Loss of Rsph9 causes neonatal hydrocephalus with abnormal development of motile cilia in mice

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Hydrocephalus is a brain disorder triggered by cerebrospinal fluid accumulation in brain cavities. Even though cerebrospinal fluid flow is known to be driven by the orchestrated beating of the bundled motile cilia of ependymal cells, little is known about the mechanism of ciliary motility. RSPH9 is increasingly becoming recognized as a vital component of radial spokes in ciliary “9 + 2” ultrastructure organization. Here, we show that deletion of the Rsph9 gene leads to the development of hydrocephalus in the early postnatal period. However, the neurodevelopment and astrocyte development are normal in embryonic Rsph9−/− mice. The tubular structure of the central aqueduct was comparable in Rsph9−/− mice. Using high-speed video microscopy, we visualized lower beating amplitude and irregular rotation beating pattern of cilia bundles in Rsph9−/− mice compared with that of wild-type mice. And the centriolar patch size was significantly increased in Rsph9−/− cells. TEM results showed that deletion of Rsph9 causes little impact in ciliary axonemal organization but the Rsph9−/− cilia frequently had abnormal ectopic ciliary membrane inclusions. In addition, hydrocephalus in Rsph9−/− mice results in the development of astrogliosis, microgliosis and cerebrovascular abnormalities. Eventually, the ependymal cells sloughed off of the lateral wall. Our results collectively suggested that RSPH9 is essential for ciliary structure and motility of mouse ependymal cilia, and its deletion causes the pathogenesis of hydrocephalus.

Hydrocephalus is a prevalent birth defect triggered by excessive accumulation of cerebrospinal fluid (CSF) in brain cavities1. Natural CSF flow is secreted by specialized ependymal cells in the choroid plexuses of lateral ventricles. In addition, the CSF carries signaling molecules, nutrients, microRNAs and exosomes2–4. It passes through the 3rd ventricles, cerebral aqueduct, and the 4th ventricles and can be absorbed by arachnoid granulations5,6. The directional flow of CSF is driven by continuous CSF secretion and by the orchestrated beating of bundles of motile cilia that are located at the apical surface of ependymal cells7.

The motile cilia are arranged structurally as a “9 + 2” axoneme, which is composed of nine interconnecting peripheral pairs of microtubules and a central pair of single microtubules. The radial spoke head protein, RSPH9, is a structural protein located at the T-shaped macromolecular configurations protruding from the nine peripheral pairs of microtubules8. Interaction between radial spoke heads and the central pair of single microtubules is the central for ciliary regulation. Previous studies showed that Chlamydomonas RSP9 mutant strains lack the entire radial spoke head complex and displacement of the central pair of single microtubules9. And zebrafish Rsph9 mutant larvae exhibit similar cilia-dysmotility defects and reduced initiation of the acoustic startle response9. However, mutations in RSPH9 cause primary ciliary dyskinesia (PCD; MIM 244400) in human, which is characterized by phenotypic heterogeneity and lacks a suitable “gold standard” diagnostic test10,11,12. In addition, it is difficult to build a model of direct protein interaction between radial spoke proteins and the central...
pair of single microtubules because of the gap between them. Thus, we further investigated the spatiotemporal developmental function of RSPH9 using mouse model.

In this study, we generated global knockout mouse models to elucidate the pathogenesis of PCD by targeting the murine Rsph9 locus. We systematically investigated the development of Rsph9−/− mice to understand the consequence of losing this gene. Our study reveals the role of RSPH9 in hydrocephalus pathogenesis and ependymal cilia motility in the developing mouse brain.

Results

Targeting Rsph9 in mice. RSPH9-associated primary ciliary dyskinesia has a wide phenotypic variability in humans. To target Rsph9 in mice, we used the CRISPR-Cas9 system and the zygote microinjection of a single-guide RNA1 targeting exon1 of Rsph9 (Fig. 1A). The strategy deleted 8 base pairs to produce producing a premature stop codon at the end of exon 1, which significantly truncated the RSPH9 protein (Fig. 1B). The truncated RSPH9 with 61 amino acid residues are much shorter compared with normal RSPH9 with 276 amino acid residues. The generated heterozygous Rsph9+/− mice were viable and fertile. We backcrossed them with C57BL/6 mice for more than five generations to obtain a more purified genetic background.

Homozygous Rsph9 mice were a slower growth rate and postnatal lethality. Rsph9−/− mice were interbred to obtain homozygous knockout mice and were confirmed by genotyping PCR (Fig. S1A). The genotyping results on the first postnatal day revealed a prospective Mendelian ratio (1:2:1) and no sex-specific differences in survival. RSPH9 is highly expressed in multiciliated cells. Immunostaining with RSPH9 showed deletion of Rsph9 in Rsph9−/− brain ependymal cilia and tracheal cilia (Fig. 1C,D). However, the knockout pups failed to grow normally, and most died during the weaning phase (Fig. 1E–G). Only a very few survived into adult (Fig. S1B). These Rsph9+/− mice are characterized by enlarged dome-shaped skulls and severe neurological symptoms, including lethargy, apathy and muscle weakness.

Rsph9+/− mice develop progressive hydrocephalus and sinusitis. PCD is usually accompanied by randomized body laterality and respiratory disease. Therefore, we investigated these phenotypes in mice. There was no situs inversus in Rsph9+/− mice, which shows RSPH9 is not associated with the determination of the left–right axis of visceral organs (Fig. S2A; n = 6). However, RSPH9−/− mice developed severe neurological disorders and sinusitis (Fig. S2B). To analyze macrocephaly in Rsph9−/− mice, the brains were isolated and were found to be enlarged (Fig. 2A). Magnetic resonance imaging revealed severe thinning of the cerebral cortex and enlarged hemispheres with massive accumulation of CSF in the lateral ventricles (Fig. 2B). The hippocampus and the hypothalamus were severely compressed. All of these are characteristic symptoms of hydrocephalus. To characterize the temporal feature of hydrocephalus in mice with the Rsph9 deletion, we compared sagittal sections of the developing brain between P0 and P7 in wild-type and Rsph9+/− mouse pups (Fig. 2C). There was no significant difference at P0. However, the area of lateral cerebral ventricular zone was comparatively increased at P3 and P7. Histological analysis of coronal brain sections revealed enlarged ventricles with abnormal brain morphology in P8 Rsph9−/− mice (Fig. 2D,E). Cerebrospinal fluid analysis showed clear CSF and no visible brain hemorrhage, which indicated that hydrocephalus was not caused by hemorrhaging in the Rsph9−/− mouse brain. Therefore, RSPH9 is not associated with situs inversus, but sinusitis. Furthermore, deletion of Rsph9 can result in the development of brain dysfunction and progressive hydrocephalus during postnatal development in mice.

Hydrocephalus is not caused by embryonic development defects in Rsph9−/− mice. Since ciliopathies are a group of genetic disorders closely related to neuronal cell fate, migration, and differentiation, as well as a host of adult behaviors13. We investigated whether hydrocephalus originates by embryonic brain developmental disorders. First, BrdU/EdU dual labeling experiments were conducted to confirm whether RSPH9 affects the neurogenesis process. The results showed that there was no significant difference in cell cycle dynamical disorders. First, BrdU/EdU dual labeling experiments were conducted to confirm whether RSPH9 affects the neurogenesis process. The results showed that there was no significant difference in cell cycle dynamics in embryos at 15 days when comparing wild-type and Rsph9−/− mice (Fig. 3A,B). Then the cortex markers CUX1 and CTIP2 were used for labeling layer II–IV and layer V of neonatal mice, which showed the typical cortical layer pattern of Rsp9+/− mice (Fig. 3C,D). Neuronal migration is also not affected by Rsph9 deletion. Immunofluorescent staining of glial fibrillary acidic protein (GFAP) was used to label astrocytes, and we found no significant change in GFAP-positive cell number or expression pattern between P0 Rsph9+/− and Rsph9−/− mice (Fig. 3E,F). Taken together, neurogenesis, neuronal migration and astrocyte development were determined to be undisturbed by Rsph9 deletion in mice. Hydrocephalus was caused by postnatal developmental defects.

CSF flow and circulation are impaired in Rsph9−/− mice. Cerebrospinal fluid is produced by the choroid plexus in lateral ventricles passing through the foramina of Monro, the 3rd ventricle, the cerebral aqueduct and the 4th ventricle. We injected Evans blue dye into the right lateral ventricle to investigate CSF flow through the ventricular system in Rsph9−/− mice. In wild-type mice, the tracer could travel through the third ventricle, central aqueduct and fourth ventricle 10 min after injection (Fig. 4A; the upper row, n = 3). In contrast, no tracer or only very little tracer could be detected at the third ventricle and the fourth ventricle in Rsph9−/− mice (Fig. 4A; the lower row, n = 4). Nissl staining results showed that the shape of the central aqueduct in P8 Rsph9−/− mice was intact, the size was unchanged, and no blockage occurred (Fig. 4B). Thus, these results indicate that the barrier of CSF flow is due to the disrupted activity of the ependymal cells.

Rsph9+/− ependymal cells display defects in motion pattern of cilia bundles. To assess the disorder of ependymal cells, whole-mount staining of the subventricular zone en-face was conducted to show...
the cilia bundles in the wild-type and Rsph9−/− mouse brain (Fig. S3A,B). Wholemount immunostaining for β-catenin revealed intact adherens junctions in Rsph9−/− mice (Fig. S3C). To evaluate the pattern of ependymal cilia beating in Rsph9−/− ependymal cilia, video microscopy experiments were conducted. The analysis results
revealed that the movement of beating cilia bundles is characterized by lower amplitude from the side views in P7 Rsph9−/− mice compared with that of wild-type mice (Movie 1, 2, Fig. 5A,F). The wild-type cilia bundles moved orderly with planar beating pattern from the top views (Movie 3, Fig. 5B). The Rsph9−/− cilia bundles, by contrast, moved disorderly with rotation beating pattern (Movie 4, Fig. 5B). Beating frequency was not significantly affected in Rsph9−/− mice (Fig. 5G). Transmission electron microscopy (TEM) was performed on ependymal cilia to investigate the ciliary axoneme ultrastructure. In wild-type ependymal cilia, all axonemes exhibited a typical "9 + 2" ultrastructure (Fig. 5C). In Rsph9−/− ependymal cilia, most axonemes exhibited normal ultrastructure and few exhibited various defects (Fig. 5C,D). The central pair of microtubules and the outer microtubule doublets may turn into single microtubules or may become vacant. Moreover, the Rsph9−/− cilia frequently had abnormal ectopic ciliary membrane inclusions (Fig. 5E). Thus, RSPH9 has no significant effect on the "9 + 2" arrangement of microtubules. Furthermore, immunofluorescence analysis showed that assembling of RSPH3 into radial spoke head complex is not affected by RSPH9 deletion (Fig. S4). The defects in Rsph9−/− cilia may due to disorders of sliding motion between adjacent microtubules. Remarkably, centriolar patch size was significantly increased in Rsph9−/− cells (Fig. 5H,I). The mechanical stress of cilia beating pattern of Rsph9−/− cells may destroy apical centriolar distribution. These results show that RSPH9 is necessary for coordinated beating.

Figure 2. Severe postnatal hydrocephalus in Rsph9−/− mice. (A) Images of Rsph9+/+ and Rsph9−/− mouse brains at postnatal day 8 (P8). The arrow indicates the enlarged brain hemispheres. Scale bar, 100 μm. (B) T2-weighted coronal and sagittal magnetic resonance images (MRI) of the brains of Rsph9+/+ and Rsph9−/− mice at P10. CSF in enlarged lateral ventricles is hyperintense. Scale bar, 100 μm. (C) Sagittal sections of P0 (top), P3 (middle) and P7 (bottom) mouse brains that were stained with Nissl. Scale bar, 200 μm. (D) Coronal sections of P8 mouse brains that were stained with Nissl. (E) Cerebral ventricular size in P8 brains (n = 5 mice per group; ***P < 0.001; data are expressed as the means ± SEs). SEM is the standard deviation divided by the square root of the sample size. Student's t test.
of ependymal cilia. Defects in Rsph9−/− ependymal cells can lead to disruption of the pattern of cilia beating and can give rise to hydrocephalus. Hydrocephalus in Rsph9−/− mice results in astrogliosis, microgliosis, cerebrovascular abnormality and myelination disorders. Hydrocephalus can damage brain tissue and cause a wide range of symptoms. Here we investigated the pathological characteristics of the thinning of cerebral cortex. The hydrocephalus caused by Rsph9 deletion was accompanied by astrogliosis and microgliosis in the cortex. Immunostaining with GFAP (astrocyte marker) antibody and DAPI (blue, cell nuclear marker) in P0 mouse brains. Scale bar, 50 μm. (F) Quantification of GFAP+ cells in the P0 Rsph9+/+ and Rsph9−/− mouse cortex (n = 3 mice per group; NS, not significant; data are expressed as the means ± SEMs). SEM is the standard deviation divided by the square root of the sample size. Student's t test.
inflammatory response. The analysis of cerebral vessels showed that vessel density and branching were reduced in P8 Rsph9−/− mice (Figs. 6E–G and S5). Hydrocephalus in Rsph9−/− mice also attenuated the expression of myelin basic protein (MBP), which is a marker of myelinating glia, but it enhanced the expression of oligodendrocyte transcription factor 2 (OLIG2), which is a marker of oligodendrocyte progenitor cells and mature oligodendrocytes (Fig. 6H,I). It turned out that myelin is damaged in Rsph9−/− mice and that enhanced OLIG2 expression may contribute to myelin repair. Immunostaining with an ependymal layer marker (S100β) showed slightly rupture of the ependymal layer in P8 Rsph9−/− mice (Fig. S6A). And then the ependymal layers were severely damaged and ependymal exfoliation was detected in P12 Rsph9−/− mice (Fig. S4B). Altogether, these results suggest that in P8 Rsph9−/− mice, hydrocephalus is associated with severe pathological reactions, inflammation reactions and myelination disorders.

Discussion

RSPH9 is known to be a component of the axonemal radial spoke head complex, which is a thin stalk attached to the outer doublet microtubule in motile cilia. In this study, we show that Rsph9-deficient mice developed severe hydrocephalus with postnatal ventriculomegaly and severe sinusitis. The characteristic feature of hydrocephalus in Rsph9−/− mice also attenuated the expression of myelin basic protein (MBP), which is a marker of myelinating glia, but it enhanced the expression of oligodendrocyte transcription factor 2 (OLIG2), which is a marker of oligodendrocyte progenitor cells and mature oligodendrocytes (Fig. 6H,I). It turned out that myelin is damaged in Rsph9−/− mice and that enhanced OLIG2 expression may contribute to myelin repair. Immunostaining with an ependymal layer marker (S100β) showed slightly rupture of the ependymal layer in P8 Rsph9−/− mice (Fig. S6A). And then the ependymal layers were severely damaged and ependymal exfoliation was detected in P12 Rsph9−/− mice (Fig. S4B). Altogether, these results suggest that in P8 Rsph9−/− mice, hydrocephalus is associated with severe pathological reactions, inflammation reactions and myelination disorders.
et al. reported that knockdown of RSPH9 by RNAi in mouse ependymal cells, which differentiated from radial glia ex vivo, resulted in a near complete CP loss26; however, our results showed that there were no defects with CP in Rsph9−/− ependymal motile cilia in vivo, and few exhibited various ultrastructure disorganizations. This may be due to the truncated RSPH9, which may still support the central pair formation, or the differences between in vivo and in vitro experiments. Furthermore, our results showed that centriolar patch size was significantly increased in Rsph9−/− cells. Structural changes of radial spoke complex in RSPH9 deletion likely disturb doublet microtubules to tolerate both tensile and compressive stresses during ciliary beating27. This may disrupt the mechanical resistance of the apical actin network around centrioles, in turn, and disrupt centriole stability28.

Hydrocephalus proceeds with reactive astrogliosis and microgliosis, which lead to the formation of glial scars. Upon activation by injury, active glial cells release chemokines and cytokines, which help recruit of microglia29,30. Recruitment facilitates the formation of glial scars, which impede neovascularization and block the growth of neuronal processes31. Our experimental data are consistent with previous results29,32–34. We have provided further evidence that vessel density and branching frequency are both decreased in mice with hydrocephalus. Furthermore, it has previously been reported that oligodendrocyte precursor migration is associated with the abluminal

Figure 5. Rsph9−/− ependymal cells display defects in motion pattern of cilia bundles. (A) Diagram of wild type ciliary beat cycle and Rsph9−/− ciliary beat cycle from the side views. Wild type ciliary beat pattern is characterized by a strong beat stroke and a recovery stroke. Rsph9−/− ciliary beat pattern is characterized by gently stroke from side to side. (B) Diagram of wild type ciliary beat cycle and Rsph9−/− ciliary beat cycle from the top views. Wild type is characterized by orderly planar beating pattern. Rsph9−/− is characterized by disorderly rotation beating pattern. (C) TEM images of ependymal cilia in wild-type and Rsph9−/− mice. WT mice presented normal axonemes with “9 + 2” ultrastructure. Some Rsph9−/− motile cilia exhibited a “9 + 2” ultrastructure, but others exhibited a disorganized axonemal structure. Scale bar, 50 nm. (D) Quantification results for TEM-cross sections of Rsph9+/+ and Rsph9−/− ependymal cilia. (E) The ectopic abnormal ciliary membrane inclusions in the Rsph9−/− (red arrows). Scale bar, 200 nm. (F) Quantification of cilia beating amplitude (n = 11 cilia per group; ***P < 0.001; and data are expressed as the means ± SEMs). (G) Quantification of cilia beating amplitude (n = 21 cilia for wild-type, n = 19 cilia for Rsph9 mutants; NS; and data are expressed as the means ± SEMs). (H) Immunofluorescence staining with antibodies for β-Catenin antibody (greed, adherens junction) and γ-tubulin (red, centrioles) in wholemounts of lateral ventricular walls at P7. Centriolar patches are outlined with dashed white lines. Scale bars, 5 μm. (I) Quantification of ratio of centriolar patch size and total cell surface (n = 34 cells for wild-type, n = 41 cells for Rsph9 mutants; **P < 0.01; and data are expressed as the means ± SEMs).
endothelial surface of nearby blood vessels, which may explain the defects of myelin and the accumulation of OLG2+ cells in the medial ganglionic eminence of Rsph9−/− mouse brains.

Dysfunction of RSPH9 can change the motion pattern of motile cilia. However, we still do not know how this change occurs in Rsph9−/− ciliary motility. Our knowledge of the mechanism of ciliary organization and motility has been very limited. In our experiments, we cannot see the ultrastructure of radial spoke complex clearly using TEM, and there remains questions of that whether RSPH9 affects localization of other radial spoke head proteins. The precise ultrastructural organization and sliding mechanism of radial spoke complex are still need to be investigated.

Methods

Mice. All mice were housed in specific pathogen-free conditions and maintained on a 12:12 h light–dark lighting cycle, with lights off at 19:00. Animal procedures were performed in accordance with experimental protocols and approved by Animal Care and Use Committees of the Institute of Zoology, Chinese Academy of Sciences. The Experimental Animal Center of the Institute of Zoology generated the Rsph9 knockout mice. The Rsph9 knockout mice were generated by C57Bl/6 × 129/SvEv zygote microinjection with CRISPR-Cas9 system. Scisences. The Experimental Animal Center of the Institute of Zoology, Chinese Academy of Sciences. The Experimental Animal Center of the Institute of Zoology, Chinese Academy of Sciences generated the Rsph9 knockout mice. The protocols and approved by Animal Care and Use Committees of the Institute of Zoology, Chinese Academy of Sciences. The Experimental Animal Center of the Institute of Zoology, Chinese Academy of Sciences.

Antibodies. For immunofluorescence analysis, the following primary antibodies were used: mouse anti-ARL13B (1:1,000 dilution, Abcam, #Ab136648), Goat anti-IBA1 (1:500, Abcam, #Ab5076), mouse anti-IB4 (1:400, Vector Laboratories, #B-1205), rabbit anti-GFAP (1:3,000, Dako, #Z0334), rabbit anti-S100β (1:1,000, Proteintech, #15146-1-AP), rat anti-BrDU (1:1,000, Abcam, #ab6326), rabbit anti-CUX1 (1:500, Santa Cruz Biotechnology, #sc-13024), rat anti-CTIP2 (1:500, Abcam, #ab18465), rabbit anti-RSPH9 (kindly gifted from Zhu Xueliang, Shanghai Institute of Biochemistry and Cell Biology, CAS), rabbit anti-RSPH3 (1:1,000, Proteintech, #17603-1-AP).

Histology and immunofluorescence confocal microscopy. Tissues were fixed in 4% paraformaldehyde (PFA) overnight and dehydrated in 30% sucrose, and 15-μm-thick cryosections were prepared. For Nissl staining, 0.1% Cresyl Violet solution was used. For immunofluorescence, sections were blocked by 5% bovine serum albumin/0.1% Triton X-100/PBS for 1 h, incubated with primary antibodies at 4 °C overnight and fluorescent-conjugated secondary antibodies for 2 h. Images were taken with a Zeiss LSM780 laser scanning confocal microscope and Leica Aperio VESA8 microscope.

Transmission electron microscopy (TEM). The median walls of P6 forebrains were fixed in 4% PFA. The samples were cut into 300 μm thick by Leica vibratome. The brain slices were shaped into cubes and fixed in electron microscopy grade 2% PFA and 2.5% glutaraldehyde in PBS (pH 7.4) at 4 °C overnight. Tissues were washed with PBS three times, and post-fixed in 1% osmium oxide for 1 h. The samples were thoroughly washed in PBS and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 100%). The samples were washed twice in 100% acetone. Soak the samples in 1:1 ratio of acetone to epoxy resin for 1 h, then in 1:3 ratio of acetone to epoxy resin for 3 h, and in pure epoxy resin for more than 5 h. The samples were embedded and polymerized in epoxy resin at 60 °C for 48 h. Ultra-thin sections of 60 nm were obtained with Leica UC7 ultramicrotome, stained with uranyl acetate and Reynold's lead citrate, and imaged using a transmission electron microscope (Tecnai G2 F20 TWIN TMP).

Video microscopy of ciliary motion. P7 wild-type and Rsph9−/− brains were collected and dissected in DMEM/F12 supplemented with L-glutamine and 2% B27 (Invitrogen) at room temperature. 200 μm-thick sections including ventromedial walls of lateral ventricle was acquired using a Leica vibratome. Images were acquired by a customized microscope with a Nikon S Plan Fluor 40x objective. The motion of cilia was captured for 7 s (380 frames/s) with a high-speed CMOS camera (PDV, MV-500C). Time-series images were captured at a resolution of 640 by 480 pixels and saved in raw format with timing information. A MATLAB (Mathworks, ver 2015b) script was used to select the region of interest (ROI) and export the ROI into PNG format. The in plane...
drifts due to the environmental vibrations were corrected by co-registering the PNG image series to the first image with TurboReg (ImageJ).

Cerebral ventricular injection of tracers (systemic CSF flow analysis). The pattern of CSF flow is practically the same as the one described by. Briefly, four Rsph9−/− and five controls postnatal day 8 mice were anesthetized. Then slowly injecting 5 μl Evans blue dye (4% in PBS) into the lateral ventricle at 1.7 mm posterior, 0.8 mm right and 1.8 mm deep from the Bregma on the head. The mice were sacrificed 10 min later. Whole brains with a part of the spinal cord were fixed in 4% PFA overnight. 1 mm-thick coronal slices were generated by Vibrating Microtomes Tissue Slicer.

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Author contributions
W.Z. conceived the project; W.Z. and Y.L. performed most of the experiments with help from H.L.; Z.L. collected time-series images and processed the data. P.X. performed transmission electron microscopy. J.J. supervised the project and acquired the funding support. W.Z. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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