Accidental encounters: the chance to solve a mystery

Masatoshi Takeichi

Cells organize into tissues by adhering to one another. Such intercellular associations can be disrupted artificially and, under the right culture conditions, the dissociated cells can re-aggregate and reconstitute their original tissue-like structures, as demonstrated by early pioneering studies. When I entered graduate school, the molecular mechanisms governing these striking cellular behaviours, including the formation of the initial cell–cell contacts, were largely unresolved.

At the beginning of my career, I was interested in lens cell differentiation. Lens epithelial cells differentiate into lens fibres, a process that was thought to depend on unidentified factors released from the retina. I set out to characterize these putative factors by culturing retinal cells and collecting the culture medium 'conditioned' by these cells, thinking that it might contain the factors I sought. But when I grew lens cells in this conditioned medium, nothing seemed to happen. After much fruitless effort, I finally noted a difference; lens cells suspended in the conditioned medium attached to the culture dish more slowly than lens cells in the control medium. This unexpected effect had nothing to do with lens differentiation, but attracted my interest nonetheless, for I felt it should be somehow possible to analyse the underlying mechanisms. But the tools needed to take a mechanistic approach to problems in cell differentiation had yet to be developed, and I eventually gave up on the lens.

I was in Tokindo Okada's laboratory at Kyoto University at this time. Although his main interest was in cell differentiation, he encouraged students to learn about morphogenesis as well. He inspired his students to gain broad insights into developmental mechanisms, and provided us with a learning environment that, I believe, was critical in developing my interest in topics such as cell adhesion, which were not widely popular among developmental biologists.

I continued studying cell adhesion and subsequently found that the mechanisms of cell–cell and cell–substrate adhesion require different divalent cations (Ca^{2+} and Mg^{2+}, respectively) and through this work I became convinced that cells must have multiple adhesion mechanisms. But as the necessary techniques remained unavailable, I still could not test this idea at the molecular level. Around this time, I went to do a postdoctoral fellowship in Richard Pagano's laboratory at the Carnegie Institution of Washington, Department of Embryology, and began to explore the mechanisms behind liposome–cell membrane interactions. Soon after the move, however, I noticed something strange. I generally used trypsin to dissociate cells, which would normally re-aggregate when cultured in suspension. But when I used the Carnegie recipe do the same thing, the trypsinized cells never re-aggregated. This surprised and interested me, and I set out to solve the mystery. It turned out that the Carnegie trypsin solution contained EDTA to remove divalent cations, whereas my usual solution did not. I confirmed that the presence or absence of Ca^{2+} in the trypsin solution was the key to the difference I had observed. This led me to hypothesize that cells have an adhesion mechanism that can be disrupted with trypsin, and that Ca^{2+} confers a protective effect against trypsin digestion. Since the re-aggregation of the cells equipped with this hypothetical mechanism also required Ca^{2+}, I called it the Ca^{2+}-dependent adhesion system. I had a strong feeling that this mechanism must be crucial for cell–cell adhesion in animal cells, as it had previously been suggested that Ca^{2+} is indispensable for the maintenance of animal tissues, and so I decided to follow up on this finding.

Identifying the molecular mechanism underlying Ca^{2+}-dependent adhesion, however, was not an easy task. One promising approach was the immunological one, which was introduced by Günther Gerisch's group to identify adhesion molecules of the cellular slime moulds. The idea behind this approach was that if I could raise antibodies that are able to block cell–cell adhesion, it would enable me to identify antibody targets, which would presumably be adhesion molecules. I tried injecting rabbits with cells, which I had used in the experiments at Carnegie, but these never led to the production of the blocking antibodies I was after. One day, however, I came across a paper by Rolf Kemler and colleagues reporting that antibodies raised against teratocarcinoma cells blocked the compaction of early mouse embryos. Given the morphological similarity between embryonic compaction and Ca^{2+}-dependent cell aggregation, I suspected the underlying mechanisms might be related as well. Indeed, when I switched to teratocarcinoma lines, I was finally able to obtain blocking antibodies. It was these antibodies that led us to identify the first of a large family of molecules now known as cadherins.

Looking back on my early research, it is clear that the struggle to account for some unexpected finding or other has often brought me to a turning point. As scientists, we need to keep ourselves attuned to the uncommon and to avoid blinkering ourselves with dogma. Admittedly, these days I tend to propose rationally designed experiments to my postdocs and students, but I always strive not to overlook any unexpected results from their experiments, and to emphasize to them the importance of this attitude for the advancement of science.

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CHAPTER TWO

Remodeling of the Adherens Junctions During Morphogenesis

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Contents

Abstract

Morphogenesis of epithelial tissues involves various forms of reshaping of cell layers, such as invagination or bending, convergent extension, and epithelial–mesenchymal transition. At the cellular level, these processes include changes in the shape, position, and assembly pattern of cells. During such morphogenetic processes, epithelial sheets in general maintain their multicellular architecture, implying that they must engage the mechanisms to change the spatial relationship with their neighbors without disrupting the junctions. A major junctional structure in epithelial tissues is the "adherens junction," which is

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composed of cadherin adhesion receptors and associated proteins including F-actin. The adherens junctions are required for the firm associations between cells, as disruption of them causes disorganization of the epithelial architecture. The adherens junctions, however, appear to be a dynamic entity, allowing the rearrangement of cells within cell sheets. This dynamic nature of the adherens junctions seems to be supported by various mechanisms, such as the interactions of cadherins with actin cytoskeleton, endocytosis and recycling of cadherins, and the cooperation of cadherins with other adhesion receptors. In this chapter, we provide an overview of these mechanisms analyzed *in vitro* and *in vivo*.

1. Introduction

Epithelial cell layers dynamically change their morphology via a number of processes, such as folding, invagination, and convergent extension, during development. Epithelial remodeling continues into adulthood, occurring on various occasions such as during cell renewal, tissue regeneration, and wound healing. Cancer growth and invasion also involve epithelial remodeling. Since an important structural function of the epithelia is to cover and seal the tissues or organs, they need to maintain persistently their cell–cell junctions during the remodeling processes. Accordingly, the epithelia must have elaborate mechanisms to coordinate the stability and yet flexibility of their junctions.

The cell–cell junctions in the simple epithelia typically comprise the tight and adherens junctions and the desmosome. These three junctional components are clustered together at the apical-most portion of the lateral cell–cell contacts, forming the apical ''junctional complex'' (Farquhar and Palade, 1963), although the adherens junctions (AJs) and desmosomes are also distributed throughout the cell–cell contacts. The AJ located at the junctional complex is specifically termed the ''zonula adherens'' or ''adhesion belt,'' which encloses the cells at a site near their apical surface, along with the circumferential actin belt.

The major adhesion receptors constituting the AJ are the classic cadherins, which are Ca^{2+}-dependent, homophilic cell–cell adhesion molecules (Fig. 2.1). They are a group of single-pass transmembrane proteins, consisting of about 20 subtypes. The classic cadherins are conserved among vertebrates and invertebrates, although the size of their extracellular domain varies between the species (Takeichi, 2007). Their cytoplasmic domain binds p120-catenin and β-catenin (or plakoglobin) at its N-terminal and C-terminal side, respectively; and β-catenin/plakoglobin, in turn, binds α-catenin (Gumbiner, 2005). Many other proteins are also associated with the cadherin–catenin complex. In addition to the classic cadherins, there are a large number of

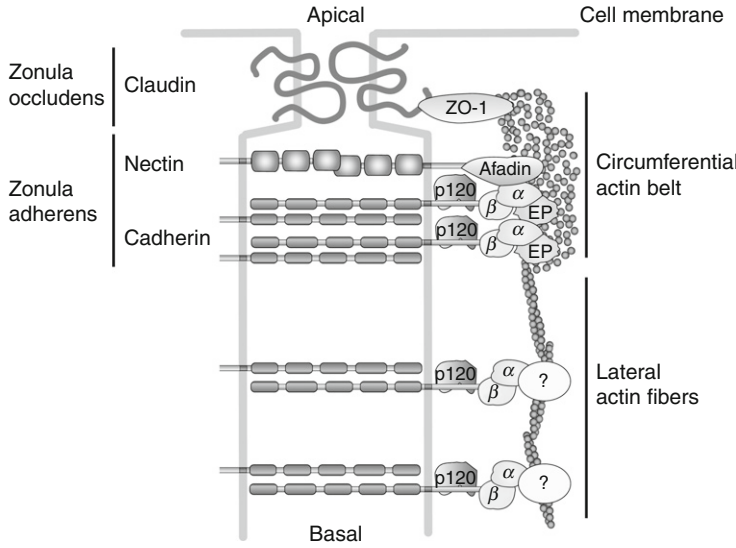

Figure 2.1 Basic molecular elements organizing the adherens junction in simple epithelial cells of the vertebrates. Tight junction (zonula occludens) is also depicted. The adherens junction adjacent to the tight junction is called zonula adherens, which is specialized by the association with the circumferential actin belt. The cadherin–catenin complexes themselves are in general distributed throughout the cell–cell contacts in vertebrate cells. Vertical section of the junctions is shown. EP, EPLIN; p120, p120-catenin; α, α-catenin; β, β-catenin; ?, unknown molecules.

nonclassic cadherins, constituting the cadherin superfamily (Takeichi, 2007). As far as studied, the nonclassic cadherins seem not to be components of the adherens junction. Many of them, however, interact homophilically as the classic cadherins do, thereby being concentrated at cell–cell contacts; and they often affect the classic cadherin-based AJs. Besides the cadherins, nectins, a family of immunoglobulin-domain membrane proteins, are also localized in the AJs (Takai and Nakanishi, 2003).

The classic cadherin-based AJs play a major role in the physical association of epithelial cells in both vertebrates and invertebrates, and therefore the regulation of them is assumed to be critical for epithelial sheet remodeling. There appear to be multiple ways to reshape the AJs. For example, in many morphogenetic processes, epithelial cells undergo shape changes or movement, not disrupting the zonula adherens (ZA), the most organized part of the AJ. In this form of remodeling, the regulation of the contractility of the ZA seems to be important. The other ways of remodeling include the downregulation of cadherins within a range that can keep the cohesion of cells, but yet allow their rearrangement within the mass. (Cells forming solid tissues always express some types of classic cadherins, even in loose tissues such as mesenchymal ones.) These mechanisms enable an epithelial layer to

rearrange its cellular elements for assuming new architecture. In this chapter, we discuss how these forms of junctional remodeling are molecularly controlled.

2. Basic Machinery of the Adherens Junction

The adhesive function of cadherins requires cytoplasmic partners, that is, the catenins. Among the catenins, α-catenin is essential for the firm associations of epithelial cells, as it is well known that α-catenin-deficient cells cannot organize the typical epithelial junctions (Hirano *et al.*, 1992; Vasioukhin *et al.*, 2001; Watabe-Uchida *et al.*, 1998). This is also the case even for neuronal contacts; the synaptic contacts in α-catenin-deficient hippocampal neurons are unstable and turn over more rapidly than those in the wild-type ones (Abe *et al.*, 2004). Thus, given that the actions of α-catenin may be regulated physiologically, this catenin might join the processes for junctional remodeling (Takeichi and Abe, 2005).

Early studies showed that α-catenin could directly bind F-actin (Rimm *et al.*, 1995). It has, thus, long been thought that a role of α-catenin is to tether the cadherin–β-catenin complex to F-actin. However, recent studies have challenged this idea, by demonstrating that the cadherin–β-catenin–α-catenin complex cannot associate with F-actin *in vitro* but that only free α-catenins can do so (Drees *et al.*, 2005; Yamada *et al.*, 2005). Despite these findings, it is widely accepted that normally functioning cadherins always colocalize with actin fibers, bringing up the question of how these *in vitro* and *in vivo* observations can be reconciled. One answer has been given by the finding of a molecule that mediates the linkage between the cadherin–β-catenin–α-catenin complex and F-actin (Abe and Takeichi, 2008). This is EPLIN, which is known to be an actin-binding and -stabilizing protein (Maul *et al.*, 2003). EPLIN can also bind α-catenin associated with the cadherin–β-catenin complex; and through this interaction with α-catenin, EPLIN serves to link this complex to the actin fibers. Depletion experiments have indicated that EPLIN is required not only for the linkage between cadherin and F-actin but also for maintaining the circumferential actin belt. These studies also suggested that there would be another mechanism(s) to link cadherin and F-actin, because these two molecules still colocalized to each other in the absence of EPLIN, although their association patterns were dramatically altered (Fig. 2.2).

A recent work on *Drosophila* E-cadherin has strengthened the concept that α-catenin functions to tether the cadherin to actin fibers (Cavey *et al.*, 2008). Only in the presence of α-catenin are homotypic cadherin clusters mobile along the cell junctions. Another work demonstrated that α-catenin is required for cadherin molecules to move together with the actin filaments

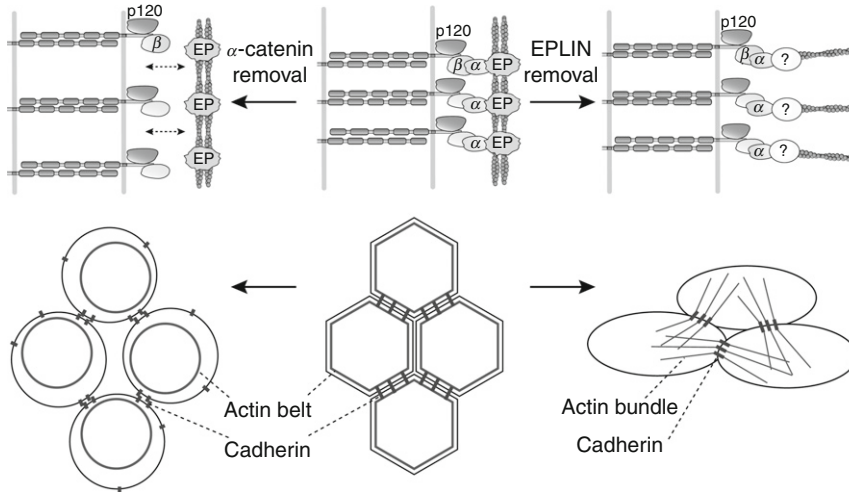

Figure 2.2 Models to explain the roles of α-catenin (α) and EPLIN (EP) in cadherin–actin interactions. Horizontal sections of the junction at the zonula adherens level are shown. See Abe and Takeichi (2008) and Watabe-Uchida *et al.* (1998) for details of EPLIN and α-catenin functions, respectively.

undergoing a retrograde flow, again supporting the idea of its tethering role (Kametani and Takeichi, 2007). The detailed mechanisms of how the α-catenin tethered to actin fibers functions to maintain the AJs still remain unclear. The actin cytoskeleton is important for the shaping, contraction, and movement of cells, while the AJs are essential for the zippering of them. The structural and functional association between these two molecular systems must be a critical way to control the complex behavior of cells forming a sheet.

It should additionally be noted that, in cells treated with actin-depolymerizing reagents, the junctional cadherins still associate with residual actin clusters (Cavey *et al.*, 2008), and this association of cadherin and actin seems to be α-catenin independent. Actually, the AJs also have other actin-binding proteins such as afadin (see below). The entire story on the interaction between the AJ and actin systems is thus still incomplete.

3. Remodeling by Small GTPase

Rho-family small GTPases, as well as their regulators such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), are known to be crucial in establishing and maintaining the AJs (Braga and Yap, 2005; Fukata and Kaibuchi, 2001), suggesting that they

may also participate in junctional remodeling. A recent addition to the list of this class of AJ regulators is a Cdc42-specific GEF known as Tuba (Otani *et al.*, 2006). Tuba is localized along the apical-most region of the cell junctions interacting with ZO-1; and at this site, Tuba activates Cdc42 and in turn regulates N-WASP, a Cdc42 effector. Depletion of Tuba and N-WASP equally disrupts the network-like organization of E-cadherin and F-actin distributed at the lateral cell–cell contacts, but not at the ZA. Concomitantly, the loss of Tuba causes the junctions to have less tension. Thus, the Tuba–Cdc42–N-WASP pathway seems to regulate the junctional tension via the regulation of actin polymerization at the lateral cell–cell contacts (Fig. 2.3). An interesting point in these observations is that Tuba is required for the lateral portions of the junction, but not for the apical-most ZA, despite the localization of Tuba in the close vicinity of the ZA. The ZA may function to regulate the other portions of the junction.

Another small GTPase, Rap1, first identified as a repressor for cell transformation by Ras, also has attracted attention as a regulator for cell junction formation (Bos, 2005; Kooistra *et al.*, 2007). In *Drosophila*, Rap1 is enriched at the AJs. In Rap1-depleted *Drosophila* cells, the AJs become condensed to one side of the cells; and the cohesion between cells is lost (Knox and Brown, 2002). C3G, a Rap1 GEF, interacts with the cytoplasmic region of E-cadherin, and then activates Rap1 (Hogan *et al.*, 2004). E-cadherin-mediated adhesion is required for the Rap1 activation; and, conversely, Rap1 activity is necessary for the localization of E-cadherin at cell–cell contacts. Rap1 rescues the Ras-transformed or HGF-induced

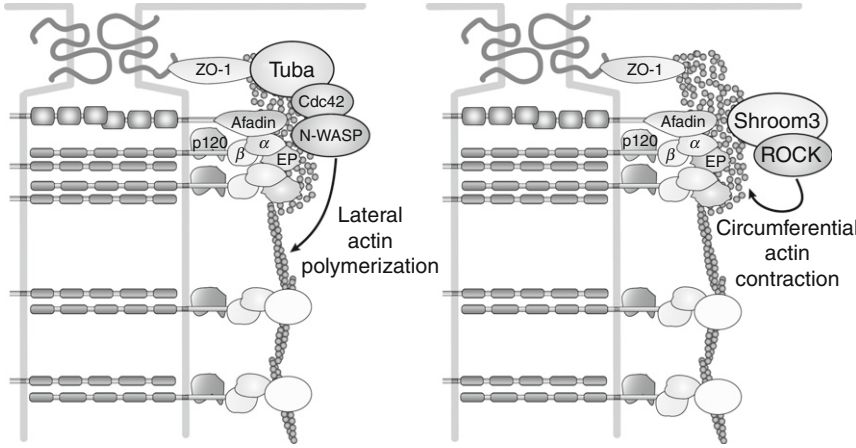

Figure 2.3 Models to explain the roles of Tuba and Shroom3 in junctional remodeling. Tuba regulates the polymerization of lateral actin fibers (Otani *et al.*, 2006), whereas Shroom3 regulates the contraction of circumferential actin belts (Nishimura and Takeichi, 2008). Vertical sections are shown.

downregulation of E-cadherin, and the effect is enhanced by the activation of another Rap GEF, Epac1 (Price *et al.*, 2004). The potential role of Rap1 in more dynamic morphogenetic systems, however, remains to be investigated.

4. Remodeling by Cadherin Turnover and Endocytosis

One of the mechanisms to modulate the AJs would be to remove/add cadherin molecules from/to the junctional sites. Several mechanisms for cadherin trafficking have been investigated and elucidated, as outlined below.

4.1. Basic processes of cadherin recycling

Cell-surface cadherins seem to be actively turned over, which would affect the adhesion activity between cells and facilitate junctional remodeling (Bryant and Stow, 2004; Yap *et al.*, 2007). Newly synthesized E-cadherin is transferred from the Golgi to specific sites on the plasma membrane with the Sec6/8 exocyst complex (Yeaman *et al.*, 2004). Once having attained the cell surface, on the other hand, E-cadherin is internalized by clathrin-mediated endocytosis, especially when cells are not in stable contacts (Le *et al.*, 1999). Internalized E-cadherin is transported to recycling endosomes colocalizing with Rab11, and then is recycled back to the cell surface with the exocyst (Langevin *et al.*, 2005) or trafficked to late endosomes and lysosomes for degradation (Palacios *et al.*, 2005). This E-cadherin trafficking from recycling endosomes to the cell adhesion sites appears to depend on the interaction of Rab11 and β-catenin with the exocyst components Sec15 and Sec10, respectively (Langevin *et al.*, 2005). The homophilic interaction of E-cadherin molecules makes them resistant to endocytosis, via the activation of Cdc42 and Rac, and then IQGAP, which enhances F-actin crosslinking (Izumi *et al.*, 2004).

4.2. p120-catenin-dependent cadherin stabilization

p120-catenin (p120), which binds the juxtamembrane region of the E-cadherin tail, plays a critical role in cadherin turnover (Xiao *et al.*, 2007). A pioneering study (Ireton *et al.*, 2002) demonstrated that exogenous expression of p120 in SW40 carcinoma cells upregulated the surface level of E-cadherin, leading them to acquire a typical epithelial configuration, which cells were otherwise loosely associated. This finding suggested that p120 is required for the stabilizing of cadherins on the cell surface, and this idea was confirmed by the experiments to deplete p120 by siRNA, which

caused elimination of cadherins from the junctions (Davis *et al.*, 2003; Xiao *et al.*, 2003). *In vivo* depletion of p120 in the salivary gland also causes E-cadherin deficiency as well as severe defects in epithelial cell adhesion and polarity (Davis and Reynolds, 2006). These effects of p120 expression on cadherin turnover are dependent on its binding to cadherins via the armadillo domain (Liu *et al.*, 2007; Xiao *et al.*, 2005). A dileucine motif on the juxtamembrane domain of E-cadherin (Miranda *et al.*, 2001) is required for its clathrin-mediated endocytosis (Miyashita and Ozawa, 2007a,b), and this motif is masked by p120 binding, explaining the mechanism of p120-dependent stabilization of E-cadherin.

4.3. MicroRNA-dependent turnover

MicroRNAs (miRNAs) are small, noncoding RNAs that modulate gene expression of target molecules. Recent screening of miRNAs identified miR-200, a family of microRNAs, whose expression was inversely correlated with E-cadherin downregulation or the epithelial-to-mesenchymal transition (EMT). Ectopic expression of miR-200 in cancer cell lines causes upregulation of E-cadherin; and conversely, inhibition of miR-200 reduces E-cadherin expression in epithelial cells, enhancing their migration (Gregory *et al.*, 2008; Park *et al.*, 2008). This action of miR-200 is elicited via suppression of the expression of E-cadherin repressors, ZEB1 (δEF1) and ZEB2 (SIP2), which are thought to be involved in EMT. In addition, a significant correlation between E-cadherin and miR-200 expression was found in primary human cancer specimens, confirming the association of miR-200 with the E-cadherin status in *in vivo* situations.

4.4. Other mechanisms

E-cadherin internalization is enhanced by tyrosine kinase activation via various pathways, such as those mediated by the HGF receptor c-Met, EGF receptor, and Src, causing the EMT featured by cell scattering and fibroblast-like morphology. Upon HGF treatment or v-Src activation, two tyrosine residues within the juxtamembrane domain of E-cadherin are phosphorylated, and then Hakai, an E3 ubiquitin ligase, ubiquitinates E-cadherin (Fujita *et al.*, 2002). Ubiquitinated E-cadherin binds to the HGF-regulated tyrosine kinase substrate and is shuttled to lysosomes in a Src-activated Rab5- and Rab7-dependent manner (Palacios *et al.*, 2005). In addition, the signaling mechanisms of HGF-induced Rab5 activation have been revealed (Kimura *et al.*, 2006): HGF treatment activates Ras, which in turn activates RIN2, a Rab5-GEF localizing at cell–cell junctions. This process leads to Rab5 activation and E-cadherin endocytosis.

5. Remodeling by Nonclassic Cadherins and Nectins

Many of nonclassic cadherins also show the activity of homophilic binding, and thereby are concentrated at cell–cell contacts. Although some of these molecules function to sustain cell–cell adhesion, others seem to serve as a modulator of the classic cadherins, or even as an inhibitor of cell adhesion, rather than as adhesion receptors. Nectins, a subfamily of the immunoglobulin superfamily, is localized in the AJs, and cooperate with the classic cadherin in junctional remodeling. Examples of the action of these molecules are outlined below.

5.1. Protocadherins

Protocadherin represents a subfamily of the cadherin superfamily, whose cytoplasmic domain is not identical to that of the classic cadherin (Redies *et al.*, 2005; Vanhalst *et al.*, 2005), thus suggesting their distinct functions. One of them, paraxial protocadherin (PAPC, protocadherin-8), has been shown to be important for gastrulation. In the developing *Xenopus* embryo, this protocadherin is expressed first in Spemann's organizer and then in the paraxial mesoderm. The expression of a dominant-negative form of PAPC or its depletion by morpholino oligos inhibits the convergent extension movement of the mesoderm (Kim *et al.*, 1998; Medina *et al.*, 2004; Unterseher *et al.*, 2004). The phenotypes observed here are similar to those for cells defective in planar cell polarity (PCP), which is regulated by the Wnt/Frizzled 7 signaling pathway. Indeed, PAPC was shown to activate RhoA and c-Jun N-terminal kinase (JNK) (Medina *et al.*, 2004; Unterseher *et al.*, 2004), which are the effectors of the Wnt/Frizzled 7 signals. Interaction between PAPC and Frizzled 7 at their extracellular domain was also observed, although these two molecules appeared to regulate the PCP signals separately. A recent report shows that ankyrin repeats domain protein 5 (ANR5) interacts with PAPC (Chung *et al.*, 2007). Depletion of ANR5 causes defects in the elongation of activin-treated animal caps and tissue separation, critical for gastrulation movement, and also inhibits PAPC-induced activation of RhoA and JNK, suggesting that ANR5 is a functional partner for PAPC. PAPC was also shown to bind Sprouty (Wang *et al.*, 2008), a receptor tyrosine kinase inhibitor protein, which has the ability to inhibit the convergent extension movement (Sivak *et al.*, 2005). It seems that PAPC promotes gastrulation by sequestering and inactivating Sprouty.

One of the biological functions of PAPC seems to be downregulation of C-cadherin activity. Overexpression of PAPC decreases the adhesive

activity of C-cadherin without changing its expression level (Chen and Gumbiner, 2006). Activin treatments induced PAPC expression, and simultaneously decreased C-cadherin activity during elongation of the animal cap. Depletion of PAPC interferes with this activin-induced downregulation of C-cadherin activity, inhibiting animal cap elongation. To elucidate how this activity of PAPC is linked with its role in PCP signaling is an intriguing future subject. It is noteworthy that arcadlin, the mammalian homolog of PAPC, also downregulates another classic cadherin, N-cadherin (Yasuda *et al.*, 2007). N-cadherin, which is localized at synaptic junctions in neurons, is endocytosed in an activity-dependent manner. The expression of arcadlin is upregulated by excitatory stimuli of hippocampal neurons, and this promotes N-cadherin internalization. During this process, arcadlin interacts with the cytoplasmic region of N-cadherin, and also interacts with TAO2β, a MAPKKK. Homophilic interaction of arcadlin molecules activates the TAO2β–MEK3 MAPKK–p38 MAPK pathway, which in turn phosphorylates the arcadlin and then accelerates the internalization of N-cadherin. These observations suggest that downregulation of classic cadherins might be a conserved function of PAPC/arcadlin.

OL-protocadherin (OL-pc, protocadherin-10) has also been shown to interfere with the classic cadherin function. In the knockout mice for OL-pc, the growth cones of striatal neurons do not normally migrate, as they lump together (Uemura *et al.*, 2007). OL-pc interacts with the Nap1–WAVE complex (Nakao *et al.*, 2008), a regulator of actin assembly, which functions downstream of Rac signaling. Although the Nap–WAVE complex is generally localized at the lamellipodia to sustain cell migration, this complex becomes redistributed to cell–cell contacts when OL-pc is expressed, because the OL-pc is concentrated there due to its homophilic interactions. As a consequence, the cell–cell contacts become unstable, and the action of classic cadherins to hold the apposing cell membranes is abrogated. Based on these observations, it has been proposed that a function of OL-pc is to upregulate the cell motile machinery at cell–cell contact sites, interfering with the classic cadherin-dependent contact inhibition of cell movement (Fig. 2.4).

Another protocadherin, NF-protocadherin (BH-protocadherin, protocadherin-7), is involved in the integrity of the deep ectoderm layer in *Xenopus* embryos (Bradley *et al.*, 1998). NF-protocadherin interacts with TAF1 via the cytoplasmic domain of the former (Heggem and Bradley, 2003). Depletion of either this protocadherin or TAF1 in embryos results in neural tube closure defects, influencing the columnar epithelial morphology as well as convergent extension movement (Rashid *et al.*, 2006). Thus, NF-protocadherin appears to contribute to maintaining the epithelial architecture and remodeling. Detailed molecular mechanisms of its action on cell–cell adhesion are not known.

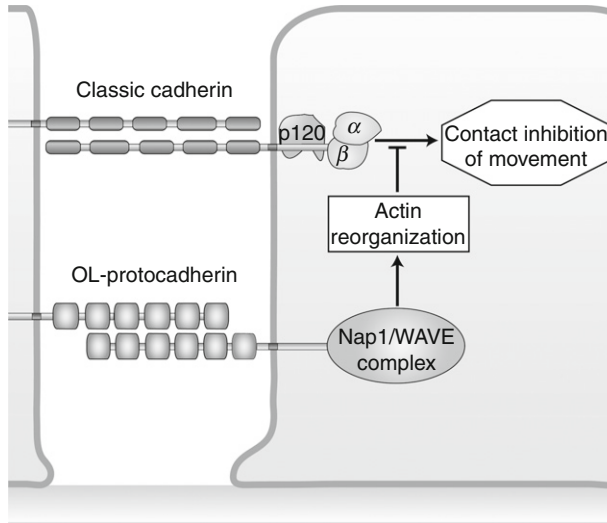

Figure 2.4 Hypothetical role of OL-protocadherin in regulation of cell motility. Classic cadherins are known to induce contact inhibition of cell movement (Bracke *et al.*, 1997; Chen and Obrink, 1991; Huttenlocher *et al.*, 1998), and this activity of cadherins might be blocked by the OL-protocadherin–associated Nap1/WAVE complex, resulting in an increase in the motility of cells within their sheets (Nakao *et al.*, 2008).

5.2. Fat cadherins

Fat cadherins, constituting another subfamily of the cadherin superfamily, are characterized by their unusually large extracellular domain (Tanoue and Takeichi, 2005). In *Drosophila*, although Fat is known to regulate the proliferation of cells, as well as planar cell polarity, the other subfamily member "Fat-like" is involved in tubular morphogenesis (Castillejo-Lopez *et al.*, 2004), suggesting that the latter might play a role in epithelial remodeling. Cytological studies of a vertebrate homologue of Fat-like, termed Fat1, showed that and this molecule was in abundance at the basal regions of cell–cell contacts, segregated from the apically concentrated classic cadherins. Nevertheless, depletion of Fat1 loosens the AJ-based junctions (Tanoue and Takeichi, 2004). Biochemical studies showed that Fat1 interacts with Ena/VASP proteins at its cytoplasmic tail, promoting the polymerization of actin fibers such as stress fibers (Moeller *et al.*, 2004; Tanoue and Takeichi, 2004). Interestingly, Fat1 loss in epithelial cells causes a reduction in not only the number of basally located stress fibers but also the amount of AJ-associated F-actin. This finding explains why the cell junctions are widely disrupted by Fat depletion. These observations suggest that Fat1 indirectly control the AJ integrity via promotion of cytoplasmic actin

polymerization. How Fat1-dependent actin polymerization affects the AJ-associated actin networks remains unknown.

5.3. Nectins

Nectins are immunoglobulin (Ig)-like cell adhesion molecules (Takai and Nakanishi, 2003). Nectin–nectin trans-interactions cause the activation of Cdc42 and Rac (Kawakatsu *et al.*, 2002), facilitating cadherin-mediated AJ formation (Fukuhara *et al.*, 2003, 2004). Nectin binds afadin at the cytoplasmic region of the former. Afadin interacts with Rap1 and further with p120-catenin, resulting in the strengthening of the binding of p120 to E-cadherin (Hoshino *et al.*, 2005), a process critical for E-cadherin stabilization. This pathway is considered to be one of the mechanisms of Rap1-dependent AJ remodeling.

An important biological function of the nectins is to recruit classic cadherins to specific junctional sites through their cross-interactions via afadin. Members of the nectin subfamily interact with each other in a heterophilic fashion more strongly than in their homophilic interactions; for example, the binding between nectin-1 and nectin-3 is stronger than that between 1 and 1, or 3 and 3 (Takai and Nakanishi, 2003). As a result, when cells expressing nectin-1 and nectin-3 are mixed, these nectins are selectively concentrated at the heterotypic cell-contact sites. This leads classic cadherins to accumulate preferentially to the heterotypic cell boundaries where the binding of nectin-1 to nectin-3 is taking place (Togashi *et al.*, 2006). This way of cooperation between classic cadherins and nectins (or other Ig-superfamily members) might play an important role in polarizing the AJ distribution, as well as serve to selectively link a specific pair of cells, in which the nectins and cadherins would function as a recognition receptor and adhesion stabilizer, respectively.

6. Junctional Remodeling During Morphogenesis

For the actual morphogenetic processes, various mechanisms of AJ remodeling, some of which have been discussed above, are assumed to operate singly or coordinately. Some examples are described below.

6.1. Remodeling through actin modulation

Given the critical role of actin filaments at the AJs, the regulation of F-actin polymerization or contractility is expected to have significant impacts on junctional remodeling. A typical example can be seen in the action of

Shroom3 (Hildebrand and Soriano, 1999). This actin-binding protein is localized along the circumferential actin belts (Fig. 2.3), which are also associated with myosin II (Hildebrand, 2005). Depending on the activity of Rho kinase (ROCK), the Shroom3-associated actin filaments contract, resulting in the apical constriction of the epithelial layers. The role of Shroom3 in this system is to recruit ROCKs to the junctions via its direct binding to them (Nishimura and Takeichi, 2008).

A more dynamic feature of junctional actin remodeling was observed in cells undergoing intercalation during the germ-band extension (GBE) in *Drosophila* embryos (Bertet *et al.*, 2004; Zallen and Wieschaus, 2004). In the intercalating cells, their junctions shrink selectively at the sides oriented toward the dorsoventral axis, resulting in the formation of vertices composed of four cells. Subsequently, the vertices elongate toward the anteroposterior direction to create new horizontal junctions. Myosin specifically localizes at the junctions to be shrunken, mediating the contraction of the actomyosin-associated junctions. Additional observations revealed that many of these intercalating cells are transiently organized into a rosette configuration (Blankenship *et al.*, 2006). These rosettes are then resolved so as to organize the anteroposteriorly oriented junctions. These ways of cellular rearrangement have been proposed to generate a driving force for the intercalation of germ-band cells (Lecuit and Lenne, 2007).

Rosette formation was also found in vertebrate ectodermal cells undergoing neural tube (Nishimura and Takeichi, 2008) or primitive streak (Wagstaff *et al.*, 2008) formation, implying that this would be a general mechanism for the epithelial cell rearrangement involving intercalation. Detailed observations of the apical surface of the closing neural tubes have clarified additional molecular events. The neural tube closure is a Shroom/ROCK-dependent event (Haigo *et al.*, 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Wei *et al.*, 2001), and these molecules are concentrated along the AJs located close to the apical surface of the columnar neuroepithelial cells (Nishimura and Takeichi, 2008), suggesting that the ROCK-dependent contraction of the AJ-associated actomyosin is responsible for the epithelial bending. However, the neuroepithelial bending is a polarized phenomenon; that is, the invaginating neuroepithelial cells bend only along the dorsoventral axis to form a tube. Correlating with this polarity, the phosphorylation of myosin light chain (MLC), which represents the active form of myosin II, is detectable preferentially along the AJs distributed toward the dorsoventral direction, which corresponds to the direction of bending (Fig. 2.5). ROCK inhibitors, or ROCK fragments which can interfere with the interaction of ROCK and Shroom3, abolish the rosette formation as well as the dorsoventrally polarized distribution of phosphorylated MLC. Concomitantly, neural tube closure fails. These findings suggest that the junctional actomyosin is locally activated in a

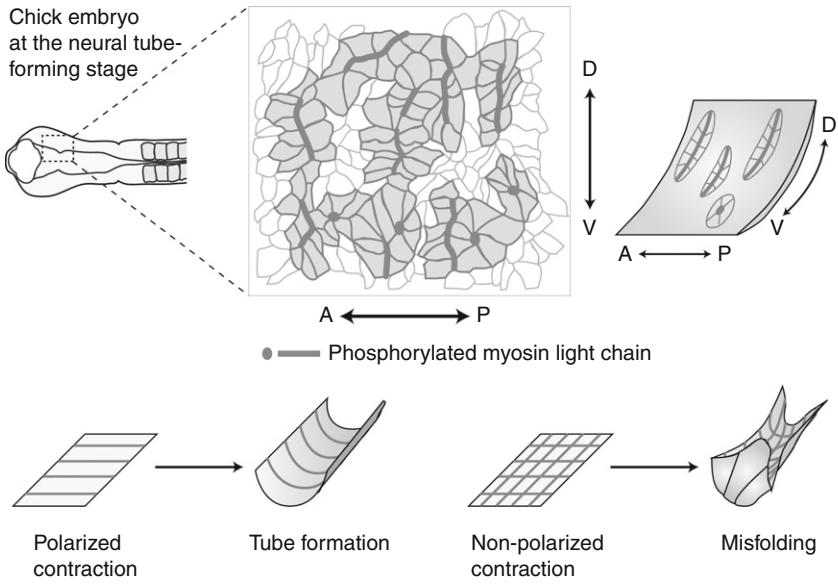

Figure 2.5 Distribution of phosphorylated myosin light chain at the apical (ventricular) surface of the closing neural tube, with reference to the polarity of neuroepithelial bending. See Nishimura and Takeichi (2008) for the original data. In the lower panels, two types of the apical junctional contraction in an epithelial sheet are compared. The contraction toward the dorsoventral axis alone would be necessary for proper tube formation; and that along random directions may result in misfolding of the neuroepithelial sheet. D, dorsal; V, ventral; A, anterior; P, posterior.

planar-polarity fashion, so as to bend the neuroepithelial layer in a restricted direction, which leads to correct neural tube formation. How the actomyosin is locally activated remains to be elucidated.

6.2. Cadherin endocytosis during epithelial cell packing

Another type of epithelial remodeling has been discovered from experiments using the developing *Drosophila* wings (Classen *et al.*, 2005). Wing epithelial cells are irregularly shaped in larvae, but most of them are reshaped to become hexagonal during the pupal stage before hair formation begins. At this time, E-cadherin is vigorously endocytosed with Rab11-positive endosomes and recycled, which may contribute to the active junctional remodeling and hexagonal repacking. E-cadherin recycling is affected by the mutation of PCP signaling molecules such as Flamingo cadherin, a cadherin superfamily member, and Frizzled, suggesting the involvement of PCP signaling in this type of junctional remodeling.

6.3. Classic cadherin regulation during gastrulation and neurulation

In *Xenopus* embryos, cadherins are involved in gastrulation movements. C-cadherin is a classical cadherin expressed in *Xenopus* embryos, and acts as a major mediator of intercellular adhesion in the blastula (Heasman *et al.*, 1994). Injection of mRNA encoding a dominant-negative form of C-cadherin into the prospective dorsal marginal zone causes gastrulation defects, such as incomplete involution and an open blastopore (Lee and Gumbiner, 1995). In the zebrafish, E-cadherin depletion impairs gastrulation movement (Babb and Marrs, 2004); and the E-cadherin-mediated cell adhesion between the deep cells and enveloping layer seems to be important for the epiboly movement (Shimizu *et al.*, 2005). On the other hand, downregulation of C-cadherin activity in *Xenopus* embryos is required for the convergent extension movement of gastrulation, as animal cap extension by activin is inhibited by a C-cadherin-activating antibody (Zhong *et al.*, 1999). Thus classic cadherins are likely essential for the collective cell migration (Friedl and Wolf, 2003) necessary for gastrulation, but their adhesive activity has to be downregulated to allow the convergent extension of the cell mass.

A number of mechanisms to downregulate the classic cadherins during gastrulation have been suggested. C-cadherin is downregulated by a TGFβ signaling pathway (Ogata *et al.*, 2007): Activin/nodal members of the TGFβ superfamily induce the expression of two genes, fibronectin leucine-rich repeat transmembrane 3 (FLRT3), a type I transmembrane protein containing extracellular leucine-rich repeats, and the small GTPase Rnd1. FLRT-3 physically interacts with Rnd1. Depletion of FLRT-3 or Rnd1 blocks the activin-induced animal cap elongation, upregulating C-cadherin-mediated cell adhesion. FLRT3 mediates C-cadherin internalization via Rab5- and dynamin-dependent endocytosis.

Wnt pathways are also involved in E-cadherin regulation during zebrafish gastrulation (Ulrich *et al.*, 2005). In Wnt11 mutants, the coordinated movement of the prechordal plate is disturbed. The mutant cells show cell cohesion defects, and lack Rab5-mediated endocytosis of E-cadherin. Enhancing Rab5c activity rescues the zebrafish from the mutant phenotypes. Thus, Wnt11 signaling plays a role in gastrulation movement through Rab5c-mediated E-cadherin endocytosis.

In the mouse, E-cadherin is transcriptionally downregulated by Snail (Cano *et al.*, 2000). This E-cadherin downregulation is accompanied by N-cadherin upregulation during gastrulation and neurulation (Takeichi, 1988); and E-cadherin proteins sharply disappear during this process. The p38 MAP kinase and a p38-interacting protein (p38IP) have been reported to downregulate the level of E-cadherin protein during gastrulation (Zohn *et al.*, 2006). In mice having a mutation in their p38IP, which interacts with

and activates p38, both the downregulation of E-cadherin protein and cell migration during gastrulation are inhibited at the posterior primitive streak; although E-cadherin transcription is unaffected. Thus, the p38 pathway regulates gastrulation by acting on E-cadherin protein levels and, in turn, cell movement. As mentioned above, since microRNAs also regulate E-cadherin expression at EMT (Gregory *et al.*, 2008; Park *et al.*, 2008), they might be involved in the regulation of E-cadherin in epithelial remodeling during gastrulation.

7. Perspectives

We have outlined various mechanisms of AJ remodeling, as summarized in Fig. 2.6. Many of these mechanisms have been revealed by cell biological studies. One of the next important challenges is to test how they are utilized *in vivo*. We need to understand how the individual events affecting the AJs are networked together in developing embryos, and how these are linked to other signal pathways controlling morphogenesis. For example, MLC in the AJs of the closing neural tube is phosphorylated at

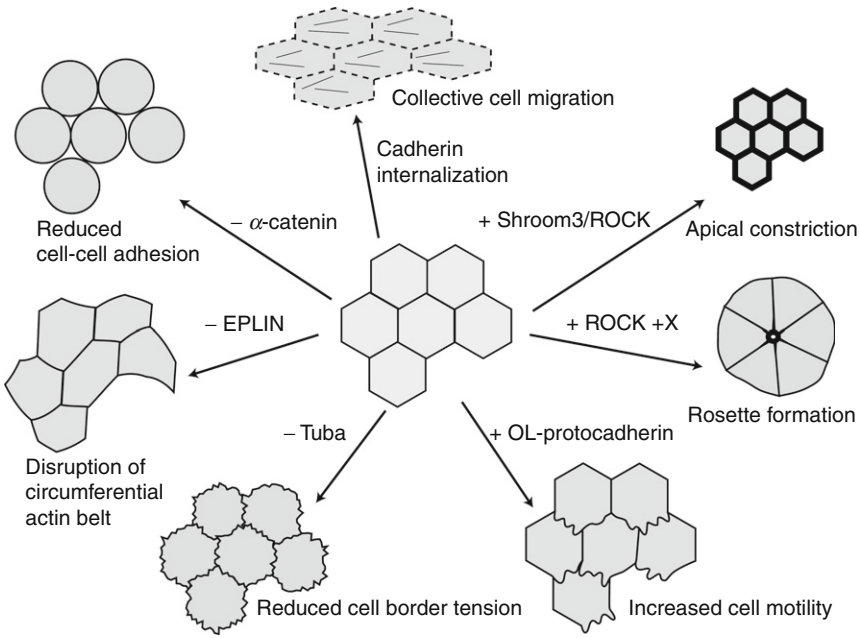

Figure 2.6 Reshaping of epithelial sheets, which can be regulated by various forms of adherens junction remodeling.

restricted parts of the junctions. This restriction strongly suggests the involvement of PCP signaling, which widely controls tissue patterns, in this regulation of phosphorylation. In this case, our goal should be to clarify the linkage between AJ modulation and PCP signals.

On the other hand, the regulatory mechanisms of cell–cell adhesion are still unclear even at the cell biological level. New components of AJ are still being identified, and the entire molecular complex organizing the AJ appears to be more complex than ever thought. The detailed functions of each component remain unresolved for many of them. Even concerning the most classic component of AJ, α-catenin, its role is not perfectly understood yet. New technologies such as proteomics analysis of the AJ components and live-cell imaging of individual components should facilitate our eventual understanding of the AJ structure and remodeling mechanisms.

One of the final goals of developmental biology is to understand the mechanisms of how the complex body structures are organized by the cells. Remodeling of the epithelial cell junctions likely plays a key role in this aspect of development. In addition, misregulation of the AJ remodeling is very likely involved in pathogenetic behavior of cells, such as cancer metastasis. Understanding the regulation of junctional remodeling is thus critical for understanding both the basic morphogenetic processes and malignant cell behavior.

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Developmental Cell
Forum

Self-Organization of Animal Tissues: Cadherin-Mediated Processes

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Animal cells are capable of self-organizing into multicellular tissues, and important players in this process are cadherin receptors. Through the homophilic interactions of cadherins, cells adhere to one another. Cells can also dynamically change shapes or positions within tissue layers via cadherin-cytoskeleton interactions and become arranged into various architectures.

One of the remarkable discoveries in developmental/cell biology in the early 1900s was that animal cells, isolated by dispersion of tissues, behave like independent organisms. Then, if cultured properly, they can eventually reassemble and reorganize into multicellular structures (Trinkaus, 1969). In vivo, although tissue-forming cells do not normally dissociate, those undergoing morphogenetic movement or rearrangement behave like liberated cells. This is particularly the case for migrating cells, such as neural crest cells and neuronal progenitor cells: they detach from neighbors, move to remote positions, and become associated with new partners. These phenomena suggest that the self-assembling ability of cells, as seen through in vitro experiments, plays a vital role in normal morphogenetic processes.

Aggregates of dissociated embryonic cells reconstitute tissues with an architecture resembling the original one (Trinkaus, 1969). Such observations led early investigators to propose that individual cells are able to self-organize to form tissues. This classic concept is being strengthened by modern observations. For example, brain cortices with typical lamina structures can develop from ES cell aggregates under purely in vitro conditions (Eiraku et al., 2008). This suggests that, once a group of cells has somehow acquired a brain-specific lineage, they can autonomously construct brain structures without passing through the normal steps of development. Needless to say, external signals or information are required for the complete patterning of tissues and organs, but what is emphasized here is that the autonomous ability of cells is sufficient to determine their local patterning.

Our knowledge about the self-assembling behavior of animal cells could facilitate technological innovations in regenerative medicine or tissue engineering, such as artificial tissue design. Since these fields are rapidly growing, this is an ideal time to take a fresh look at early observations in this area of biology and to pinpoint what needs further clarification. Of critical importance to tissue construction, and the focal point of this commentary, is cell-cell adhesion and how cell-cell adhesion molecules contribute to the self-organization of tissues.

Autonomous Cell-Cell Adhesion

How do cells adhere to each other during tissue formation? In vertebrates, cell-cell contact is mediated through three specialized structures: tight junctions (TJs), adherens junctions (AJs), and desmosomes (Franke, 2009). The TJs form the permeability barrier across epithelial sheets, and desmosomes are thought to serve as the junctions for resisting mechanical stresses. As a result of having these specific functions, the TJs and desmosomes develop in particular groups of cells. On the other hand, AJs are detected in essentially all tissue-forming cells. Invertebrates also have AJs, although their junctional organization is a little different from that of the vertebrates. Thus, the AJs are highly conserved across different cell types and species, suggesting their general importance in animal cell-cell adhesion and body formation.

The major cell-cell adhesion receptors functioning at the AJs are called cadherins, and desmosomes comprise similar molecules. Since the cadherins constitute a superfamily, and the functions of its members are diverse, those that function at the AJs are sometimes called "classic cadherins." Here, for convenience, they are simply referred to as cadherins. The cadherins are a group of transmembrane proteins, and the homophilic interactions between their extracellular domains generate the forces that hold the apposed membranes together. They associate with cytoplasmic molecules, collectively called catenins (α-, β-, γ-, and p120-catenins), at the intracellular domain. In the absence of catenins, cadherins cannot function normally, indicating that their adhesive functions are supported by these cytoplasmic proteins. The extracellular domain of cadherins assumes a rod-like shape with a length of about 20 nm, and their homophilic dimers bridge a 10–20 nm gap present between the apposed plasma membranes (Shapiro and Weis, 2009), a hallmark of the AJs. Curiously, the classic cadherins of invertebrates are much larger than their vertebrate homologs. It remains unknown how these large cadherins are accommodated in the intercellular spaces at the AJs, whose dimensions appear to be roughly conserved among vertebrates and invertebrates.

When cells are artificially dissociated, cadherins diffuse on the cell membranes or are internalized into the cytoplasm. However, as soon as cells touch one another, cadherins become concentrated at the cell-cell interfaces via their homophilic interactions, and they hold the cells together. Owing to this nature of cadherins, vertebrate embryonic cells are able to self-assemble. In the absence of cadherins, tissues are disorganized or cells are unable to form firm contacts with others (Figure 1A).

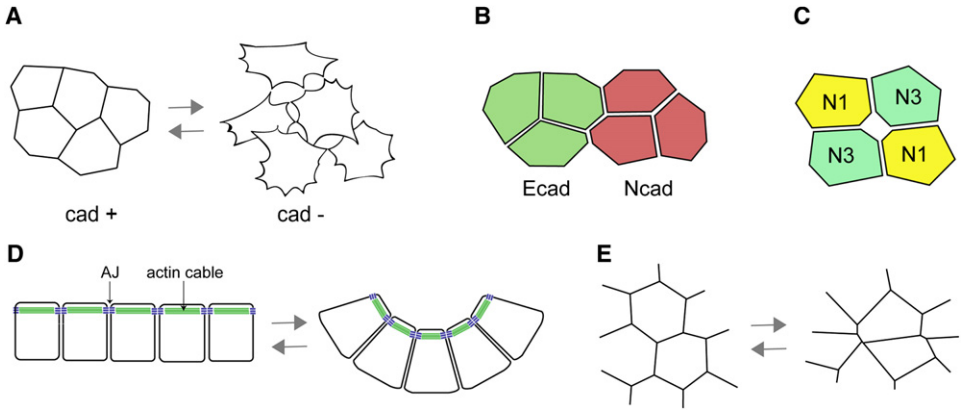

Figure 1. Multiple Roles of Cadherins or Cadherin-Based Adherens Junctions
(A) Cadherins are required for firm and ordered cell-cell associations.
(B) Cadherins take part in cell sorting. In a mixture of cells expressing different cadherin subtypes, cells preferentially associate with those expressing the identical subtype. Depending on the combinations of the subtypes, the two cell populations form chimeric aggregates via their heterophilic interactions (Katsamba et al., 2009). Ecad, E-cadherin; Ncad, N-cadherin.
(C) Nectin-dependent cell patterning. Heterophilic interactions between different nectin subtypes, e.g., nectin-1 (N1) and nectin-3 (N3), are stronger than homophilic interactions between the respective subtypes. Cadherins are recruited to the N1–N3 interacting sites, stabilizing the mosaic cell arrangements (Togashi et al., 2006).
(D and E) AJs constrict via the interactions between the cadherin-catenin complex and F-actin, resulting in various forms of cell rearrangement, such as epithelial sheet bending (D) and cell interaction (E) (Nishimura and Takeichi, 2009; Rauzi et al., 2008). Vertical (D) or horizontal (A–C, E) views of cells are shown.

Cadherins in Cell Sorting

A tissue generally comprises multiple cell types, and the different cell types are neatly segregated from each other. This segregation is an essential process for tissue organization. How do cells recognize their neighbors and obtain their proper positions in a tissue? This has been a long-standing question, and we still do not have conclusive answers. Multiple processes must be involved: not only the control of cell adhesion but also those of cell movement, polarity, and so on. The differential adhesiveness hypothesis (1963) explains the segregation of two cell types in an aggregate by assuming that cells with a higher adhesive strength position themselves inside the aggregate, pushing other cell populations with weaker adhesiveness outward (Steinberg, 1963). Such behavior of cells was indeed proven by using a mixture of cells expressing high and low levels of a cadherin (Steinberg and Takeichi, 1994). However, it still remains unclear how this strategy of cell sorting is used in morphogenesis in vivo.

Another view of cell sorting is based on qualitative differences in cell adhesiveness. Early studies showed that cells prefer homotypic adhesions to heterotypic ones (Roth and Weston, 1967). For example, liver and retinal cells selectively attach to their own cell types in mixtures. This phenomenon can be well explained in terms of the cadherin binding specificity. There are multiple subtypes of cadherins, such as E-cadherin (uvomorulin/LCAM) and N-cadherin (ACAM), and each subtype is expressed in particular groups of cells (e.g., E-cadherin in epithelial cells and N-cadherin in neural cells), although there is no strict tissue specificity in their distributions. In general, a single cell type expresses multiple cadherin subtypes, but the combinations and proportions of them differ among the cell types. In mixtures of cells expressing different cadherin subtypes, cells preferentially adhere to those expressing the same cadherin subtypes (Figure 1B), although the degree of selectivity varies with the combination of the subtypes (Katsamba et al., 2009).

During development, the cadherin expression profile changes in correlation with tissue segregation events. A well-known example is during neural tube development, when the overlying ectoderm expresses E-cadherin, but this E-cadherin is replaced with N- and other cadherins in the invaginating neural plate, while E-cadherin remains in the ectoderm (Takeichi, 1988). Such observations suggest that a switch in cadherin subtype expression might contribute to the segregation of tissues. Indeed, when N-cadherin is ectopically overexpressed in the ectoderm, the separation of the future epidermis and neural tube does not take place normally (Fujimori et al., 1990). In other cases, however, the switch in cadherin expression is not so simple; for example, during the segregation of lens from the future cornea, E-cadherin expression is not turned off in the invaginating lens epithelium, although N-cadherin is newly added to the lens-forming cells (Takeichi, 1988). This type of incomplete switching occurs widely. A question here is whether this ''addition'' alone is sufficient to promote the segregation of two cell groups. To obtain conclusive answers, it would be important to look at the effects of genetic perturbation of cadherin expression patterns in vivo. For example, what happens if E-cadherin expression persists without the addition of N- or other cadherins in the invaginating neural plate during neural tube formation? In addition, it should be noted that cadherin subtypes are not functionally equivalent, as N- and E-cadherins are not completely substitutable for the maintenance of tissues (Libusova et al., 2010). The biological roles of cadherin subtype switching thus need to be further investigated.

Cell sorting is also controlled by cooperation of cadherins with other adhesion molecules such as nectins, a subfamily of immunoglobulins that also localize in

the AJs. Nectins prefer heterophilic interactions to homophilic ones, in contrast with the homophilic nature of cadherin interactions. Therefore, the combination of nectins and cadherins results in the generation of complex cell-adhesive behavior. For example, when cells expressing nectin-1 and -3 are mixed, these cells are arranged in a mosaic pattern due to their heterophilic interactions (Figure 1C), and cadherins work to stabilize this pattern of cell adhesions via their later interactions with these nectins (Togashi et al., 2006). This type of cooperation between multiple cell-cell adhesive systems likely contributes to the production of complex cell sorting patterns, which cannot be achieved by a single mechanism.

Cadherins in Tissue Morphogenesis

One of the critical factors in determining tissue morphology is the polarity of cells. In epithelia, the AJs are localized near the apical end of cell-cell contacts, and this polarized localization of AJs is likely critical in determining the epithelia-specific 2D structures. Mesenchymal cells, on the other hand, do not exhibit such polarized AJ distributions, and their association patterns tend to be more irregular. Whether or not cells have polarized AJs thus affects cell patterning. This can be seen from the beginning of mouse development, where blastomeres of mouse preimplantation embryos begin to polarize at the late 8-cell stage with the generation of the apical junctional complex. This junctional polarization, however, occurs only in the outer layer of the embryo, and unpolarized cells occupy the inner portions. These differences in junctional organization in turn regulate the fates of the respective cell population: the outer cells differentiate into the trophectoderm and the inner cells become the inner cell mass (Nishioka et al., 2009). Thus, the polarization of the AJs is involved not only in segregation of cell populations but also in their differentiation.

Evidence has accumulated that cadherins interact with actin cables via α-catenin. This means that the cell-cell adhesion and motile/contractile machineries can closely cooperate with one another. In the epithelial AJs, cadherins associate with the circumferential actomyosin ring. This ring contracts due to the action of Rho kinases, causing a constriction of the apical portion of the cells (Figure 1D). This mechanism of cell shape changes is important for morphological modulations of epithelial layers (Nishimura and Takeichi, 2009). The actomyosin-associated AJs are also used in more sophisticated ways to control cell rearrangements. In the process of germband elongation of *Drosophila* embryos, cell junctions in the overlying epithelial layer contract locally. This local contraction of the junctions leads the cells to intercalate (Figure 1E), which in turn promotes the convergent extension of tissues (Rauzi et al., 2008). What is important in these observations is that the local changes in the junctions of individual cells regulate the global pattern of cell collectives. This is a critical point in explaining the tissue-organizing behavior of animal cells.

The cadherin-catenin complex interacts not only with F-actin but also with many other proteins, including microtubules and signaling factors (Nishimura and Takeichi, 2009). Studies of the molecular and biological roles of these interactions are currently producing findings that will further disclose the novel morphogenetic functions of AJs or cadherins. In conclusion, cell-cell adhesion mechanisms are more complex and dynamic than previously thought, and individual cells seem to ingeniously use the cadherin and associated systems for organizing highly ordered multicellular structures.

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