RhoA-mediated apical actin enrichment is required for ciliogenesis and promoted by Foxj1

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Accepted 11 April 2007

Journal of Cell Science 120, 1868-1876 Published by The Company of Biologists 2007
doi:10.1242/jcs.005306

Summary

Programs that direct cellular differentiation are dependent on the strict temporal expression of regulatory factors that can be provided by Rho GTPases. Ciliogenesis is a complex sequence of events involving the generation and docking of basal bodies at the apical membrane, followed by ciliary axoneme generation. Although a cilia proteome has been assembled, programs that direct ciliated cell differentiation are not well established, particularly in mammalian systems. Using mouse primary culture airway epithelial cells, we identified a critical stage of ciliogenesis requiring the temporal establishment of an apical web-like structure of actin for basal body docking and subsequent axoneme growth. Apical web formation and basal body docking were prevented by interruption of actin remodeling and were dependent on RhoA activation. Additional evidence for this program was provided by analysis of Foxj1-null mice that failed to dock basal bodies and lacked apical actin. Foxj1 expression coincided with actin web formation, activated RhoA and RhoB, and persisted despite RhoA inhibition, suggesting that Foxj1 promoted RhoA during ciliogenesis. Apical ezrin localization was also dependent on Foxj1, actin remodeling, and RhoA, but was not critical for ciliogenesis. Thus, temporal Foxj1 and RhoA activity are essential regulatory events for cytoskeletal remodeling during mammalian ciliogenesis.

Key words: Basal bodies, Cilia, Airway epithelial cells, GTPase, Mouse

Introduction

Mutations in genes with roles in ciliogenesis have been increasingly implicated in human disease (Ibanez-Tallon et al., 2003). For example, polycystic kidney disease is caused by mutations in genes expressed in sensory cilia (Nauli et al., 2003), whereas primary ciliary dyskinesia (leading to chronic respiratory infections) is caused by mutations in dynein motor proteins in motile cilia (Olbrich et al., 2002). The Bardet-Biedl syndrome (BBS) has also been associated with mutations in proteins identified in cilia and manifest with a constellation of both rare and common disorders including retinopathy, polycystic kidneys, obesity, polydactyly, male hypogonadism, cognitive impairment, diabetes mellitus and hypertension (Blacle et al., 2004). Environmental factors also play roles as evidenced by cilia loss due to respiratory viruses and cigarette smoke, requiring intact mechanisms of ciliogenesis for repair (Ibricevic et al., 2006; Look et al., 2001; Sisson et al., 1994). Together, these disorders suggest a role for cilia in a wide range of physiologic and pathologic processes. Despite the identification of these mutations, relatively little is known about the biochemical pathways for ciliogenesis, particularly in mammalian systems.

Groundbreaking work in ciliogenesis has been accomplished in Chlamydomonas, leading to identification of homologues in mammals, including those causing human disease (Li et al., 2004; Pazour et al., 2005). A large number of genes and proteins expressed in the ciliary apparatus have recently been compiled from several organisms and tissues to form a ciliome library (Inglis et al., 2006). Having catalogued genes with putative roles in cilia assembly, attention must now shift to the biochemical steps required for ciliogenesis. In this regard, the axoneme assembly process of intraflagellar transport has recently been found to involve hedgehog-dependent pathways (Scholley and Anderson, 2006). However, regulatory factors that are required for earlier steps of ciliogenesis are not defined (reviewed in Dawe et al., 2007). In multi-ciliated cells, one of the earliest known steps marking the onset of ciliogenesis is the generation of centrioles through a process shown to require γ-tubulin nucleation (Dutcher, 2003). The centrioles become basal bodies upon docking at the apical membrane of the cell, where axoneme assembly ensues. Mechanistically, in the mammalian lung, much of what is understood about the pathways of ciliogenesis between the stages of basal body generation and axoneme growth is based on analysis of elegant sets of transmission electron photomicrographs of developing ciliated airway cells provided over a quarter century ago (Dirksen, 1991; Dirksen and Crocker, 1966; Sorokin, 1968; Steinman, 1968). How regulatory factors fit into this schema remains to be determined.

One clue for understanding ciliogenesis comes from observations of changes in cytoskeletal structures during differentiation. In ciliated cells, this has been supported by several studies, including the association of cytoskeletal components (e.g. microfilaments, actin) with basal bodies in electron photomicrographs of ciliated cells in non-mammalian organisms (Hoey and Gavin, 1992; Reed et al., 1984; Sandoz et al., 1988). Altered expression of cytoskeleton-associated genes resulting in the formation of cysts has also been described as a feature of interrupted ciliogenesis in polycystic kidney disease (Charron et al., 2000; Nauli et al., 2003). Our
Role of RhoA in ciliogenesis

Fig. 1. The apical localization of actin in ciliated airway epithelial cells during differentiation. Differentiation was induced in primary culture mTECs under ALI conditions. (A) Photomicrographs of mTEC cultures stained for filamentous actin (phalloidin, red) and for the cilia marker β-tubulin-IV (green) on the indicated day of ALI culture. Reconstructed confocal images show z-axis view below. An apical web-like structure is seen at ALI day 3 in ciliated cells. (B) Photomicrographs obtained as in A stained for actin (phalloidin, red) and basal body marker γ-tubulin (green). γ-tubulin is additionally present in paired nuclear centrioles within the microtubule organizing center as seen at ALI day 2. Bars, 10 μm.

Evaluation of mTEC cultures at ALI day 2-5 using confocal microscopy showed that formation of the apical actin web was coincident with basal bodies docking at approximately ALI day 3 (Fig. 1B). The temporal relationship between the appearance of the apical net and localization of the centrioles suggested that basal bodies colocalize with the enriched apical actin web during ciliogenesis.

The stage-specific effect of pharmacologic inhibition of actin assembly on the differentiation of ciliated cells

To determine the role of actin assembly for basal body docking during ciliogenesis, we treated cells with either cytochalasin D, an inhibitor of F-actin polymerization (Fox and Phillips, 1981) or latrunculin B to inhibit F-actin polymerization and induce disassembly of rapidly turning over microfilaments (Morton et al., 2000). To determine the critical actin-dependent

Results

Actin is enriched at the apical membrane of ciliated cells during differentiation of airway epithelial cells

Previous observations that ezrin was selectively expressed in ciliated airway cells, and binds to actin when localized to the apical membrane led us to examine the temporal and spatial localization of actin during epithelial cell differentiation. As a model system, we used primary mouse tracheal epithelial cells (mTEC) in culture for induction of differentiation upon initiation of air-liquid interface (ALI) conditions and assessed filamentous actin (F-actin) expression by phalloidin binding (Fig. 1). This culture system provided a high-fidelity model of ciliogenesis that could be staged during differentiation (Huang et al., 2003; You et al., 2004; You et al., 2002). During early differentiation (ALI day 0-2), actin was present along the basolateral aspect of the cells, particularly at regions of cell-cell junctions. At ALI day 3, we observed that a web-like structure of actin developed at the apical membrane of ciliated cells, which were identified by the ciliary axoneme protein β-tubulin-IV (Fig. 1A). The development of the apical actin web was restricted to ciliated cells. The relationship between apical actin localization and early stages of ciliogenesis was next determined using γ-tubulin expression as a marker for centrioles and ciliary basal bodies that serve as organization sites for assembly of the axonemes (Dutcher, 2003; You et al., 2004). The basal body precursors (centrioles) initially appear as a dense cluster in the cytoplasm, then form a punctate array at the apical membrane when docking occurs (You et al., 2004).
stage(s) of ciliogenesis, cultures were treated for 4 hours on each ALI day (day 0 to day 4) and assayed 24 hours later (i.e. treated on ALI day 0 then assayed on ALI day 1, treated on ALI day 1 then assayed on ALI day 2, and so on). Preservation of tight junctions following drug treatment was manifest by maintenance of an air-liquid interface. We observed that only treatment on days 2 or 3 interrupted ciliogenesis by preventing basal body docking (Fig. 2A,B). This was temporally coincident with the failure of apical-actin-web formation (Fig. 2A,B). As expected, treatment during these stages of differentiation was accompanied by inhibition of apical cilia growth as indicated by an absence of apical β-tubulin-IV expression and decrease in cilia observed in SEM samples (Fig. 2A,C). The effect of actin interruption on ciliogenesis was consistent with a requirement for the actin structures to dock basal bodies. Earlier treatment (at ALI day 0 or ALI day 1), or late treatment (at ALI day 4), did not alter ciliogenesis (Fig. 2B,C). This suggested a temporally regulated process of apical actin enrichment and actin-dependent ciliogenesis.

Rho protein inhibition interrupts the differentiation of ciliated cells

Small GTPase Rho family proteins regulate actin and cytoskeleton organization, affecting cell shape and differentiation (Hall, 1998). To determine whether these proteins are required for ciliogenesis, we treated mTEC cultures on ALI day 0-4 with Rho inhibitors, then assayed the cells the following day (using a protocol similar to the treatment of mTECs with actin inhibitors described above). Cells were treated with the HMG-CoA reductase inhibitor mevastatin to inhibit post-translational isoprenylation of small G proteins or Clostridium difficile toxin B to inactivate the Rho family of small GTP-binding proteins (e.g. Rho, Rac and Cdc42) (Just et al., 1995). These inhibitors effectively interrupted ciliogenesis when cultures were treated on ALI day 2 or day 3 (Fig. 3). Similar to the effect observed with agents that interrupted actin directly, inhibition of ciliogenesis by Rho inhibitors was also associated with a failure of apical localization of basal bodies (Fig. 3A,C). Cells otherwise maintained

Fig. 2. The effect of pharmacological inhibition of actin assembly on ciliogenesis. (A) First nine panels from left: photomicrographs showing actin (phalloidin), basal body (γ-tubulin) and cilia (β-tubulin-IV) localization in mTEC cultures at ALI day 3, obtained 1 day after treatment at ALI day 2 with vehicle, cytochalasin D or latrunculin B. Bar, 10 μm. Three panels on right: representative scanning electron micrographs (SEMs) of mTEC cultures on ALI day 4 obtained 1 day after treatment at ALI day 3. Bar, 1 μm. (B) Percentage of cells containing basal bodies localized at the apical membrane as a proportion of total cells containing basal bodies at the apical membrane or within the cytoplasm (identified by immunostaining for γ-tubulin). Data are the mean ± s.d.; *P<0.05, significance compared with vehicle. (C) The percentage of cells with apical cilia (identified by immunostaining for β-tubulin-IV) as a proportion of all cells (identified by nuclear staining with DAPI). Data are the mean ± s.d.; *P<0.05, significance compared with vehicle.

Fig. 3. The effect of Rho GTPase inhibition on epithelial cell differentiation. (A) mTEC cultures treated with vehicle, mevastatin, or toxin B for 24 hours on ALI day 2 were harvested one day later and stained for actin (phalloidin, red) and basal bodies (γ-tubulin, green). Different fields are shown for each marker assayed. Bar, 10 μm. (B) Scanning electron micrograph of mTEC on ALI day 4 after treatment with mevastatin on ALI day 3 showing microvilli but absent cilia. (C) Transmission electron photomicrograph of mTEC treated with toxin B as in B. Vehicle treated cells show normal basal bodies docked at the apical membrane with ciliary axonemes (arrowheads). In toxin B treated cells, apical basal bodies are absent, but centrioles are present within the cytoplasm (magnification shown in the box). Microvilli are intact at the apical membrane (arrows) Bar, 1 μm in B,C.
features of polarized epithelial cells, including apical microvilli (Fig. 3B,C). However, the microvilli were notably sparse, consistent with reports that the development of these structures is dependent on members of the Rho family of GTPases (Gauthier-Rouviere et al., 1998). Importantly, during treatment with the Rho inhibitors there was loss of apical phalloidin staining, suggesting a disruption in the formation of an actin-docking network. Again, as with actin-active agents, treatment with Rho inhibitors at ALI day 4 or in older cultures (ALI day 7 or later) did not alter ciliogenesis as assessed by immunostaining (data not shown). Thus, Rho proteins are required for normal differentiation of airway epithelial cells in a stage-dependent manner; however, a specific Rho family member required for ciliogenesis has not been identified.

RhoA is required for apical actin localization and ciliogenesis

Earlier studies have shown that RhoA is required for the localization of ERM protein at the cortical membrane of cell lines (Hirao et al., 1996; Kotani et al., 1997; Shaw et al., 1998; Yonemura et al., 2002) and may play a role in establishment of actin polarity in yeast (Nakano et al., 1997). Also, in developing *Drosophila*, RhoA is required to organize the localization of actin along the apical membrane of the tracheal cells (Lee and Kolodziej, 2002; Matussek et al., 2006). To determine a role for RhoA in mammalian ciliogenesis, the mouse trachea cell cultures were treated with the *Clostridium botulinum* C3 exotoxin which specifically inactivates RhoA through ADP-ribosylation (Rubin et al., 1988). Similar to the effects of non-specific Rho inhibitors, treatment with C3 exotoxin on ALI day 2 resulted in impaired basal body docking, ciliary axoneme production and apical actin localization when assayed on ALI day 3 (Fig. 4A,B). Again, treatment with these agents did not disrupt the monolayer. Furthermore, there were no effects on β-catenin localization, suggesting that apical membrane organization was selectively altered and the basolateral cell junction complexes remained intact (Rajasekaran et al., 1996) (Fig. 4C). Similar to non-specific Rho interruption, treatment with C3 exotoxin on ALI day 4 or later did not alter ciliogenesis. Taken together, these data indicate that RhoA regulates a specific stage of ciliogenesis to direct apical actin for basal body docking.

A role for Foxj1 in RhoA-mediated ciliogenesis

We have previously reported that Foxj1 expression is first detected (by RNA and protein) at ALI day 2, just prior to basal body docking (Brody et al., 2000; You et al., 2004). ALI day 2 represents a stage of ciliogenesis that is characterized by the appearance of multiple centrioles within the cytoplasm. As we have shown in Fig. 1, apical web formation occurs after ALI day 2. To determine whether Foxj1 plays a role in RhoA-mediated apical web localization, we first compared phalloidin staining in mTECs cultured from wild type (WT) with those obtained from Foxj1–/– mice. Foxj1–/– mTECs that were cultured at ALI for longer than 10 days showed a striking absence of the apical actin web, together with a failure of basal body docking (Fig. 5A,B). The 3D reconstruction of confocal microscopy images of WT compared with Foxj1–/– mTEC cultures clearly show the defect in apical actin with preserved cortical actin (Fig. 5B).

A role for Foxj1 in the activation of RhoA-dependent differentiation was further substantiated when increased active RhoA (Rho-GTP) was observed following Foxj1 overexpression in undifferentiated cells. Foxj1 expression is normally restricted to differentiated, ciliated epithelial cells. Adenovirus-mediated expression of Foxj1 (but not control GFP) in a HEK293 cell line and undifferentiated mTECs (cultured by submersion on plastic dishes to block Foxj1 expression and ciliogenesis) resulted in increased RhoA determined by rhotekin pull-down assay (Fig. 5C). Using the rhotekin assay which recognizes several Rho family members (Ren et al., 1999), and a RhoB-specific antibody, we found that RhoB was also activated by Foxj1 (Fig. 5D). A pan-Rho antibody did not reveal RhoC expression in the mTEC cultures (data not shown). Also, although hepatocyte growth factor activated Rac1 in mTEC (Ren et al., 1999), Foxj1 did not activate Rac1-GTPase (Fig. 5E). Thus, specific Rho family GTases may function with Foxj1 during ciliogenesis.

To additionally establish that active RhoA was required for ciliogenesis we demonstrated that a dominant-negative form of RhoA (N19), delivered by adenovirus vector (AdRhoAN19), interrupted basal body docking and ciliary axoneme formation. AdRhoAN19 was coinfected with an adenovirus that constitutively expresses the tetracycline repressor-VP16 fusion protein (AdtTA) providing activation or, in the presence of doxycycline, repression (Neering et al., 1996). RhoAN19, identified by immunostaining as previously described (Kalman

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**Fig. 4.** The effect of C3 exotoxin on ciliogenesis. (A) Confocal photomicrographs of actin stained with phalloidin (red) and of γ-tubulin (green) in mTEC cultures on ALI day 3 and ALI day 5 following treatment with vehicle or C3 exotoxin initiated on ALI day 2 and day 4, respectively. Reconstructed z-axis images are below. (B) The percentage of apical γ-tubulin in all cells with basal bodies (apical or cytoplasmic) and apical β-tubulin in total cells treated as in A. Data are the mean ± s.d. (n=3 experiments); *P<0.05, significance compared with vehicle. (C) Basolateral β-catenin (red) and DAPI-stained cell nuclei (blue) in ALI day 3 cultures following treatment with vehicle or C3 exotoxin at ALI day 2. Bars, 10 μm.
et al., 1999), was colocalized with the cilia marker Sp17 (Grizzi et al., 2004; Wen et al., 2001) in the absence of doxycycline (Fig. 6A). AdRhoAN19 transfection interrupted basal body docking and normal β-tubulin-IV expression in axonemes, as well as apical ezrin localization (Fig. 6B,C).

However, transfection of cells with AdRhoN19 did not diminish expression of Foxj1, indicating that ciliated cells did not de-differentiate and that the RhoA pathway was downstream or parallel to a Foxj1 pathway (Fig. 6B,C). As expected, transfection of Foxj1−/− cells with vectors that expressed constitutively active RhoA (RhoAV14) did not induce ciliogenesis (data not shown). This is consistent with reports that overexpression of constitutive active RhoAV14 in polarized cell lines disrupt cell cytoskeleton function where highly regulated transient activation is required for normal differentiation (Desai et al., 2004).

Fig. 5. The effect of Foxj1 on actin assembly and RhoA activation. (A) Confocal photomicrographs of actin (phalloidin, red) and basal body (γ-tubulin, green) localization in mTEC cultures (ALI longer than day 7) from wild-type (WT) or Foxj1−/− mice. Reconstructed confocal z-axis images are below. (B) 3D reconstruction of confocal microscopy images from A showing the loss of apical actin in Foxj1−/− compared to WT mTECs. (C) Western blot showing RhoA and RhoB GTPase activation in non-differentiated mTECs infected with AdGFP or AdFoxj1. GTP-bound RhoA and RhoB were detected in the same membrane re-probed with specific antibodies. (D) Scanning densitometry of immunoblots from C showing active relative to total Rho. (E) Western blot showing Rac1 GTPase activation in mTECs cultured as in C, treated with hepatocyte growth factor (HGF, 50 ng/ml, 4 hours) or transfected with adenovirus vector expressing GFP or Foxj1. Rac1-GTP was isolated from cell lysates by binding to P21-binding-domain-bound agarose and immunoblots analyzed. (F) Scanning densitometry of blots shown in E.

Fig. 6. The effect of dominant-negative RhoA on ciliogenesis and ezrin localization. (A) Adenovirus-mediated overexpression of dominant-negative RhoA (AdRhoAN19) in cells co-expressing cilia marker Sp17 (arrows). At ALI day 0, mTEC cultures were infected with a tetracycline regulated (tet-off) adenovirus vector system (AdtTA) to control the expression of AdRhoAN19 by continuous incubation without or with doxycycline (Dox), then immunostained at ALI day 5. Bar, 10 μm. (B) β-tubulin-IV, γ-tubulin, ezrin and Foxj1 in mTEC cultures infected with RhoAN19 and AdtTA as in A treated without or with doxycycline. (C) The percentage of cells transfected with RhoAN19 with apical localization of ciliated cell markers (β-tubulin, γ-tubulin, ezrin) and Foxj1 (nuclear) in the absence or presence of doxycycline. Data are the mean values ± s.d.; *P<0.05, significance in the absence of doxycycline. (D) Western blots showing expression of ezrin and moesin in mTEC cultured on plastic dishes treated with siRNA containing scrambled (Scr) or three pooled ezrin-specific (Ezr) sequences. (E) β-tubulin-IV and ezrin in mTEC cultures treated with medium, Scr or pool ezrin siRNA sequences at ALI day 2 through ALI day 5, then immunostained for cilia (β-tubulin-IV, green) and ezrin (red) at ALI day 5. (F) The percentage of cells co-expressing β-tubulin-IV and ezrin (mean ± s.d.) following treatment with media, scrambled sequence (scram) siRNA or ezrin-specific siRNA. Shown are the mean values ± s.d. (from three independent experiments); *P<0.05, significance compared with control conditions.
Ciliogenesis occurs independently of ezrin expression

Ezrin serves to link the actin cytoskeleton with scaffold and transmembrane proteins (Bretscher et al., 2002). Ezrin is expressed in ciliated cells where apical membrane localization is regulated, in part, by Foxj1 (Huang et al., 2003). Accordingly, apical ezrin has been suggested to have a function in basal body docking (Bossinger and Bachmann, 2004; Gomperts et al., 2004; Huang et al., 2003). To test this, we used an siRNA knock-down strategy to inhibit ezrin expression in mTEC cultures then evaluated ciliogenesis. We used a pool of three ezrin siRNA sequences, and confirmed that these siRNA sequences specifically inhibited ezrin expression in NIH3T3 cells (data not shown) and in mTEC cultures (Fig. 6D). In mTECs transfected with a control scrambled siRNA sequence (at ALI day 2 to ALI day 5), ezrin was uniformly expressed at the apical membrane of ciliated cells as previously demonstrated (Fig. 6E, top row) (Huang et al., 2003). By contrast, mTEC cultures transfected with pooled ezrin siRNA had several regions of cells with absent ezrin expression, but abundant apical β-tubulin-IV in normal appearing cilia (Fig. 6E, bottom row). Moesin is also normally expressed in the apical membrane of ciliated cells but could not compensate for any ciliogenesis role in the Foxj1-null mouse, as we previously reported (Huang et al., 2003), and is not upregulated when ezrin was inhibited by siRNA in mTEC (Fig. 6D). The presence of normal ciliogenesis following ezrin siRNA-mediated silencing was also consistent with the report of normal lung development and epithelial differentiation in the ezrin knockout mouse (Saotome et al., 2004). Thus, it is likely that the presence of the apical actin web, rather than the presence of ezrin per se, is required for ciliogenesis.

Discussion

Recent proteomic and genomic analyses of non-mammalian ciliated organisms have identified a conserved group of proteins that are components of cilia and basal bodies (e.g. Avidor-Reiss et al., 2004; Pazour et al., 2005). The current studies were directed toward understanding dynamic processes essential for mammalian ciliogenesis using a high-fidelity primary culture model of mouse tracheal epithelial with well-characterized stages of differentiation (You et al., 2004; You et al., 2002). In this model, we found that early steps in ciliogenesis require an active remodeling of the cytoskeleton to provide a dense actin net or web at the apical membrane for basal body docking and subsequent axoneme production. By treating cells with inhibitors and using genetic models, we demonstrated that the small GTPases, and particularly RhoA, have an essential role in the establishment of this actin net in ciliated airway epithelial cells. Foxj1, a forkhead transcription factor previously shown to be essential for mammalian ciliogenesis appears to promote RhoA activity and is also required for apical actin localization. These observations are in agreement with a role for small GTPases in regulating unique features of cell differentiation (Van Aelst and Symons, 2002), in part, through actin localization, but here in concert with the ciliated epithelial cell-specific factor Foxj1.

The localization of an actin web on the apical aspect of mammalian ciliated cells is consistent with studies of ultrastructure in multi-ciliated invertebrates. For example, the epithelial cells at the border of the gill of the freshwater mussel have rows of cilia (alternating with microvilli) that are similar to those found in respiratory epithelia and basal bodies contained in a dense gird of actin and microtubules, as identified using electron microscopy (Reed et al., 1984). Similarly, detailed immunolocalization studies in Tetrahymena thermophylia described a cage of actin around each basal body that was docked at the apical membrane of ciliated cells (Hoey and Gavin, 1992). Further, in accordance with an observed requirement for actively remodeled actin during ciliogenesis, treatment with cytochalasin D in a quail oviduct model was found to impair basal body migration and inhibited ciliogenesis (Boisvieux-Ulrich et al., 1990).

Small GTPases are switch proteins that can control cell phenotype through actin assembly/remodeling pathways, microtubule organization, and activation of transcription factors required for cell differentiation (Hall, 1998; Van Aelst and Symons, 2002). Our studies using first broad, then specific inhibitors of RhoA supported a role for these small G-proteins to regulate the organization of actin during differentiation of ciliated airway epithelial cells. The role of RhoA in directing apical actin in mammalian airway cells was analogous to that reported in the Drosophila trachea cells (Lee and Kolodziej, 2002; Matuske et al., 2006). Although the Drosophila trachea cells lack cilia (and Foxj1), actin was also found to be required for apical microtubule positioning in these cells (Lee and Kolodziej, 2002). This is reminiscent of defects in β-tubulin-IV organization that we observe in the ciliated cells treated with Rho inhibitors, where tubulin failed to localize properly and the microtubule-dependent ciliary axonemes did not form. Understanding the additional role of RhoB activation by Foxj1 will require further studies; however, RhoB is expressed in the endosomes where it also may have a crucial role in differentiation by regulating EGF receptor trafficking (Wherlock et al., 2004) and coordinating actin polymerization (Sandilands et al., 2004). Rho and actin polymerization inhibitors prevented basal body docking and axoneme formation only when applied at a specific stage of differentiation, however, removal of the drugs resulted in restoration of a program for cilia formation. This indicates that a key set of signals led to actin assembly for ciliary axoneme at a centrally control point, which – we propose – is regulated, in part, by Foxj1.

The function of Foxj1 was specifically related to cytoskeleton organization, and several observations support the possibility that Foxj1 regulates RhoA activation required for actin reorganization. First, there was a marked defect in the formation of the actin net at the apical membrane of cells from Foxj1−/− mice. Second, Foxj1 overexpression could activate RhoA in cultured cells. The overexpression studies were performed in mouse tracheal cells that were prevented from full differentiation (and induction of ciliogenesis) by culture on plastic dishes and submergence in media, suggesting that activation was the result of Foxj1, rather than other factors that may function during ALI-induced differentiation. Third, when we inhibited RhoA by expression of a dominant-negative RhoA prior to Foxj1 expression at ALI day 0, Foxj1 expression was not subsequently extinguished (e.g. at ALI day 2-5). Thus, a biochemical pathway for Foxj1 regulation of RhoA may be indirect via activation of a growth factor that subsequently activates RhoA, direct through transcriptional activation of a GTPase regulatory factor (e.g. a guanine exchange factor, GEF), or by means of a broad program that alters the balance of Foxj1–/– mice. Second, Foxj1 overexpression could activate RhoA in cultured cells. The overexpression studies were performed in mouse tracheal cells that were prevented from full differentiation (and induction of ciliogenesis) by culture on plastic dishes and submergence in media, suggesting that activation was the result of Foxj1, rather than other factors that may function during ALI-induced differentiation. Third, when we inhibited RhoA by expression of a dominant-negative RhoA prior to Foxj1 expression at ALI day 0, Foxj1 expression was not subsequently extinguished (e.g. at ALI day 2-5). Thus, a biochemical pathway for Foxj1 regulation of RhoA may be indirect via activation of a growth factor that subsequently activates RhoA, direct through transcriptional activation of a GTPase regulatory factor (e.g. a guanine exchange factor, GEF), or by means of a broad program that alters the balance of Foxj1–/– mice. Second, Foxj1 overexpression could activate RhoA in cultured cells. The overexpression studies were performed in mouse tracheal cells that were prevented from full differentiation (and induction of ciliogenesis) by culture on plastic dishes and submergence in media, suggesting that activation was the result of Foxj1, rather than other factors that may function during ALI-induced differentiation. Third, when we inhibited RhoA by expression of a dominant-negative RhoA prior to Foxj1 expression at ALI day 0, Foxj1 expression was not subsequently extinguished (e.g. at ALI day 2-5). Thus, a biochemical pathway for Foxj1 regulation of RhoA may be indirect via activation of a growth factor that subsequently activates RhoA, direct through transcriptional activation of a GTPase regulatory factor (e.g. a guanine exchange factor, GEF), or by means of a broad program that alters the balance of
of GEF and GTPase dissociation inhibitors (GDI) during cell differentiation (Etienne-Manneville and Hall, 2002). In this regard, the expression of a GEF protein PSec7 has been identified during ciliation in Paramecium tetraurelia (Nair et al., 1999) and a GEF domain is present in the retinitis pigmentosa GTPase regulator (RPGR) protein that is expressed in the connecting zone of cilium of rods and cones (Linari et al., 1999) as well as in the motile cilia of airway epithelial cells (Hong et al., 2003).

Additional candidates for regulation of the Foxj1-RhoA pathway include planar cell polarity (PCP) genes regulated by the Wnt pathways (Adler, 2002; Montcouquiol et al., 2006). The PCP cascade has also recently been implicated in apical actin organization required for ciliogenesis. In Xenopus laevis, disruption of Drosophila PCP genes Inturned or Fuzzy resulted in decreased apical actin and cilia formation (Park et al., 2006). This phenotype was strikingly similar to that observed in the Foxj1−/− and RhoA inhibited cells. Interestingly, RhoA is a downstream component of the PCP pathway (Adler, 2002). Preliminary analysis suggests that the transcriptional expression of PCP genes in mTEC cultures from Foxj1−/− and WT mice are similar, based on microarray analysis of RNA expression during ALI day 0-7 (S.L.B. and Y.Y., unpublished observation). This may be because apical and basolateral domains in the mTEC culture system are established by ALI day 0 (You et al., 2002), and suggests that Foxj1 could be placed downstream within the Wnt/planar cell polarity pathway. The lack of effect of RhoA inhibition (Fig. 4C) or Foxj1 deficiency (data not shown) on β-catenin localization suggests that apical membrane organization was selectively altered and that perhaps the non-canonical Wnt pathway is involved in motile ciliogenesis, as described in cilia of the inner ear (Montcouquiol et al., 2006; Montcouquiol et al., 2003).

In summary, we have identified a temporal sequence of actin remodeling that occurs in a subpopulation of epithelial cells during differentiation toward a ciliated phenotype. The actin forms a thick apical net that is required for basal body docking and subsequent axoneme production. Comparative studies across several species suggest that this is a conserved process that is highly regulated by multiple signals, and our data indicate that Foxj1 and RhoA are fundamental signals for mammalian ciliogenesis. These findings provide further support for a central role for Foxj1 in ciliated airway cell differentiation and maintenance in the mammal, and now link small GTPase RhoA to that program. Furthermore, these findings may implicate pathologic mediators that interrupt GTPase factors that can perturb ciliated cell repair in infections such as Pseudomonas aeruginosa type III exotoxins (Sun and Barbieri, 2004). Studies that can associate RhoA- and Foxj1-mediated events with proteins in the growing set of cilia-specific genes will further elucidate pathways for ciliogenesis that are important for understanding the newly recognized ciliopathies in humans.

Materials and Methods

Cell culture

HEK293 and NIH3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum. Cultures of mouse tracheal epithelial cells (mTECs) were established on membranes using air-liquid interface (ALI) conditions as described previously (You et al., 2002). Briefly, cells were harvested from tracheas by pronase digestion and epithelial cells selected by differential adherence. To initiate cultures, cells were seeded on supported polyester semipermeable membranes (12-mm diameter, Transwell, Costar-Corning, Corning, NY) and proliferated in growth-factor-containing medium until cells were confluent, and electrical resistance was increased across the cell layer (You et al., 2002). Subsequently, the ALI condition was established for induction of differentiation by aspirating apical chamber medium and changing the medium in the lower compartment to one supplemented with 2% NucSerum (BD Biosciences, San Diego, CA). To culture cells without inducing ALI differentiation, mouse tracheal epithelial cells were cultured on plastic tissue culture plates coated with 50 µg/ml rat tail collagen, submerged in mTEC-Plus medium (You et al., 2002).

Inhibitors and treatment protocols

Cells were treated with cytchalasin D (10 μM, 4 hours, Sigma, St Louis, MO), latrunculin B (1 μM, 4 hours, Calbiochem, San Diego, CA), mevastatin (50 μM, 24 hours, Sigma), toxin B (5 nM, 4 hours, Calbiochem), or botulinum exotoxin C3 (5 μg/ml, 24 hours, Calbiochem) for indicated times by addition of the agent to the basal and apical compartments of the Transwell filters. Exotoxin C3 delivery was facilitated by Lipofectin (Invitrogen, Carlsbad, CA) as described (Kreisberg et al., 1997). In preliminary studies, dose toxicity in differentiated mTEC was determined by titrating each agent and evaluating for maintenance of a confluent cell layer with tight junctions, manifest by preservation of the ALI condition. At least three independent cell preparations were used for each inhibitor experiment.

Immunofluorescence and microscopy

Cells on Transwell membranes were processed for immunodetection as described (Huang et al., 2003; You et al., 2004; You et al., 2002). Primary antibodies and dilutions used were: mouse anti-β-tubulin-IV, 1:250 (BioGenex, San Ramon, CA); rabbit anti-α-tubulin, 1:1000 (Sigma); rabbit anti-SP17, 1:1000 (a gift from M. O’Rand, University of North Carolina, Chapel Hill, NC) (Wen et al., 2001), mouse anti-RhoA (sc-418), 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-Rac1 1:1000 (Chemicon, Temecula, CA); rabbit anti-ezrin, 1:400, (Upstate Biotechnology), rabbit anti-moesin 1:500 (Upstate Biotechnology), rabbit anti-β-catenin, 1: 250 (Calbiochem), and mouse anti-Foxj1, 1: 500 (clone 2A5, produced by immunizing mice with a GST fusion protein containing amino acids 1-117). Antibody binding was detected using secondary antibodies conjugated with Alexa Fluor-555 or Alexa Fluor-488 (Molecular Probes, Carlsbad, CA). Membranes were mounted on slides with medium containing 4', 6-diamidino-2-phenylindole (DAPI) to stain intracellular DNA. Microscopy was performed using a Zeiss LSM 510 META laser scanning confocal instrument (Zeiss, Thornwood, NY) and an Olympus BX51 (Melville, NY) for reflected fluorescent imaging with a charged-coupled device camera interfaced with MagnaFire software (Olympus). Images were composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA). Protein localization was quantified in photomicrographs by overlaying images of different fluorofors within the same field to enumerate cells containing each specific signal as detected by immunostaining. A minimum of three photographs were evaluated for each experiment. The position of centrioles and basal bodies expressing γ-tubulin was recognized to be in the cytoplasm or apical membrane based on prior studies and compared immunostaining with electron micrographs (You et al., 2004).

Electron microscopy

Cells on membranes were prepared for electron microscopy (EM) as previously described (You et al., 2004). Scanning-EM samples were visualized on a Hitachi S-450 microscope (Tokyo, Japan). For transmission EM, samples were visualized on a JEOL 1200EX electron microscope (JEOL., Inc., Peabody, MA).

Protein blot analysis

Cells were lysed in a modified RIPA buffer (1× PBS pH 7.4; 1% IPEGAL CA630; 0.5% sodium deoxycholate; 0.1% sodium dodecylsulfate) containing proteinase inhibitors (complete mini cocktail, Roche, Mannheim, Germany) for immunoblot analysis, which was performed as previously described (Huang et al., 2003). Primary antibodies mouse anti-RhoA (3 μg/ml, Upstate Biotechnology), rabbit anti-β-catenin (1:1000) (Blatt et al., 1999) or mouse anti-β-actin (1:2000, Sigma) were incubated for 1 hour at 25°C. Horseradish-peroxidase-labeled secondary antibody binding was detected by enhanced chemiluminescence (ECL, GE Healthcare UK, Buckinghamshire, UK).

GTPase activation assays

To detect active RhoA (RhoA-GTP) or Rac1 (Rac-GTPase), lysed cells were immediately incubated with agarose-bound rhokin (Upstate Biotechnology) or the p21-binding domain of p21-activated kinase (PAK1) (Chemicon), respectively, according to the manufacturer’s instructions (Upstate Biotechnology). The agarose beads were resuspended in Laemmli sample buffer separated on SDS-PAGE, and analyzed by protein blot using anti-RhoA or Rac1 antibodies. Treatment of mTEC with hepatocyte growth factor (HGF) purified from HeLa cells infected with an HGF adenovirus vector (Gao et al., 1999) (kindly provided by Kathy Ponder, Washington University) was used as a control for Rac1 activation (Wells et al., 2005). An aliquot of lysate retained prior to pull-down assay was subjected to...
immunoblotting to determine total Rho or Rac1. Representative immunoblots were scanned and subjected to densitometry to compare activated with total GTPase activity by using Multi-analyst software (Bio-Rad, Hercules, CA).  

**Adenovirus vector transfection**

Adenovirus-mediated gene transfer in mTEC cultures was performed at ALI day 0 by incubation with adenovirus vector for 4 hours following treatment with sodium caprate (C10) according to a previously described method (Coyne et al., 2000). To overexpress Fojx1, cells were infected with a recombinant adenovirus AdFojx1 or control AdGFP that have been described previously (You et al., 2004). To interrupt RhoA, mTEC cultures were infected with recombinant adenovirus (AdRhoAN19) expressing a dominant-negative RhoA (N19) under control of tetracycline repressor elements. This was co-transfected with an adenovirus (AdTA) that constitutively expressed a tetracycline-repressor–VP16 fusion protein (Neering et al., 1996) that was required to activate expression of RhoAN19 (both vectors kindly provided by Daniel Kalman, Emory University, Atlanta, GA) (Kalman et al., 1999). Cells were continuously treated with or without 2 μg/ml of doxycycline (to inhibit RhoAN19 gene expression). Cells expressing RhoAN19 were identified by immunostaining with an anti-RhoA antibody (sc-418, Santa Cruz Biotechnology) as previously described (Kalman et al., 1999).

**RNA interference**

Three 21-nucleotide ezrin-specific siRNA sequences (Ez-siRNA-1, 2, 3) were designed using an open-source protocol (www.ambion.com/techlib/basics; Ambion, Austin, TX) as the following oligonucleotide sequences were synthesized (Invitrogen) as templates used for siRNA construction: Ezrin-siRNA-1, sense, 5′-AATCCGAGAGAGAGAGGGGGGTCTGTC-3′; ezrin-siRNA-1, antisense, 5′-GCACTCTTCTCTCTCAGCGTCTGTC-3′; ezrin-siRNA-2, sense, 5′-AAAGATTCTACCTTGCGGACCTGTC-3′; ezrin-siRNA-2, antisense, 5′-AACGACCTGTCCTGATGCTGCCCTGTCTC-3′. An adenovirus vector transfection activity by using Multi-analyst software (Bio-Rad, Hercules, CA).

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**Statistical analysis**

Means of cell numbers were subjected to student’s t test for modulation of tight junctions with sodium caprate. Am. J. Respir. Cell Mol. Biol. 23. 602-689.

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