COMMEMORATIVE LECTURE

Mechanisms of morphogenetic cell assembly

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Animal cells isolated from tissues can reaggregate *in vitro*. Within the aggregates, cells reconstruct tissue structures, in which ''cell sorting'' is an essential process. It is thought that this self-assembling ability is a basic property of animal cells. More than 25 years ago, I became attracted to this phenomenon, and began to identify molecules involved in the tissue reconstruction processes. Yesterday, I explained the early story of my studies, and so I am going to skip over this part of the story today, and focus on our recent progress.

Through the studies from 1974 into the 1980's, we identified a group of adhesion molecules designated as the cadherins.[1] Cadherin is a so-called ''homophilic'' adhesion molecule, connecting cells via a like-like molecular interaction (Fig. 1). Without cadherin, cells in tissues tend to come apart, although they generally still maintain weak associations, because many other adhesion proteins are also present on the cell surface. Today, in the initial part of my talk, I would like to summarize the general properties of the cadherin molecule, and in the rest of the talk, tell you about our recent findings on the role of cadherin in synaptic junction and neural network formation.

The functions of cadherin molecules can be divided into two categories. First, these molecules are essential for tight cell-cell associations. Second, cadherins are involved in specific cell adhesion or sorting. Both functions are regulated through the actin-based cytoskeleton. The next slide indicates how cadherins are important for tight cell-cell adhesion. These are early mouse embryos (Fig. 2). During development, they undergo so-called ''compaction.'' At the early 8-cell stage, the cells are round, showing clear boundaries between themselves, but their boundaries gradually become invisible during the late 8-cell stage of development. However, when the cadherin system is blocked in em-

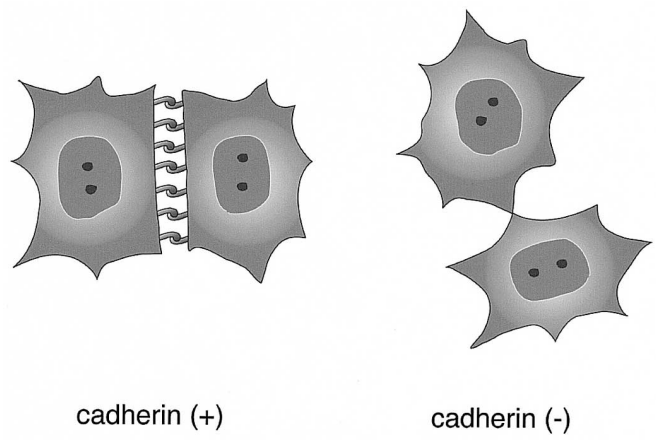

Fig. 1 Cadherin molecules are essential for tight cell-cell association.

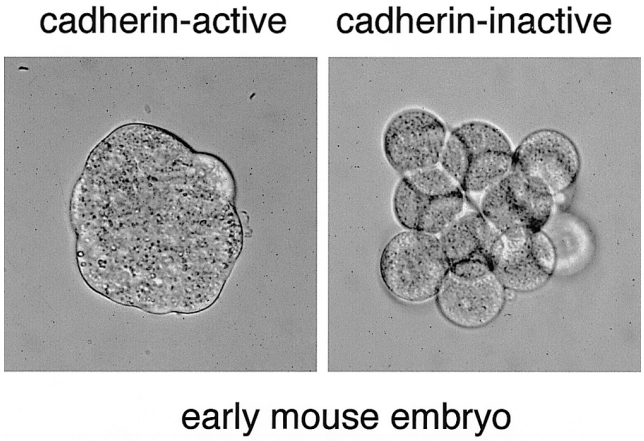

Fig. 2 Compaction of early mouse embryos is blocked by antibodies to E-cadherin.

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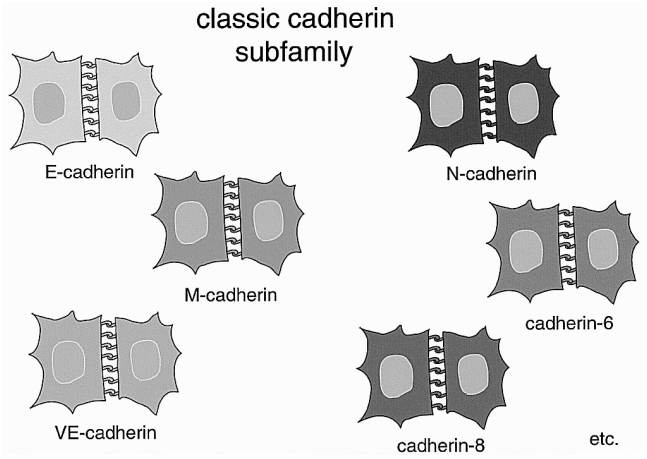

Fig. 3 The classic cadherin subfamily. Each cadherin undergoes a type-specific homophilic interaction.

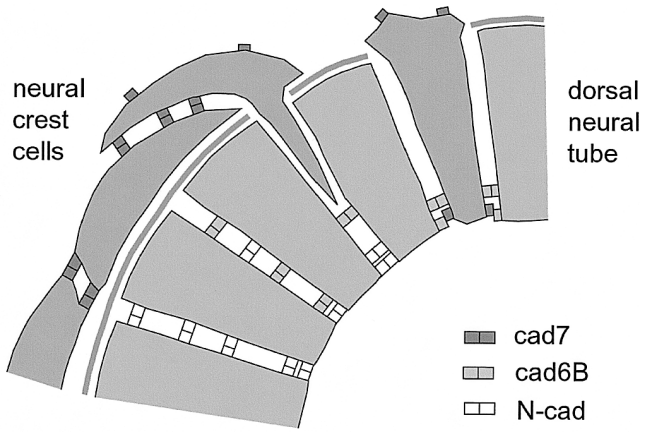

Fig. 4 Cadherin type changes during neural crest emigration from the neural tube.

bryos at this late stage, individual cells again become round, indicating that their associations have become very loose. One can do similar experiments using more differentiated tissues, such as embryonic retinal fragments. If retinal fragments collected from early embryos are cultured in the presence of anti-cadherin antibodies, these fragments become disassociated into small cell clusters or single cells. Such experiments clearly demonstrate the importance of cadherins in maintaining cells together in a variety of cellular systems.

Next, I should emphasize that there are a number of different cadherin molecules, such as E-cadherin (E-cad), N-cadherin (N-cad), and M-cadherin (Fig. 3). Some cadherins are called only by numbers, such as cadherin-6, -8 and -11. More than 15 cadherins have been identified from each vertebrate species including human beings, and are now called ''classic cadherins.'' *In vitro*, cells expressing different cadherins are sorted from each other based on their properties of homophilic interaction.

Then, a question arises as to the role of this variety of cadherins *in vivo*. When we look at the expression of a given cadherin during development, we notice that its pattern dynamically changes. Interestingly, such changes, in general, correlate with morphogenetic events. A typical example is seen during neuronal tube formation and neural crest differentiation. In the early chicken embryo, the overlying ectoderm expresses L-CAM, a chicken homologue of mammalian E-cad, but when the neural tube forms, L-CAM gradually disappears from the forming tube, and instead N-cad begins to appear. Another cadherin, cadherin 6B (cad6B), is also added to the dorsal portion of the neural tube. When neural crest cells begin to migrate, these cells stop expressing N-cad and cadh6B, and instead start to express cadherin-7 (cad7). Migrating crest cells seem to express only cad7.

What is the biological role of these changes? Let me focus on the neural crest-forming region. Dorsal neural tube cells use N-cad and cad6B for their mutual connections. Crest cells leaving the neural tube, however, lose these two cadherins, and instead begin to express cad7 (Fig. 4). To understand what kind of cell behavior is controlled by these changes, we did experiments to perturb their expression pattern by overexpressing N-cad or cad7 at the dorsal neural tube. What we found was that, when either N-cad or cad7 had been overexpressed in the dorsal neural tube, crest cells expressing this ectopic molecule became unable to migrate out of the neural tube.[2] This result suggests that the switching of cadherin types during neural crest development is essential for the crest cells to leave the tube. Under the overexpression of N-cad or cad7, presumably, crest and tube cells stick together, being unable to separate from one another. These observations are an example of how the differential expression of multiple cadherins is important for morphogenetic processes.

Now I would like to discuss the regulatory aspects of the cadherin system. The activities of cadherins are regulated through cytoplasmic components. In epithelial cells, the cadherins organize so-called ''adherens junctions,'' part of the junction complex, to which a number of cytoplasmic proteins, including ''catenins'', become directly or indirectly associated. The cytoplasmic domain of cadherin can be subdivided into two portions, the N-terminal ''juxtamembrane (JM)'' and C-terminal half domains (Fig. 5). To the latter, β-catenin binds directly, and α-catenin binds to this β-catenin; this molecular complex further associates with the actin filaments via the α-catenin. To the JM domain, another catenin, p120-catenin, binds.[3] What are the roles of these catenins? The β-catenin/α-catenin complex is well known to be essential for the cadherins to function as

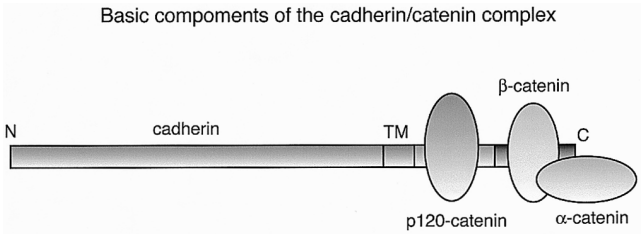

Fig. 5 A cadherin-catenin complex.

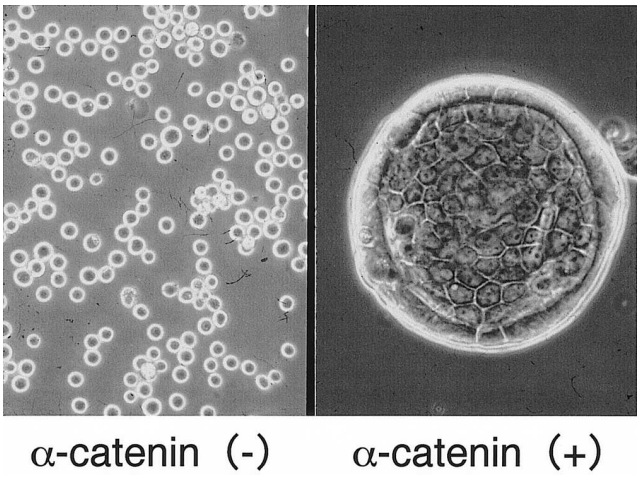

Fig. 6 PC12 cells before and after transfection with the α-catenin cDNA.

adhesion molecules. Here is a nice example. PC12 is a carcinoma cell line derived from a lung tumor. These cells lose their α-catenin gene, and grow as single cells. However, once α-catenin cDNA has been introduced into these cells, they begin to aggregate strongly, organizing epithelioid spheres (Fig. 6).[4] Thus, the introduction of α-catenin was sufficient to restore epithelial cell-cell junctions in these mutant cells, indicating that the C-terminal half domain, as the β-catenin/α-catenin complex binding site, plays a critical role in junction formation.

What about the JM domain? We have evidence that the JM domain is a very important regulatory portion of the cadherin molecules. This idea came from the analysis of the adhesive properties of another tumor cell line, called Colo205, derived from a colon carcinoma. These cells grow just like the PC12 cells; they never show tight associations, and grow as loose aggregates. However, very interestingly, this cell line, different from PC12, expresses all the important components for the cadherin system, E-cadherin, α-catenin, and β-catenin.[5] Nevertheless, they cannot normally aggregate. This was a very curious phenomenon for us, prompting us to determine why their cadherin system could not work.

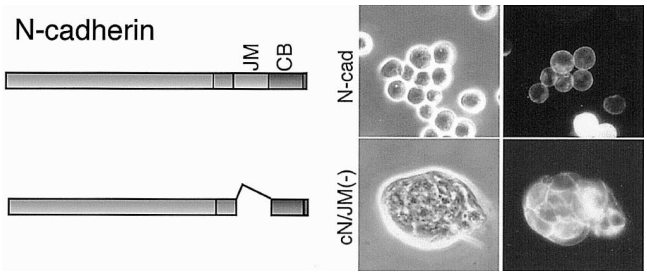

Fig. 7 Colo205 cells transfected with the control full-length N-cadherin or mutant N-cadherin in which the JM domain had been deleted. Left, phase-contrast micrographs; right, immunofluorescence staining for the introduced molecules. (Prepared by Shinji Aono)

In the course of various series of experiments, we found that, when a mutant cadherin, in which the JM domain had been artificially removed, was introduced into Colo205 cells, their cadherin-dependent adhesion was restored (Fig. 7), indicating that cadherin molecules can normally function in this cell line, if they lack the JM domain. To explain this interesting phenomenon, we hypothesized that the JM domain has some inhibitory signals to suppress the cadherin function, assuming that in normal cells, these hypothetical inhibitory signals are inactive, whereas in Colo205 cells, the inhibitory system is abnormally activated. If such inhibitory signals indeed exist, these signals might be used for the regulation of cell adhesion, *e.g.*, for promoting cell detachment necessary for cell relocation or rearrangement.

To test this idea, we chose somite development as a model system. Somite development involves a number of cell rearrangement processes, and among them we focused on myotome morphogenesis.[6] Myotome cells arise from the expanding dermomyotome, and are rearranged to form the myotome cell sheets. We injected the JM domain-deficient cadherin into somites using adenoviral expression vectors, and found that myotome cells expressing these mutant molecules could not undergo normal relocation; they became stuck at a lateral position of the body forming clusters, never expanding to organize two dimensional cell sheets (Fig. 8). This phenomenon indicated that without the JM domain, cell-cell adhesion could not be regulated, causing cells to clump. The JM domain is thus indeed necessary for the regulation of cell relocation; it perhaps temporarily inhibits cadherin-mediated cell adhesion to allow cells to relocate. We do not know what kind of inhibitors are working with the JM domain, and identification of them is an important future goal.

Now I would like to move to the second story. How the neural network is formed is a central question in neural biology. Neural network formation can be considered as a problem of cell-cell adhesion, as the net-

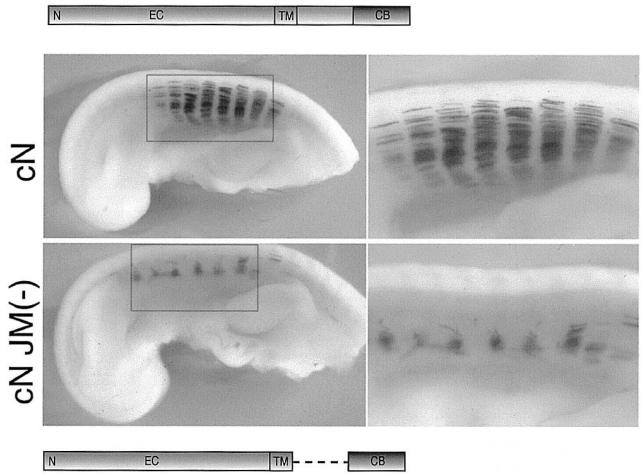

Fig. 8 Effects of the ectopic expression of the control full-length N-cadherin (cN) or JM-domain-deleted mutant N-cadherin (cN JM (-)) in somites of chicken embryos. cDNAs encoding these molecules and β-galactosidase were used for co-transfection, and cells expressing the latter were stained. Myotomes are visualized by this staining. The images in the right panels are a larger magnification of the enclosed portions of the left panels. (Prepared by Kazuki Horikawa)

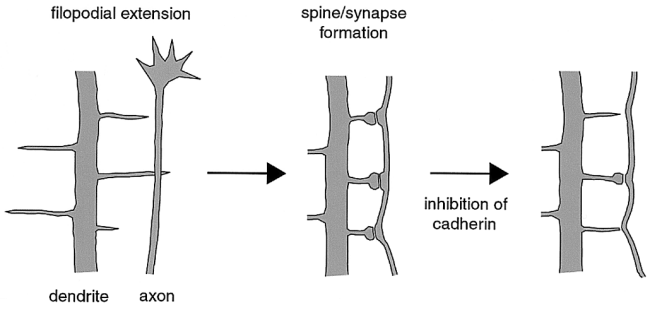

Fig. 9 Processes of synapse formation, and the effect of cadherin blockade.

work is established by a sequential connection of neurons. If we could understand how neurons recognize each other and become connected with one another, we should be able to answer important questions concerning the mechanisms of neural network formation.

The synapse is the site for interneuron connections. There are two types of synapses, excitatory and inhibitory. Many of the excitatory synapses are formed by contacts between dendritic spines and axons, and others, by direct contacts of the axons with the dendrtic shaft. In textbooks of neural biology, you see receptors, channels, and transmitters in the illustrations of synapses, but may not see any molecules connecting the synaptic plasma membranes in apposition. This omission means that this aspect of the synapse has been poorly studied. We became interested in uncovering the mechanisms for the recognition and connection between the plasma membranes constituting synapses.

Several years ago, we found that cadherin and catenins were localized in synaptic junctions, although these molecules avoided the transmitter release zones, suggesting that the cadherin system may be involved in interneuronal synaptic connections in some specific ways.[7] We are testing this idea using various approaches. Today I want to tell you about some of our recent results obtained from experiments using *in vitro* cultures of hippocampal neurons. Hippocampal neurons can form synapses *in vitro*. The cellular processes of excitatory synapse formation are the following: Neurons initially extend dendrites and axons. From the dendrites, fine filopodia protrude, and when the filopodia meet an axon, their tips attach to it, swelling at the contact portion (Fig. 9). Subsequently, synaptic vesicles start to accumulate in the presynaptic sites, and postsynaptic density proteins also accumulate, leading to the maturation of synapses.

Using such cultured neurons, we attempted to inhibit cadherin by two different methods, expression of a dominant-negative cadherin construct,[8] and genetic knockout of the αN-catenin gene, whose presence is essential for cadherin function.[4] Now I show you the results of the first approach (manuscript in preparation). When the dominant negative cadherin was overexpressed in neurons, their dendritic morphology was dramatically altered (Fig. 9). Many of the dendritic spines were transformed into filopodium-like processes, and others were irregularly deformed, losing the typical mushroom shape, although they tended to maintain contacts with axons. Next, we studied the intracellular organization in these treated neurons. Synapsin, a synaptic vesicle protein, shows a punctate distribution in normal neurons, each punctum representing a cluster of synaptic vesicles. When the dominant-negative cadherin was expressed, their distribution became diffuse, suggesting that synaptic vesicle accumulation had been blocked. Consistently, the uptake of FM-64, a fluorescent dye, into synapses was greatly reduced, indicating the suppression of synaptic vesicle recycling in cadherin-blocked neurons. Furthermore, we examined the distribution of PSD-95, a postsynaptic density protein, and again found that its accumulation into synapses had been perturbed.

As another approach, we collected hippocampal neurons from αN-catenin KO mice, and cultured them *in vitro*. The results were consistent with the observations I just mentioned. Dendritic spines elongated in the mutant neurons. Thus, both experiments, genetic and *in vitro* overexpression experiments, showed similar effects of cadherin blockade on synapse formation. To summarize, when cadherin-mediated adhesion is blocked, synaptic contacts destabilize, and dendritic spine shape is altered (Fig. 9). Intracellularly, the pre- and post-synaptic protein organization became dis-

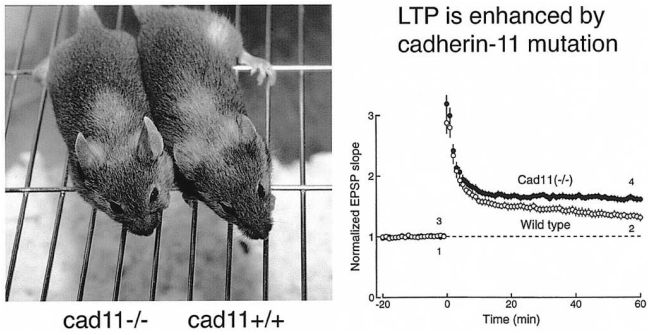

Fig. 10 LTP in cadherin-11 knockout mice. (The photograph was prepared by Osamu Chisaka.)

rupted. These findings strongly suggest that cadherin plays a crucial role in synaptic junction formation.

Finally, we are asking whether cadherin is also important for the physiological functions of synapses. The spine elongation phenotype is reminiscent of that seen in the Fragile-X syndrome causing mental retardation and other neurological defects. It should also be stressed that synapse morphology is dynamically changed during the establishment of long-term potentiation (LTP). As I have shown you, cadherin blockade affects spine shape. We can, therefore, speculate that this adhesion molecule could be an important regulator for synaptic physiological activities. In fact, we previously found that in the hippocampus of cadherin-11 KO mice, LTP was enhanced,[9] suggesting that synapses becomes more plastic when this cadherin was deleted from the hippocampus (Fig. 10). This finding together with the other observations suggests that cadherin may act as a modulator of synapse physiology by altering synaptic contact structures.

In sum, cadherin was identified as a molecule essential for the adhesion of fibroblasts and epithelial cells, and found to be important for the regulation of morphogenetic cell behavior. Recent studies have now shown that this molecular family is also involved in synaptic cell contacts. Thus, a common adhesion mechanism is operating over a wide variety of spectrum ranging from fibroblastic adhesion to synaptic neuronal contacts, suggesting that the principles for the control of cell-cell adhesion may be identical throughout such a wide variety of adhesion systems. It would be an intriguing future subject to understand how such variations in cell-cell contact have been generated during the evolution of complex multicellular organisms.

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