Planar polarity of multiciliated ependymal cells involves the anterior migration of basal bodies regulated by non-muscle myosin II

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SUMMARY
Motile cilia generate constant fluid flow over epithelial tissue, and thereby influence diverse physiological processes. Such functions of ciliated cells depend on the planar polarity of the cilia and on their basal bodies being oriented in the downstream direction of fluid flow. Recently, another type of basal body planar polarity, characterized by the anterior localization of the basal bodies in individual cells, was reported in the multiciliated ependymal cells that line the surface of brain ventricles. However, little is known about the cellular and molecular mechanisms by which this polarity is established. Here, we report in mice that basal bodies move in the apical cell membrane during differentiation to accumulate in the anterior region of ependymal cells. The planar cell polarity signaling pathway influences basal body orientation, but not their anterior migration, in the neonatal brain. Moreover, we show by pharmacological and genetic studies that non-muscle myosin II is a key regulator of this distribution of basal bodies. This study demonstrates that the orientation and distribution of basal bodies occur by distinct mechanisms.

KEY WORDS: Planar cell polarity, Ependymal cell, Cilia, Dvl2, Non-muscle myosin II, Mouse

INTRODUCTION
Motile cilia are membrane protrusions that move rapidly to generate fluid flow or to move materials present in the extracellular space. To generate directional fluid flow over the surface of a tissue, ciliary beating must be coordinated in the same direction. Recent studies have shown that the planar cell polarity (PCP) pathway, a downstream branch of Wnt signaling, plays important roles in directing the ciliary beating of multiciliated cells in *Xenopus* larval skin and mouse ependymal cells (Ganner et al., 2009; Guirao et al., 2010; Mitchell et al., 2007; Mitchell et al., 2009; Park et al., 2008; Tissir et al., 2010). In addition, fluid flow-mediated mechanisms and functional ciliary axonemes are involved in establishing the planar polarity of ciliary orientation (Guirao et al., 2010; Mitchell et al., 2007). Recently, a novel ependymal cell PCP, characterized by the anterior positioning of basal bodies, was reported and termed ‘translational’ polarity (Mirzadeh et al., 2010). Another planar polarity indicated by the direction of the basal foot was termed ‘rotational’ by the same group. Recent studies have revealed the cellular and molecular mechanisms underlying the rotational polarity of ependymal cells (Guirao et al., 2010; Mirzadeh et al., 2010; Tissir et al., 2010). By contrast, the mechanisms of translational polarity formation during postnatal ependymal development have not been elucidated.

Recently, non-muscle myosin II (NMII) was shown to play important roles in PCP formation in invertebrates and vertebrates (Bertet et al., 2004; Skoglund et al., 2008; Yamamoto et al., 2009). NMII is a hexameric protein consisting of two heavy chains (non-muscle myosin heavy chains, NMHCs) that form a dimer, a pair of regulatory light chains (RLCs), and a pair of essential light chains. The disruption of NMII causes hydrocephalus (Ma et al., 2007), suggesting that NMII is involved in the maturation of ependymal cilia. However, the exact role of NMII in the differentiation of ependymal cells remains unknown.

We show here that translational polarity is established by the anterior migration of basal bodies in the apical membrane during the differentiation of ependymal cells. We also show that NMII is a key regulator of this process.

MATERIALS AND METHODS
Animals
Wild-type ICR mice were purchased from SLC. Centrin 2::GFP (Cent2GFP) transgenic mice were described previously (Higginbotham et al., 2004). All animal experimental procedures complied with national regulations and guidelines, were reviewed by the Institutional Laboratory Animal Care and Use Committee, and were approved by the President of Nagoya City University.

Histological analysis
Immunostaining of whole-mount lateral ventricular walls was performed as described previously (Hirota et al., 2007). The following antibodies were used: rabbit anti-pericentrin (Pcnt); (1:500; Covance, PRB-432C); goat

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anti-γ-tubulin (1:100; Santa Cruz, sc7396); mouse CTR453 (1:10) (Baillie et al., 1989); mouse anti-β-catenin (1:200; BD 610154); chick anti-GFP (1:500; Aves, 2-P004-07D); rat anti-GFP (1:500; Nacalai, GF090R); rabbit anti-GFP (1:1000; Sigma, T6793); rabbit anti-Myoh9 (1:300; Covance, PRB-440P); rabbit anti-Myh10 (1:300; Covance, PRB-445P); rabbit anti-Myh14 (1:300; Covance, PRB-444P); rabbit pMLC2 (My9) (1:200; Cell Signaling, 3674); rat R4109 (1:2) (Hagiwara et al., 2000); mouse anti-ZO1 (1:300; Zymed, 33-9100); and species-specific secondary antibodies (Invitrogen or Jackson Immunoresearch). Confocal images were obtained using LSM5-Pascal and LSM710 (Zeiss) microscopes. Scanning electron microscopy analyses were performed as described previously (Sawamoto et al., 2006) using an S-5000 (Hitachi) electron microscope. Immunotransmission electron microscopy (TEM) analyses using rabbit anti-GFP antibody were performed as described previously (Yamashita et al., 2006) using a JEM-1200EX (JEOL) electron microscope.

Ependymal cell culture
The detailed protocol for the primary culture of ependymal cells and the device used to create the artificial fluid flow have been described previously (Guirao et al., 2010). For transfections, ependymal cells in 24-well plates were transfected with Lipofectamine (Invitrogen) and 500 ng of plasmid DNA per well. For the visualization of beating cilia, ependymal cells were labeled using the PKH26 Fluorescent Cell Linker Kit (Sigma) or Fluosphere carboxylate-modified microspheres (Invitrogen, F8801). The movement of the beads was recorded using a BX51 microscope (Olympus).

Quantification of the rotational orientation of cilia
To quantify the direction of ciliary beating on cultured ependymal cells, the movement of beads attached to the cilia was traced (lines in Fig. 4H-J, 11–25 cilia in each cell), and the angle of the beating direction of each cilium from the mean direction was determined for each cell. Using Oriana 2.0 (Kovach Computing Services) circular statistics software, the circular standard deviation (CSD) in each cell was calculated as described previously (Guirao et al., 2010; Mitchell et al., 2007; Park et al., 2008) and compared using a two-tailed, unpaired Student’s t-test. The basal body orientation in the ventricular walls was analyzed as described previously (Guirao et al., 2010) with modifications. Briefly, 5–11 basal feet in each ependymal cell from 2–5 ultrathin sections were analyzed. A vector was drawn from the center of the basal body to the protrusion of the basal foot, and the angle of this vector from the anterior direction (defined as 0°) was determined using ImageJ software. These data were analyzed using Oriana 2.0 software, and the output was expressed on a circular plot as a vector with an angle that denotes the mean cilary direction and a length that denotes the complement of the circular variance of these cilia around the mean. The circular distributions of the basal feet within each cell were compared with a random distribution using Rayleigh’s test.

Quantification of basal body distribution
The positions of the centroids of the ciliated region (shown by a basal body marker) and the whole cell (shown by a cell border marker) were calculated using ImageJ software. Ependymal cells with the entire cell border within the focal plane were selected and analyzed. Ependymal cells in which the centroid of the ciliated region was within 90° of the anterior (rostral) direction relative to the centroid of the whole cell were classified as having an ‘anterior’ localization of the basal body. For the transfection of dominant-negative forms of Dvl2 and the knockdown of NMII, 5–12 cells per animal were analyzed. For the inhibitor experiments, 15–53 cells per animal were analyzed.

Time-lapse imaging of basal bodies
Lateral walls from P5–8 Cent2GFP mice were included in 80% Matrigel (BD Biosciences) diluted in L-15 on glass-bottomed dishes. Time-lapse recordings were performed as described previously (Hirota et al., 2007), using a 63× oil-immersion objective lens.

High-speed live imaging of ciliary beating
Brain slices (200 μm thick) from P4–6 or P14–19 Cent2GFP mice were labeled using the PKH26 Fluorescent Cell Linker Kit (Sigma). The optical system for observing the fluorescence consisted of an epifluorescence microscope (IX-71, Olympus) equipped with a 60× water-immersion objective (UPLSAPO60XW, 1.20 NA; Olympus), a Nipkow disk-type confocal unit (CSU22, Yokogawa) and an electron multiplying CCD camera (iXon860BV, Andor Technology). For the inhibitor experiments, we used an inverted Zeiss LSM5 LIVE scanner. The images of beating cilia were recorded at 917 or 542 frames per second at room temperature (24–26°C).

DNA constructs and lentivirus expression vector
The mouse Dvl2ΔPDZ construct was described previously (Habas et al., 2001). The C-terminus of Dvl2 (Dvl2-C), spanning amino acids 508–736, was amplified by PCR. Plasmids encoding GFP fusion proteins (Dvl2ΔPDZ-GFP, Dvl2-C-GFP) were constructed using pEGFP-N1 (Clontech), which was also used as the control vector. Dvl2ΔPDZ-GFP was subcloned into self-inactivating lentivirus vector CSII-EF-MCS (provided by Dr H. Miyoshi, Riken Tsukuba BioResource Center) (Miyoshi et al., 1998) to produce CSII-EF-Dvl2ΔPDZ-GFP. Lentiviruses were produced as described previously (Kojima et al., 2010). For the knockdown experiments, the targeting sequences of Myh9, Myh10 and Myh14 (miR RNAi Select, Invitrogen) and lacZ (control) were cloned into a modified Block-iT Pol II miR RNAi expression vector (Invitrogen). DNA cassette containing DsRed-Express (Clontech) and the targeting sequence were cloned into pCAGGS vectors (Niwa et al., 1991) using the Gateway system (Invitrogen).

Injection and electroporation
Injection of lentivirus was performed as described previously (Kojima et al., 2010). For the inhibitor experiments, 1.5 μl of 5 mM (high dose) or 2.5 mM (low dose) inhibitor or vehicle was stereotaxically injected into the lateral ventricle of P4–5 mice [relative to lambdoid suture of the skull, anterior, lateral, depth (mm): 2.0, 1.0, 1.8]. The following inhibitors were used: blebbistatin (dissolved in 50% methanol in PBS, Research Chemicals); ML-9 (dissolved in 50% methanol in PBS, Calbiochem); Y27632 (dissolved in PBS, Calbiochem); and SP600125 (dissolved in 25% DMSO in PBS, Calbiochem). Electroporation of P1 mice was performed as described previously (Boutin et al., 2008).

Statistical analyses
All data are expressed as the mean ± s.e.m. Differences between means were determined by unpaired two-tailed Student’s t-test. For the inhibitor experiments, the data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. For the circular analysis, we used the Oriana 2.0 program. *P<0.05 was considered significant.

RESULTS
Basal bodies of ependymal cilia gradually translocate to the anterior region of ependymal cells during differentiation
To examine how translational polarity is established during the differentiation of ependymal cells, whole-mount lateral ventricular walls were prepared from mice of different ages. The anteroposterior (A-P) orientation of tissues was determined by the asymmetrical shape of the whole-mount ventricular walls (Fig. 1). They were then immunostained for Pent and ZO1 (Tip1 – Mouse Genome Informatics), markers for basal bodies and tight junctions, respectively. To standardize our analysis, we used only the dorsal part of the medial lateral ventricle (Fig. 1). In the early stages of ciliogenesis, the basal bodies were broadly distributed in the apical membrane of immature ependymal cells (Fig. 1A). The basal bodies gradually became asymmetrically localized within the apical membrane, accumulating at the anterior region of each ependymal cell (i.e. downstream in terms of the ciliary beating direction) (Fig. 1B). By P9, the basal bodies had formed a dense accumulation in the anterior region of most ependymal cells (Fig. 1C). In the adult,
the basal bodies were not as densely packed as at P9. However, they were still asymmetrically distributed, with more in the anterior region of each ependymal cell, in agreement with a recent report on translational polarity (Fig. 1D) (Mirzadeh et al., 2010).

We next compared the centroid position of the area containing basal bodies with that of the whole cell, by dividing each cell into quadrants (dorsal, ventral, posterior, anterior), and we counted the number of cells with a ciliated area centroid that lay in each quadrant (Fig. 1E). In P5 tissue, the basal bodies were dispersed, and the centroid of the ciliated area was found in all four quadrants. From P7 onwards, however, the number of cells with the centroid of the ciliated area in the anterior quadrant was significantly larger than of cells with the centroid in the other three quadrants ($\chi^2$ analysis; P5, 105 cells; P7, 91 cells; P9, 109 cells; adult, 137 cells; from three mice in each case). The percentage of cells in which the centroid of the ciliated area was in the anterior quadrant peaked at P9 (Fig. 1F). The density of the basal bodies also changed during differentiation. Whereas the cell area was slightly larger at P9 than at P7 and the adult stage (Fig. 1G), the area of the ciliated region was transiently and significantly reduced between P9 and P15 compared with P7 (Fig. 1H; data not shown), suggesting that the basal bodies became highly concentrated at the anterior of the ependymal cells during P9-15. We therefore focused on this period in subsequent experiments. Together, these data show that ependymal cells acquire their translational polarity between P5 and P9.

**Basal bodies move to the anterior region of ependymal cells**

To observe how the translational polarity of ependymal cells emerges between P5 and P9, we performed time-lapse recordings of basal bodies in the living ventricular wall. The lateral wall of the lateral ventricle of P5 Cent2GFP transgenic mice, which express a centrin 2-GFP fusion protein that labels the basal body (Higginbotham et al., 2004), was embedded in Matrigel, and the movement of the basal bodies was studied by time-lapse image analysis over 10 hours (Fig. 2; see Movie 1 in the supplementary material). At the beginning of the recording, the basal bodies were broadly distributed at low density in the apical membrane of ependymal cells (0 min in Fig. 2). All the basal bodies in a single cell migrated simultaneously in this process, and they gradually became asymmetrically distributed and formed a dense accumulation at the anterior apical membrane (Fig. 2; see Movie 1 in the supplementary material). We concluded that the basal bodies change their distribution within the apical membrane of ependymal cells along the A-P axis of the ventricular wall to accumulate in the anterior-most part of the cell membrane.

**Translational polarity and rotational polarity are established at around the same developmental stage**

To further characterize the timing of basal body migration during the period of ependymal cilia maturation, we used the two-color imaging of beating cilia and basal bodies to simultaneously observe
Here, we examined whether fluid flow and PCP signaling are also suggesting that fluid flow and PCP signaling both play important roles in establishing the rotational polarity of ependymal cilia. To test the role of fluid flow in the establishment of translational polarity, we compared the distribution of basal bodies between cultures with and without flow. The basal bodies of ependymal cells cultured with flow did not show a polarized distribution along the direction of flow (Fig. 4A-D). This suggested that fluid flow alone is not sufficient to induce translational planar polarity of basal bodies in primary culture.

Next, we examined the involvement of PCP signaling in the establishment of translational polarity. Immunostaining of the Cent2GFP ventricular wall revealed that Dvl2 accumulated at high levels near the basal bodies in differentiating ependymal cells (Fig. 4E). A similar localization of Dvl has been reported for Xenopus larvae (Park et al., 2008). Double immunostaining with R4109, a ciliary rootlet marker (Hagiwara et al., 2000), indicated that the Dvl2 was localized to the ciliary rootlets (Fig. 4F). To assess the role of Dvl2 in planar polarity formation in ependymal cells, we used a deletion construct, Dvl2ΔPDZ, that lacks the PDZ domain (Habas et al., 2001), and Dvl2-C, which contains only the conserved C-terminus of Dvl2. These constructs function as dominant-negative forms of Dvl2 that inhibit the PCP signal (Park et al., 2008) (Fig. 4G).

First, to test whether the inhibition of Dvl2 would affect rotational polarity in vitro, cultured ependymal cells were transfected with plasmids encoding a Dvl2-C-GFP or Dvl2ΔPDZ-GFP fusion protein. We then labeled the cilia with a fluorescent dye and performed high-speed confocal imaging. The ciliary beating of the single control cells was unidirectional, as shown in our previous report (Guirao et al., 2010), whereas that in cells transfected with one of the dominant-negative forms of Dvl2 was disturbed (see Movies 4-6 in the supplementary material). To quantify the ciliary orientation, we added small fluorescent latex beads to the medium. The movement of beads attached to the cilia was traced and is shown as lines indicating the trajectory of ciliary beating (Fig. 4H-J; see Movies 7-9 in the supplementary material). The beating direction of the cilia was quantified by measuring the angle to the mean angle in each cell, using the trace image made by the fluorescent beads. The coordinated ciliary beating was disturbed in both the Dvl2ΔPDZ-GFP-expressing and Dvl2-C-GFP-expressing cells (Fig. 4K) (control, 147 cilia from 18 cells; Dvl2ΔPDZ, 80 cilia from 11 cells; Dvl2-C-GFP, 49 cilia from six cells), suggesting that Dvl2 is required for rotational polarity in ependymal cells generated in vitro.
Regulation of ependymal cell planar polarity

Next, to examine the effect of inhibiting Dvl2 on the differentiation of ependymal cells in vivo, a lentivirus expression vector for Dvl2ΔPDZ-GFP was injected into the lateral ventricle of neonatal mice, and the brains were fixed at P10. Staining with a cilia marker revealed that ciliogenesis was not impaired in the Dvl2ΔPDZ-GFP-expressing cells (data not shown). By performing immunolectron microscopy using GFP-positive cells, the ciliary orientation was quantified based on the orientation of the basal bodies. The number of cells with anteriorly localized basal bodies (‘localized’) was significantly greater among cells with anteriorly localized basal bodies (‘dispersed’) \( (P<0.001) \). The cilia were significantly longer in the localized than dispersed cells \( (P<0.001) \).

Non-muscle myosin regulates the translational, but not the rotational, polarity of basal bodies

Emerging evidence suggests that NMII regulates planar polarity formation in several tissues in mammals. To examine whether NMII is involved in the planar polarity of basal bodies, we first analyzed the expression pattern of the NMHCs in differentiating ependymal cells. In mammals, three NMHC isoforms have been identified: Myh9, Myh10 and Myh14. Myh9 was highly expressed and broadly distributed in the ependymal cells, but it was not detectable in neural stem cells with primary cilia (Fig. 5A). Myh10 and Myh14 were distributed in the cytoplasm of ependymal cells and highly localized in the vicinity of basal bodies (Fig. 5B,C). At the apical surface of cells, the localization of Myh14 in the vicinity of basal bodies was even more evident (Fig. 5D). The activity of NMII is regulated mainly by the phosphorylation of its RLCs by Rho-associated protein kinase (ROCK; Rock1 and Rock2 – Mouse Genome Informatics) and myosin light chain kinase (MLCK; Mylk – Mouse Genome Informatics) (Adelstein and Conti, 1975; Amano et al., 1996). We therefore examined myosin activation using an antibody specific for the phosphorylated form of myosin light chain (pMLC), and found that the pMLC signal was localized to the vicinity of basal bodies (Fig. 5E). Thus, NMII was highly expressed and activated in differentiating ependymal cells, indicating that NMII could be involved in the planar polarization of ependymal cilia.

To determine whether NMII plays a role in basal body distribution, we examined the effects of three small-molecule inhibitors: blebbistatin, which inhibits myosin II (Straight et al., 2003); ML-9, which inhibits MLCK (Saithoh et al., 1987); and Y27632, which inhibits ROCK (Uehata et al., 1997). We injected the inhibitors or vehicle alone into the lateral ventricles of P5 mice and fixed the brains at P10. In the control animals, the basal bodies accumulated in the anterior region of the ependymal cells (Fig. 6A). The basal bodies were dispersed in the apical membrane of the ependymal cells in brains injected with blebbistatin or ML-9 as compared with mock-injected controls (Fig. 6A-C), but Y27632 did not affect basal body distribution (Fig. 6D). The treatment with blebbistatin or ML-9 significantly decreased the number of cells with anteriorly localized basal bodies in a dose-dependent manner (control for blebbistatin and ML-9, 172 cells from five mice; lower-dose blebbistatin, 142 cells from four mice; higher-dose blebbistatin, 65 cells from four mice; lower-dose ML-9, 158 cells from five mice; higher-dose ML-9, 157 cells from five mice; control for Y27632, 51 cells from three mice; Y27632, 45 cells from three mice) (Fig. 6I). In these experiments, staining for acetylated tubulin showed that the cilia were elongated in the inhibitor-treated brains, indicating that ciliogenesis was largely
maintained (Fig. 6E-H). These results suggested that the activation of NMII via MLCK is important for the establishment of translational polarity.

Next, to examine whether the inhibition of NMII disturbs the ciliary beating pattern, we recorded side views of the beating ependymal cilia using mock- or blebbistatin-injected Cent2GFP mice. We did not detect any obvious abnormality in the orientation (see Movies 10, 11 in the supplementary material) or frequency of the ciliary beating (control, 19.1±0.87 Hz, 22 cilia from three mice; blebbistatin, 19.0±1.05 Hz, 25 cilia from three mice; P=0.9254) in tissues from mice treated with blebbistatin, indicating that the inhibition of NMII did not affect ciliary beating. These results...
Regulation of ependymal cell planar polarity

To further examine the role of NMII in the establishment of translational polarity, knockdown experiments with vector-based RNAi were performed. Plasmids containing knockdown target sequences for Myh9, Myh10 and Myh14, or lacZ (as a negative control), together with DsRed-Express were introduced into the lateral ventricles of P1 mice by electroporation and the brains fixed at ~P12. Immunostaining for Myh9, Myh10 and Myh14 was efficiently reduced in the DsRed-Express-positive cells in the NMII-knockdown samples compared with the controls (see Fig. S1 in the supplementary material). The basal bodies docked normally at the apical membrane of the NMII-knockdown cells (Fig. 6J,K,M,N), and the DsRed-Express signal was detected in the ciliary axonemes in the NMII-knockdown cells, as in the controls (Fig. 6L,O), suggesting that the extension of the ciliary axonemes was not notably affected by the knockdown of NMII. However, in the Myh9/Myh10/Myh14-knockdown ependymal cells, the basal bodies were dispersed in the apical membrane (Fig. 6J,K,M,N) and the number of cells with anteriorly localized basal bodies was significantly decreased (control, 29 cells from five mice; NMII knockdown, 32 cells from three mice) (Fig. 6P). This result suggested that NMII regulates the distribution of basal bodies and the establishment of the translational polarity of ependymal cells in a cell-autonomous manner.

DISCUSSION
Basal bodies migrate within the apical cell membrane of differentiating ependymal cells after docking

The results presented here demonstrate that ependymal cells acquire translational polarity by the migration of basal bodies to the anterior of the apical cell membrane. For this study, we developed a method for time-lapse imaging of the cultured, whole-mount lateral wall of the lateral ventricle by modifying conventional methods for brain slice culture. Using this approach, we found that the dispersed basal bodies moved towards, and accumulated at, the anterior edge of the ependymal cells within 10 hours. Live imaging also revealed that all the basal bodies in a single cell migrate simultaneously. Thus, our time-lapse imaging of fluorescently labeled basal bodies clearly revealed their behavior after docking, providing the first evidence that basal bodies indeed move towards the anterior border of the apical membrane of ependymal cells along the A-P axis of the brain.

What is the functional significance of the translational polarity of ependymal cells? The normal beating behavior of the dispersed cilia on ependymal cells after treatment with an inhibitor of NMHCs suggested that translational polarity is dispensable for the directional and rapid beating of cilia (see Movies 10-11 in the supplementary material). Since the anterior positioning of the basal bodies causes a posterior tilt of nodal cilia, which produces a leftward flow (Nonaka et al., 2005; Okada et al., 2005), the anterior localization of the ependymal cilia might also affect the generation of fluid flow. The basal bodies did not exhibit translational polarity in other multiciliated cells, such as those of the trachea and oviduct (data not shown), suggesting that it is a unique feature of ependymal cells. Differences in the viscosity and amount of fluid might account for the differences in the alignment pattern of the basal bodies in these cells. The viscosity of cerebrospinal fluid (CSF) is lower than that of the mucus in the oviduct or trachea, and the amount of liquid is different: the lateral ventricles are filled with CSF, whereas only the epithelial surfaces of the trachea and oviduct are covered with mucus, which exists as a thin layer.

PCP signals regulate the rotational, but not the translational, polarity of basal bodies

Our recent study indicated that the orientation of ependymal cilia is regulated by the PCP protein Vangl2 (Guirao et al., 2010). It was also recently reported that the PCP components Celsr2 and Celsr3, which are members of the non-classical cadherin family, are involved in establishing rotational polarity and the membrane distribution of Vangl2 in ependymal cells (Tissir et al., 2010). Here, we found that the inhibition of Dvl2, a cytoplasmic effector of the PCP pathway, also disturbs rotational polarity. The proper membrane distribution of Dvl2 depends on the function of Vangl2 in the inner ear (Wang et al., 2005). It is possible that these three PCP components (Celsr proteins, Vangl2 and Dvl2) act cooperatively to establish the rotational polarity of ependymal cilia. By contrast, we found that translational polarity was not affected.
3044 RESEARCH ARTICLE

Development 137 (18)

Fig. 6. Inhibition of NMII disrupts the translational, but not the rotational, polarity of the basal bodies in ependymal cells. (A-H) Vehicle control (A,E) or inhibitors of NMII (B,F; higher dose blebbistatin), MLCK (C,G; higher dose ML-9) or ROCK (D,H; Y27632) were injected into the lateral ventricles of P5 mice. Five days after injection, the ependymal cells were immunostained for Pcnt (red) and β-catenin (green) (A-D) or acetylated tubulin (green in E-H, white in E’-H’). (I) Injection of blebbistatin or ML-9 resulted in a significant and dose-dependent decrease in the number of cells with the centroid of the ciliated area in the anterior quadrant. By contrast, Y27632 did not affect the anterior localization of the basal bodies. *P<0.05, control versus lower dose blebbistatin; **P<0.01, lower dose blebbistatin versus higher dose blebbistatin; P<0.01, control versus lower dose ML-9; P<0.05, lower dose ML-9 versus higher dose ML-9; P=0.0186, control versus Y27632. (J-O) Knockdown of NMII. Ependymal cells from Cent2GFP mice electroporated with plasmids expressing a control (J-L) or a Myh9/Myh10/Myh14-knockdown (KD) vector (M-O) that co-expressed DsRed (red) were stained with β-catenin (blue). (J’-M’,J’-M’) Three-dimensional reconstruction images. Basal bodies in the apical cell membrane of DsRed-expressing cells are indicated by arrows (J’,M’). (K,N) β-catenin (pseudocolored red) and Cent2GFP (green) signal in the boxed regions indicated in J and M. DsRed-expressing cells are indicated by asterisks. (L,O) DsRed-labeled cilia (arrows) in the boxed regions indicated in J and M. (P) The number of cells with the centroid of the ciliated area in the anterior quadrant was significantly reduced in the Myh9/Myh10/Myh14-KD samples compared with controls (CT) (P=0.0316). *, P<0.05; **, P<0.01. Scale bars: 10 μm in A-D,J-O; 2 μm in E-H’.

by the inhibition of Dvl2. The PCP pathway is known to regulate various cellular processes through the activation of ROCK and JNK (Mapk8 – Mouse Genome Informatics). We found that the pharmacological inhibition of both of these kinases did not affect translational polarity (Fig. 6D; data not shown), similar to the lack of effect from inhibiting Dvl2. Together, our results suggest that translational polarity is not regulated by the PCP signaling pathway, although PCP signaling does regulate rotational polarity (Guirao et al., 2010; Tissir et al., 2010); therefore, these two planar polarities are regulated by distinct molecular mechanisms.

Recent papers have reported that the PCP pathway regulates the position of the basal bodies of nodal cilia in mice and zebrafish (Antic et al., 2010; Hashimoto et al., 2010; Borovina et al., 2010). Similarly, PCP signaling is important for the polarization of the primary cilium, or kinocilium, in the hair cells of the organ of Corti (Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006), suggesting that the PCP pathway is required for positioning primary cilia but not the multiple cilia of ependymal cells. In addition, cilia are required for the normal positioning of the basal body of the kinocilium (Jones et al., 2008) but not the basal body of ependymal cilia (Mizrazi et al., 2010). In contrast to the single basal body of nodal cells or hair cells, the basal bodies of ependymal cilia migrate in a group and over a longer distance during translational polarization. It is possible that these differences account for the different requirements for PCP signaling in basal body positioning.
NMII regulates the translational polarity of basal bodies in differentiating ependymal cells

In this study, we showed that the inhibition of NMII greatly disturbs translational polarity, indicating that NMII is required for the anterior migration of basal bodies in the apical membrane of ependymal cells. Recently, the NMII motor was shown to be important for controlling the movement of the centrosome, which is identical to the basal body, into the leading process of migrating neurons (Solecki et al., 2009). Thus, it is reasonable that NMII would regulate the movement of the basal bodies in ependymal cells.

It is still unclear what signal activates NMII regulation of basal body migration. Our inhibitor experiments suggested that the activation of NMII by MLCK is important for translational polarity. An increase in the intracellular calcium concentration initiates smooth muscle contraction through the phosphorylation of RLC by MLCK downstream of the calcium/calmodulin signal in several tissues, including the renal collecting duct (Chou et al., 2004). Previous studies indicate that the calcium/calmodulin-dependent protein kinase II and calmodulin superfamily proteins are expressed in ependymal cells (Fukunaga et al., 1988; Mailleux et al., 1993), suggesting that calmodulin signaling in these cells could activate NMII to establish the translational polarity.

In conclusion, the results presented here demonstrate that basal bodies migrate anteriorly in the apical membrane and establish the translational polarity of ependymal cells. Taking into consideration recent findings (Guirao et al., 2010; Mirzadeh et al., 2010; Tissir et al., 2010) and our present results, the two distinct polarities are probably formed during ependymal cell differentiation as follows. In neonates, the formation of rotational polarity is triggered by fluid flow (Guirao et al., 2010), possibly generated by the secretion of CSF from the choroid plexus and its absorption in the foramen of Monro in the lateral ventricles, via mechanisms involving motile cilia and PCP signaling (Guirao et al., 2010; Tissir et al., 2010) (Fig. 4). By contrast, translational polarization is initiated by the anterior localization of the basal body of the primary cilium of radial glial cells (Mirzadeh et al., 2010) and is established after the transformation of radial glial cells to ependymal cells by the anterior migration of the basal bodies (Figs 1-3). These two polarities are determined independently by distinct cellular and molecular mechanisms (Fig. 4). Our results identify NMII as a key regulator of translational planar polarity (Figs 5, 6) and provide new insight into how this PCP is established in multiciliated tissue.

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Competing interests statement
The authors declare no competing financial interests.

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References
Bailly, E., Doree, M., Nurse, P. and Bornens, M. (1989). p34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO J. 8, 3985-3995.

Development


