Chapter 4
Apical Cytoskeletons Help Define the Barrier Functions of Epithelial Cell Sheets in Biological Systems

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4.1 Introduction

Epithelial cells adhere to each other by tight junctions (TJs) to form cell sheets, which is a critical step in epithelial barrier creation and the morphogenesis of vertebrate tissues (Fleming et al. 2000; Tsukita et al. 2001; Anderson et al. 2004; Furuse and Moriwaki 2009; Van Itallie and Anderson 2014; Tanaka et al. 2017). The apical surface of an epithelial cell sheet faces the outer environment, such as the lumen in the intestinal tract or the environment outside the skin surface. In the sheet, the cells' apical membranes are regarded as a continuous, connected surface, in which the cell-cell adhesion sites are cemented by TJs. Notably, each epithelial cell exhibits basolateral polarity, and therefore the apical surface of an epithelial cell sheet differs from the basolateral one, and possesses specific features that relate to its roles in a particular biological functional system (Nelson 2009; Apodaca 2017). Apical differentiation is a popular topic of study, and includes microvilli, cilia, circumferential rings (Nelson 2009; Apodaca 2017), and ratchet structures, which have been described in *Drosophila* but not yet in vertebrates (Martin et al. 2009).

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Consistent with the critical and varied roles of the apical surface, we recently identi-
fied a 3-layered cytoskeletal network of actin filaments, intermediate filaments, and
microtubules that exists just below the apical membrane of epithelial cell sheets.
The apical cytoskeletons are presumably organized under the control of the TJs and
regulate epithelial morphogenesis and barrier functions in conjunction with TJ for-
mation and TJ-based cell signaling. Thus, we propose to define this set of structures
as a system called the "TJ-apical complex" (Yano et al. 2017).

A natural question is why this apical cytoskeletal system was not discovered
until recently. It is a very thin-layered structure mainly composed of actin filaments,
intermediate filaments, and microtubules, which appear as a continuous structure
extending horizontally just beneath the apical plasma membrane. We discovered
this network using an advanced imaging system, including ultrahigh voltage elec-
tron microscopic tomography and confocal super-resolution microscopy, that we
developed (Kunimoto et al. 2012; Yano et al. 2013; Tateishi et al. 2017). Although
the molecular mechanisms and physiological roles of the "TJ-apical complex"
remain to be elucidated, we recently identified four TJMAPs (TJ Microtubule-
associated Proteins; previously called J-MAPs), including cingulin which are
TJ-associated proteins that bind to the apical cytoskeletons (Yano et al. 2013).
During the morphogenesis of epithelial cell sheets, apical constriction and the size
of the apical area defined by TJs must be kept in balance. Our recent data suggest
that TJMAPs may constitute a platform on which the apical cytoskeletons and TJs
associate to organize biological systems, although more study is needed to examine
this possibility (Fig. 4.1).

Regarding the functions of the "TJ-apical complex," multiciliated cells represent
a highly specific case in which the apical microtubules are particularly highly devel-
oped. In tracheal multiciliated cells (MCCs), the apical microtubules have a specific
role in establishing the regular arrangement of basal bodies (BBs), which generate
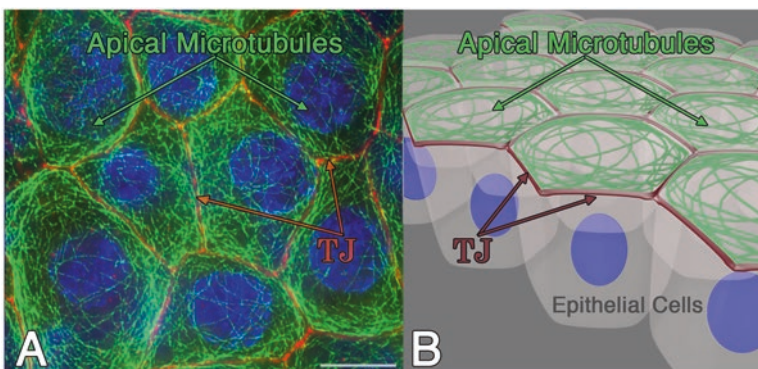cilia in the apical membranes (Kunimoto et al. 2012; Herawati et al. 2016; Tateishi

Fig. 4.1 Immunofluorescence image (**a**) and schematic drawing (**b**) of the apical microtubule
network existing beneath the apical membrane of epithelial cells. Microtubules (α-tubulin-
staining), green; Tight junctions (ZO1-staining) red; Nuclei (DAPI-staining), blue. Scale, 10 μm.
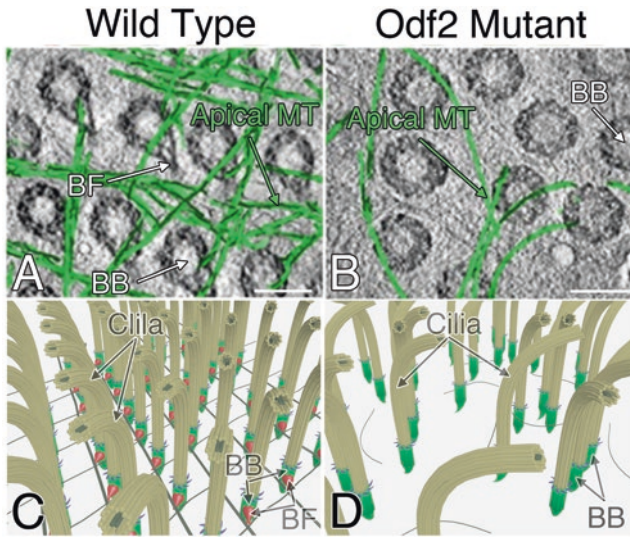TJ, tight junction. Bar, 10 μm

Fig. 4.2 Ultra high-voltage electron-microscopy images (**a**, **b**) of basal bodies and schematic drawing (**c**, **d**) of multi-ciliated epithelial cells. The apical microtubules (green) between basal bodies (BBs) in the wild-type mice and Odf2-mutant (Kunimoto et al. 2012). In the Odf2 mutant mice, the BB (basal body) alignment was perturbed by the loss of BFs (basal feet: the accessory structure of BBs) and of the regular apical microtubule network. Bar, 100 nm

et al. 2017). To explore the differentiation mechanism leading to the regular BB alignment, we examined tracheal MCCs expressing GFP-centrin, a BB-associated protein, using our new long-term, high-resolution, live-imaging system. The microtubule-dependent regular arrangement of BBs is critical for the synchronous beating and metachronal waves of hundreds of motile cilia on the apical membrane of MCCs (Guirao and Joanny 2007; Elgeti and Gompper 2013). Thus, the physiological role of the apical cytoskeleton that we revealed in this case is likely to be essential for tissue function (Fig. 4.2).

By comparing the findings for various epithelial cells, both the common and unique characteristics of the apical cytoskeletal structures are being revealed. In the following sections, we discuss the roles of the apical cytoskeletons, especially microtubules, in epithelial cell sheets that have specific functional relevance (Fig. 4.3).

4.2   The Apical Cytoskeletons in General Epithelial Cells

In confluent epithelial cell sheets, each cell is highly polarized in the apico-basal direction, and the cells' apical membranes are regarded as a continuous surface connected by TJs. Although the mechanism by which TJs are positioned at the most apical part of the lateral membrane is not understood, the TJs determine the edges
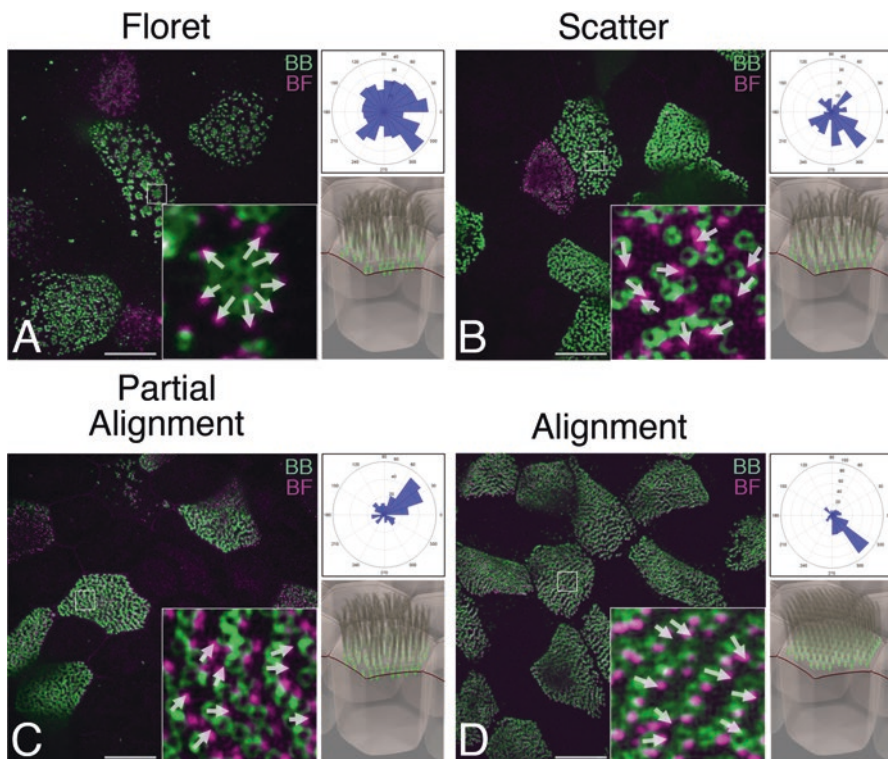
Fig. 4.3 Immunofluorescence images, circular diagrams, and schematic drawing of basal bodies (BBs) and basal feet (BF) during BB pattern development of Floret (**a**), Scatter (**b**), Partial Alignment (**c**) and Alignment (**d**). Orientation of each BB is shown by an arrow connecting the center of a BB marker (green) to the center of a BF marker (red) (insets) and by the circular diagrams of ciliary orientation (direction of white arrows in the immunofluorescence image). (Bottom) Illustration of cilia. Bar, 10 μm

of each cell's apical membrane when the cell sheet is viewed from the top (the apical view). Distinct, specific differentiation mechanisms occur in the apical area. Well-known classical examples of apical differentiation are the circumferential ring at cell-cell boundaries and the terminal web (Leblond et al. 1960; Hull and Staehelin 1979; Owaribe et al. 1981; Burgess 1982; Keller et al. 1985). In *Drosophila* epithelial cells, a "ratchet structure" consisting of actin filaments exists in the medial area of apical membranes (Martin et al. 2009), although no similar structure has been clearly identified in mammalian epithelial cells. By applying our super-resolution microscopy system to cultured epithelial cell sheets, we discovered the detailed structure of the apical cytoskeleton, which was previously unknown and uniquely distributed beneath the apical membrane like a shell (Yano et al. 2013). This location led us to propose that the apical cytoskeletal network is associated with TJs, which are located at the most apical part of the lateral membrane, and that these

structures form a system called the "TJ-apical complex." However, to establish this new point of view, we needed to acquire evidence at the molecular level for the association of the apical cytoskeleton with the TJs.

We further examined the apical cytoskeleton in detail, by performing ultra-high voltage electron microscopy experiments in which the microtubules and intermediate filaments were tracked. In general epithelial cells in culture, the apical microtubules and intermediate filaments were distributed in the apical plane in rather uniformly scattered patterns and partly overlapped each other, without any specific patterns in their distribution (Tateishi et al. 2017). In this respect, it is notable that we identified four microtubule-associated proteins, which also associate with TJs, in our recent findings, TJ-associated microtubule-binding proteins. We believe that analyses of the TJ-associated microtubule-binding proteins, which may form the platform for associations among the apical cytoskeletons, TJs, and cell signaling molecules, represent a unique direction for studying epithelial cell sheets and epithelial barriers.

4.3   The Apical Cytoskeletons in Multiciliated Cells, a Possible Extreme Example of a "TJ-Apical Complex" with a Clear Function

MCCs drive fluid transport through coordinated ciliary beating, the direction of which is established by the BB orientation of hundreds of cilia on one cell (Salathe 2007). In airway MCCs, the BBs are uniformly oriented and linearly aligned by an unknown mechanism. To explore the mechanism for BB alignment, we observed GFP-centrin2-labeled BBs in mouse tracheal MCCs in primary culture using our long-term, high-resolution, live-imaging method (Herawati et al. 2016). We found that the differentiating BB arrays sequentially adopted four stereotyped patterns: a clustering "Floret," "Scatter," "Partial alignment," and linear "Alignment" pattern. During this acquisition of regularity, we particularly noted that the patterns and densities of microtubules in the apical plane of the MCCs were well correlated with the BB patterning. In addition, the BB alignment was perturbed by disrupting the apical microtubules with nocodazole or by a basal foot (BF)-depleting Odf2 mutation. Based on these experimental results, we explored the development of BB alignment from random to the final well-ordered pattern biotheoretically. We found that the self-organization could be explained by applying hydrodynamic theories in which the apical cytoskeletons were treated as a two-dimensional viscous fluid that underwent a contractile force mediated by cytoskeletal motors and filament polymerization (Marchetti et al. 2013; Prost et al. 2015; Herawati et al. 2016). These results revealed the functional importance of the cytoskeletal components that exist in the apical plane of the epithelial cell sheet in tracheal MCCs. Although the relationship between the apical cytoskeletons and TJs remains to be elucidated, their

locations and binding molecules suggest that they are closely related both physically and functionally in MCCs. How the apical cytoskeleton is built by the TJ and its related signaling is another critical issue that remains to be explored.

4.4 Perspective

Epithelial cell sheets with a differentiated apical side are formed and organized by mechanisms involving apicobasal polarity, the details of which have been well addressed in other reviews (Shin et al. 2006; Nelson 2009; Rodriguez-Boulan and Macara 2014; Apodaca 2017). In general, to establish the apicobasal polarization in epithelial cell sheets, polarity proteins generate asymmetric membrane domains that form the basis for establishing the cell–cell adhesive TJs and adherens junctions (AJs), which combine to form apical junctional complexes (AJCs). In addition, planar cell polarity (PCP), which forms in the apical plane of epithelial cell sheets, is arranged perpendicular to the basolateral polarity. The actin filaments of the circumferential ring also lie horizontally along the apical membrane at cell boundaries. The "TJ-apical complex" expands horizontally below the apical membrane in an almost evenly scattered pattern. Since it includes TJs, it is likely to play a role in the paracellular barrier. On the other hand, since it is directly or indirectly associated with the apical membranes of epithelial cells, it probably also has a role in the transcellular barrier. Thus, the epithelial barrier is created and regulated by the combination of paracellular and transcellular barriers, which are determined, at least in part, by the "TJ-apical complex." Our continued investigation of the "TJ-apical complex" in ciliated and non-ciliated epithelial cells is expected to unveil its physiological significance in a variety of biological epithelial barrier systems.

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