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The complex morphogenesis of organisms is achieved by consecutive cell-to-cell interactions during development. Recent studies suggest that growth factors play crucial roles in controlling such intercellular communications in a variety of organisms. In addition to secretory factors that trigger intracellular signaling, transcription factors that act in the nucleus to regulate gene expression are thought to be essential for the determination of cell fates. Our main interest is to know how pattern formation and morphogenesis during development is regulated by these growth and transcription factors. We address this problem using several model animals, including frogs, mice and ascidians, and by employing embryology, genetics, molecular and cellular biology, and biochemistry.

I. Establishment of cell polarity during vertebrate embryogenesis

Gastrulation is one of the most important processes during the morphogenesis of early embryos, involving dynamic cell migration and change in embryo shape. In spite of its importance, the mechanism underlying the event has just begun to be studied at the molecular level. During *Xenopus* gastrulation, mesodermal cells migrate to the inside of the embryo and move on the blastocoel roof. One of the important mechanisms for this process is the cell movement called "convergent extension (CE)". As convergent extension begins, cells are polarized and aligned mediolaterally, followed by the mutual intercalation of the cells that acquired planar cell polarity (PCP). In the regulation of vertebrate convergent extension, the Wnt/PCP pathway is implicated.

We have recently knocked out one of two *prickle*-related genes, *mpk1*, in mice and found that the $mpk1^{-/-}$ mutants are early embryonic lethal and die between E5.5 and E6.5. The mutants show arrested development with failure of primitive streak and mesoderm formation and failure of distal visceral endoderm migration. At the cellular level, the $mpk1^{-/-}$ epiblast tissue is disorganized, with a clear defect in cell polarization.
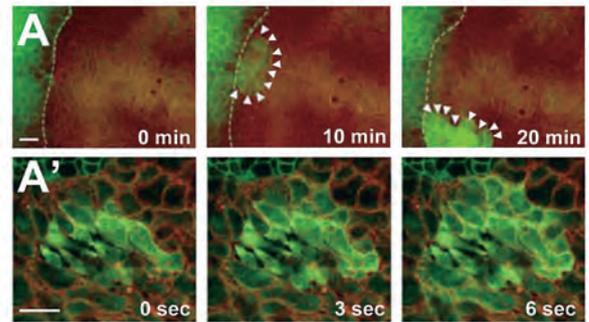

Figure 1. Intracellular Ca^{2+} increase at the border of mesoderm and ectoderm in the conjugate culture. During the culture, several islands of transiently elevated Ca^{2+} detected by a calcium indicator G-CaMP4 were observed in the single microscopic field (A). The Ca^{2+} islands appeared to be formed by a propagation of Ca^{2+} wave from fewer cells with higher Ca^{2+} levels (A').

We also study how PCP is established within the cells using explants of *Xenopus* embryonic tissues and found that heterogenous combination culture of tissues such as mesoderm and ectoderm triggers the cell polarity, as revealed by the live-imaging analysis of microtubule growth orientation. We have been able to demonstrate that intracellular Ca^{2+} is increased prior to the oriented growth of microtubules (Figure 1), and the transient Ca^{2+} increase can be triggered not only by tissue-tissue interactions, as we reported previously, but also by physical forces experimentally applied (A. Shindo et al., PLos ONE, 5, e8897, 2010). We propose that mechanical forces generated by the tissue-tissue interactions are essential for the initiation of the cell polarity and are currently investigating to identify molecules involved in the sensing of the physical forces and transmission of the signal to the cells.

II. Protein ubiquitination system involved in the Wnt/PCP pathway

Although Wnt/PCP signaling has been shown to play an essential role in the regulation of gastrulation movements, the molecular mechanisms of how Wnt signals intracellularly and how it regulates the tissue movements remain elusive. We have shown that Wnt/PCP signaling activates the protein ubiquitination/degradation system, which is essential for cell motility during *Xenopus* gastrulation. In order to clarify how the ubiquitination system is involved in the Wnt/PCP pathway, we focused on β-TrCP (transducin-repeat containing protein), a component of E3 ubiquitin ligase complex. β-TrCP has been shown to regulate the canonical Wnt pathway by ubiquitinating β-catenin, but it is not known whether β-TrCP is involved in the noncanonical Wnt/PCP pathway. Our study revealed that β-TrCP plays an essential role in this signaling pathway. Dishevelled (Dsh) is a cytoplasmic protein, which plays pivotal roles in the canonical and noncanonical Wnt pathways. When the Wnt/PCP pathway is activated, Dsh is translocated to the plasma membrane. β-TrCP colocalized with Dsh both before and after the activation of Wnt/PCP signaling (Figure 2). Furthermore, knockdown of β-TrCP by specific antisense morpholino oligonucleotides inhibited gastrulation

movements and Dsh translocation to the plasma membrane. This translocation is essential for Dsh to activate the downstream signaling pathway, and thus, our results strongly suggest that β-TrCP plays a key role in this signaling pathway. We are currently investigating the regulatory mechanisms of β-TrCP activity and its substrates to regulate gastrulation movements in *Xenopus* embryo.
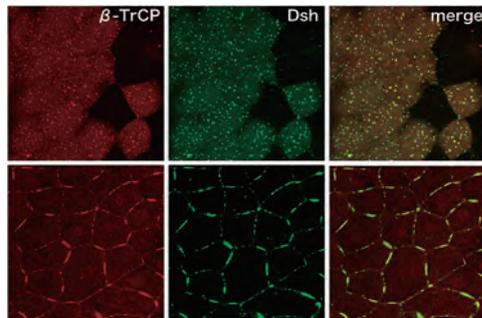

Figure 2. Colocalization of β-TrCP with Dishevelled (Dsh) in ectodermal cells of *Xenopus* embryo. Upper panels; Dsh showed punctate localization (green) and colocalized with β-TrCP (red). Lower panels; When Wnt/PCP signaling was activated, β-TrCP and Dsh were translocated to the plasma membrane.

III. Cellular morphogenesis during neural tube formation

Closure of the neural tube, like the morphogenesis of all epithelial tissues, fundamentally requires the change and maintenance of cell shape. Neural tube closure requires at least two coordinated morphogenetic events, cell elongation and apical constriction. Although cytoskeletal elements are responsible for such changes, how actin filaments and microtubules are regulated in this process *in vivo* is unknown.

We found that neural tube closure in *Xenopus* critically depended on two proteins, MID1, the responsible protein for Opitz G/BBB syndrome (OS) in humans, and MID2. OS is characterized by midline malformations, including hypertelorism, hypospadias, cleft lip/palate, and brain abnormalities. Depletion of the *Xenopus* MIDs disrupted epithelial morphology in the neural plate, leading to neural tube defects. In the MID-depleted neural tube, the normal apical accumulation of the cadherin/β-catenin complex in the cells and formation of the basal lamina were perturbed (Figure 3). Furthermore, the xMID knockdown destabilized and disorganized apicobasally-polarized microtubules in the neural plate, and these abnormalities accounted for the abnormal epithelial cell morphologies and neural tube closure. We also found that the xMIDs and their interacting protein Mig12 were coordinately required for microtubule stabilization during remodeling of the neural plate. Since the depletion of the xMIDs also affected the optic vesicle, cement gland, and kidney, all epithelial organs, we propose that similar mechanisms underlie the normal morphogenesis

of epithelial tissues and organs where MIDs are expressed, including the tissues affected in Opitz G/BBB syndrome patients.
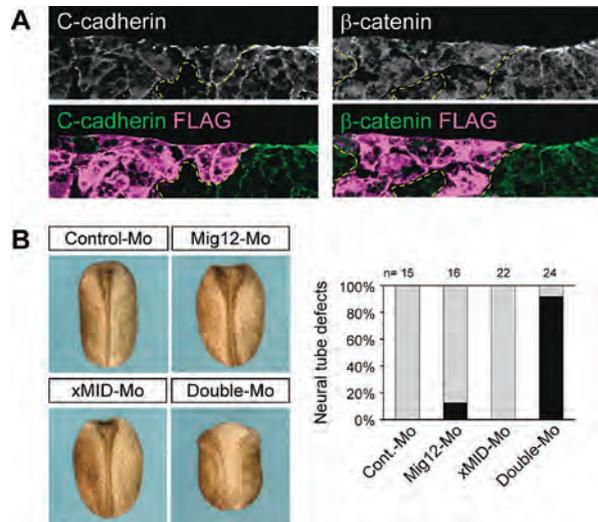

Figure 3. A, Transverse sections of the neural plate containing MID-depleted cells (magenta), stained with antibodies against C-cadherin and β-catenin. B, Functional interaction of Mig12 and MIDs. Mild-knockdown of Mig12 or MIDs by antisense Morpholino (Mo) caused slight delays in neural tube closure. Double-knockdown of Mig12 and MIDs (Double-Mo) induced severe neural tube defects.

We also investigated roles of an adhesion molecule nectin, which belongs to the immunoglobulin-like cell adhesion molecule in the neural tube formation of *Xenopus*. Depletion of nectin-2, one of the nectin family members, from early embryos resulted in incomplete neural fold formation. Cellular analyses revealed less accumulation of F-actin at the apical surface, causing an aberrant apical constriction, a cell-shape change that is required for neural tube folding. Furthermore, we found nectin-2 functionally cooperates with N-cadherin to synergistically enhance apical constriction, highlighting the cooperative action between spatiotemporally upregulated nectin-2 and N-cadherin in the neural plate.

IV. *Brachyury*-downstream gene sets in a chordate, *Ciona intestinalis*

Brachyury plays a pivotal role in the notochord formation in ascidian embryos. *Ciona intestinalis Noto4* (*Ci-Noto4*) was isolated as a gene downstream of *Ci-Bra*. This gene encodes a 307 amino-acid protein with a C-terminal phosphotyrosine interaction domain (PTB/PID). Expression of *Ci-Noto4* commences at the neural plate stage and is specific to notochord cells. Suppression of *Ci-Noto4* levels with specific antisense morpholino oligonucleotides resulted in the formation of two rows of notochord cells owing to a lack of midline intercalation between the bilateral populations of progenitor cells. In contrast, injection of a *Ci-Bra*(promoter):Ci-Noto4-EGFP construct into fertilized eggs disrupted the localization of notochord cells in the

embryonic trunk (Figure 4). Ci-Noto4 overexpression did not affect cellular differentiation in the notochord, muscle, mesenchyme, or nervous system. Analysis of Ci-Noto4 regions that are responsible for its function suggested significant roles for the PTB/PID and a central region, an area with no obvious sequence similarity to other known proteins. Genes involved in planar cell polarity, such as *Ci-prickle*, did not show similar functional effects when compared with *Ci-Noto4*. These results suggested that PTB/PID-containing Ci-Noto4 is essential for midline intercalation of notochord cells in chordate embryos.
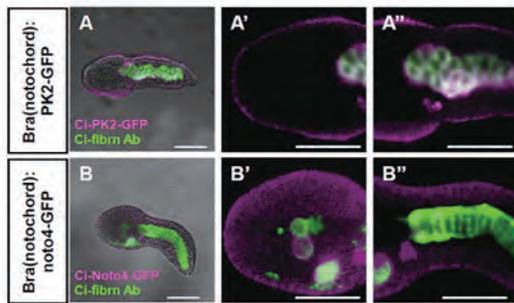

Figure 4. Effects of Ci-prickle and Ci-Noto4 overexpression. (A-A'') A Ci-Bra(promoter):Ci-Prickle-EGFP construct was injected into fertilized eggs. Ci-Prickle-EGFP overexpression did not cause the notochord cells to disperse in the trunk region as was observed in Ci-Noto4 overexpressing embryos. (A-A'', B-B'') Magenta marks Ci-Bra(promoter):Ci-Prickle-EGFP expressing or Ci-Bra(promoter):Ci-Noto4-EGFP expressing notochord cells with anti-GFP antibody. Green marks endogenous Ci-fibrn protein localization with anti-Ci-fibrn antibody. Scale bars = 50μm

Publication List

〔**Original papers**〕

● Goda, T., Takagi, C., and Ueno, N. (2009). *Xenopus* Rnd1 and Rnd3 GTP-binding proteins are expressed under the control of segmentation clock and required for somite formation. Dev. Dyn. *238*, 2867-2876.

● Hida, N., Awais, M., Takeuchi, M., Ueno, N., Tashiro, M., Takagi, C., Singh, T., Hayashi, M., Ohmiya, Y., and Ozawa, T. (2009). High-sensitivity real-time imaging of dual protein-protein interactions in living subjects using multicolor luciferases. PLoS ONE *4*, e5868.

● Sugiura, T., Tazaki, A., Ueno, N., Watanabe, K., and Mochii, M. (2009). *Xenopus* Wnt-5a induces an ectopic larval tail at injured site, suggesting a crucial role for noncanonical Wnt signal in tail regeneration. Mech. Dev. *126*, 56-67.

● Tao, H., Suzuki, M., Kiyonari, H., Abe, T., Sasaoka, T., and Ueno, N. (2009). Mouse *prickle1*, the homolog of a PCP gene, is essential for epiblast apical-basal polarity. Proc. Natl. Acad. Sci. USA *106*, 14426-14431.

● Yakushiji, N., Suzuki, M., Satoh, A., Ide, H., and Tamura, K. (2009). Effects of activation of Hedgehog signaling on patterning, growth and differentiation in *Xenopus* froglet limb regeneration. Dev. Dyn. *238*, 1887-1896.

● Yamada, S., Hotta, K., Yamamoto, T.S., Ueno, N., Satoh, N., and Takahashi, H. (2009). Interaction of notochord-derived fibrinogen-like protein with Notch regulates the patterning of the central nervous system of *Ciona intestinalis* embryos. Dev. Biol. *328*, 1-12.