse and depended on degree of affected cilia and tissue specificity. How does single structure disruption affect differently? There is no clear answer and it remains to be resolved. One possibility is that ubiquitous primary cilia are not affected exactly same fashion by one out of about 3000 ciliary genes mutation. Study from Hedgehog signaling regulator, Tectonic showed that it associated MKS and JBTS ciliopathies proteins and disrupt ciliogenesis in a tissue-specific measure (47). In nodal and neural tube cilia affected severely by losing ciliogenesis but limb bud mesenchymal ciliogenesis is mildly affected. However, limb bud ciliary membrane translocation of Smo is disrupted. It implicates that the mechanisms of ciliogenesis might exist in multiple schemes and different tissues contain tissue-specific mechanism to build up cilia and context variable ciliary functions. The degree of ciliopathy symptoms is highly dependent on the identity of affected gene and its role in ciliogenesis and ciliary function.

CONCLUSION

Times changed in cilia biology as boring to splash subject for a past century. Research on primary cilia biology expands rapidly and draws immense interest in animal development and disease focusing on processing of intracellular signaling. However we just begin to collect an ambiguous pieces of evidences and try to figure out the mystery of roles of primary cilia in cell biology. Many answers are awaiting to deepen our understanding of primary cilia. First, primary cilia are dynamic structures responding

to environmental changes in cells and integrating multiple intracellular signaling. What is the physiological implication of modulating the length of cilia? Is kinetics of signaling process in primary cilia affected by changes in length? Or is it just by-product of changes in cell state? Second, many ciliary transition zone proteins are involved in human genetic disease, ciliopathies. How do primary cilia selectively allow the entry of cargo proteins through transition zone? What are the roles of ciliopathies genes in transition zone? Finally, the ultrastructural organization and mode-of-action of ciliary tip which is distal end of primary cilia remains enigmatic. The key signaling mediators localize at the tip of cilia and are processed to relay the signal transduction (Fig. 1B). What might be the role of tip localization? Is it excluding signaling molecules or bringing together to facilitate the metabolism of signaling molecules? In the end, more advanced cutting-edge imaging technology and biochemical methods overcoming challengeable tiny cellular structure of primary cilia will lead us to unveil the secret life of primary cilia near future.

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