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The complex morphogenesis of organisms is achieved by dynamic rearrangements of tissues during embryogenesis, in which change in cellular morphology as well as orchestrated cell movements are involved. For cells to know how they should change their shape and where they should move, information called "cell polarity" is essential. How then is the cell polarity established within cells? Is it intrinsically formed within the cells or triggered by extracellular cues? Furthermore, little is known as to how coordinated and complex cell movements are controlled in time and space. We attempt to understand the mechanisms underlying these events using several model animals, including frogs, fish, mice and ascidians, taking physical parameters such as force in consideration, in addition to conventional molecular and cellular biology.

I. Spatio-temporal regulation of traction force during the collective chemotractic migration

During embryogenesis, the arrangement of multicellular tissue is dramatically changed to establish properly shaped embryos. These movements of group of cells are often highly organized and collective. Investigating the mechanism of collective cell migration is therefore essential for understanding embryogenesis. *Xenopus* leading edge mesoderm (LEM) is one of the suitable models for studying this morphogenetic movement, because of easy micromanipulation of tissue excised from the embryo. During *Xenopus* gastrulation, LEM moves into the blastocoel ahead of the axial mesoderm, which forms the notochord and muscles in the future. In our previous studies, we have shown that LEM generates the driving force of mesodermal migration, and measured the physical value of this force with the explant. Following this, we have started to address how each single LEM cell generates the force for collective migration in the explant. In order to understand this, we have decided to establish Traction Force Microscopy (TFM) for *Xenopus* LEM explant migration. Eventually, we completed the TFM system for this multi-cellular movement with a combination of acrylamide gel and chemoattractants. Our TFM observation revealed that traction force was generated by the cells, which were located at the anterior part of explants. We are investigating how each LEM cell generates traction force in a spatiotemporally regulated manner.

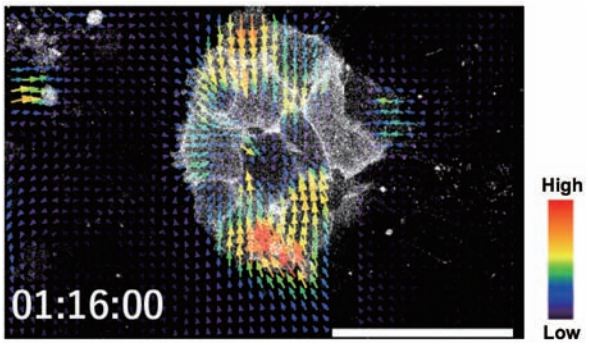

Figure 1. A snapshot of TFM observation of LEM explant migration. LEM explant expressing membrane GFP to visualize the plasma membrane migration toward the left side. The direction of arrow shows that of traction force, size and color indicate the intensity of force.

II. Mechanotransduction in *Xenopus* embryonic cells

During early embryogenesis, a variety of dynamic morphogenetic movements occur, which include convergent extension of the axial mesoderm, epiboly of the ectoderm, and neural tube formation. These movements must generate physical forces at the levels of cells and tissues. However, it is still not fully understood how these forces influence morphogenetic processes. This project is to elucidate the mechanosensing and mechanoresponse of *Xenopus* embryos, and to clarify the roles of mechanotransduction in embryogenesis.

Cells sense mechanical stresses in several ways, for example, with TRP channels, F-actin, cadherins, and/or focal adhesions. Physical stimuli sensed by these molecules are converted to intracellular chemical signals, which in turn induce cellular response. Phosphorylation is thought to be one of the earliest responses to mechanical stresses. Therefore, we attempt to profile protein phosphorylation upon mechanical stimuli and identify the repertoire of target proteins in *Xenopus* embryonic cells. In order to comprehensively analyze levels of protein phosphorylation, we took a phosphoproteomic approach. So far, we have confirmed that a mechanical stress applied to *Xenopus* embryos changes phosphorylation levels of some protein kinases and cytoskeletal regulators. Particularly, some proteins that localize in the cell cortical actin network seem to be the targets of phosphorylation. These results suggest that mechanical stresses may regulate activities of protein kinases and/or protein phosphatases in *Xenopus* embryonic cells, and that the cortical actin network may be involved in this mechanotransduction.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2016. The former title is indicated by an asterisk (*).

III. Mathematical analysis of neural tube formation

During early development of the central nervous system, neuroepithelial cells increase their height by microtubule rearrangement. This cell shape change is called cell elongation, which drives the tubular morphogenesis, called neural tube formation. We previously showed that cell elongation during *Xenopus* neural tube formation depends on orthologs of two microtubule-associated proteins: MID1, which is responsible for Opitz G/BBB syndrome in humans, and its paralog MID2. In this study, to further investigate the role of the cell elongation, we developed a three-dimensional multi-cell-based mechanical model and used it to simulate the process of neural tube formation. Computational simulation of our model suggests that cell elongation drove the rapid folding of the neural plate, and reduced the lumen size of the neural tube. Then we investigated the *in vivo* neural tube by knocking down MID proteins, and found that cell elongation actually reduces the lumen size of the neural tube. These data highlight the function of MID proteins in regulation of the lumen size of the neural tube, and demonstrate the advantage of mathematical analysis for understanding the mechanical basis of morphogenesis.

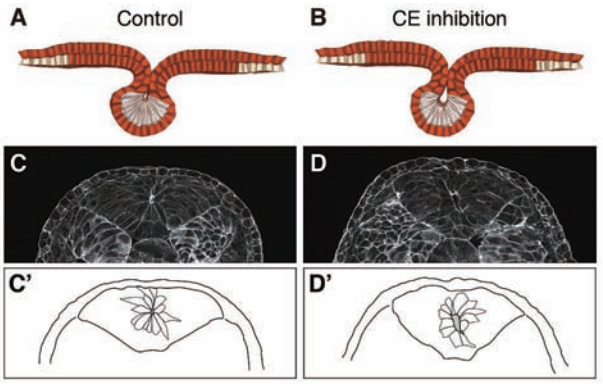

Figure 2. Effects of cell elongation (CE) on modeled epithelial sheets and *in vivo* neural tube.

(A,B) Modeled epithelial sheets at the end of each simulation with cell elongation (A) and without cell elongation (B). (C,D) F-actin staining of control embryo (C) and MID-Mo injected embryo (D). (C') and (D') indicate outlines of neural cells and lumen (gray) of (C) and (D), respectively.

IV. A novel membrane structure orchestrates centrosome positioning and the orientation of cell division axis.

The positioning of the centrosome is critical for the polarity of cilia and the orientation of the mitotic spindle, which are important events during morphogenesis. Previously, in ascidian, *Ciona intestinalis* embryos, we found a unique centrosome-targeting of the membrane structure (membrane invagination) in epidermal cells, which divide stereotypically along the anterior-posterior (A–P) axis. We also reported that membrane invaginations showed the A–P polarity and a tensile force toward the posterior end of cell. By injection of antisense morpholino oligo (MO), we revealed that Dishevelled (Dsh), a core component of Planar Cell Polarity (PCP) pathway was involved in the A–P polarity of membrane invaginations. In the Dsh-depleted embryos, the direction of the invagination as well as mitotic spindle orientation was randomized. In addition, epidermal cilia normally localized in the posterior side of the cell was anteriorized. These results support our hypothesis that the membrane invagination controls the direction of cell division as well as ciliary positioning via the tethering centrosome at the posterior side of the epidermal cell and highlight physiological importance of this unique structure.

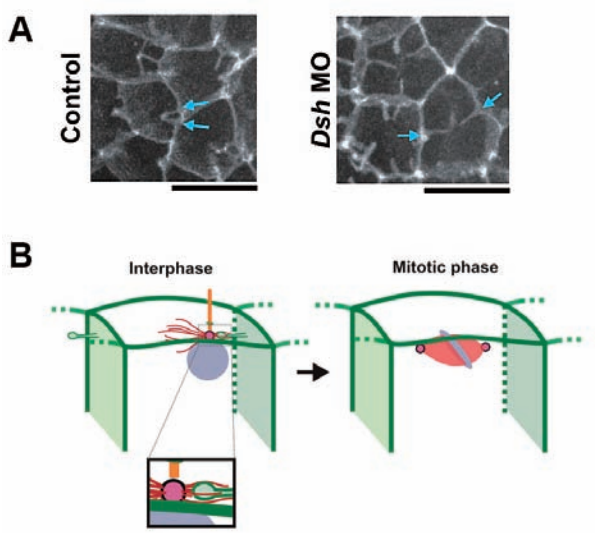

Figure 3. (A) The direction of membrane invagination is disturbed in Dsh-deficient epidermal cells. The plasma membrane was labeled with PH-GFP.

In the epidermal cells of control MO injected *Ciona* embryo, the membrane invaginations show the A-P polarity (Control). In the epidermal cells of *Dsh* MO injected embryo, the direction of invagination is randomized (*Dsh* MO). Blue arrows indicate representative membrane invaginations. Anterior: left. Bar: 10 μm.

(B) Graphical summary of our hypothesis. Anterior is left. Interphase: nuclei (purple), cell membrane (green), centrosome (magenta), microtubule (red) and cilium (orange). The membrane invagination pulls the centrosome/cilium toward the posterior in the interphase, and then mitotic spindle forms aligned along the A-P axis.

V. Notochord and evolution of chordates

A T-box family transcription-factor gene, *Brachyury*, has two expression domains with corresponding functions during animal embryogenesis. The primary domain, associated with the blastopore, is shared by most metazoans, while the secondary domain restricted to the notochord, is specific to chordates.

Therefore, how *Brachyury* acquired its secondary expression domain at the mid-dorsal region of the blastopore and how it is related to the emergence of the notochord is a big question of evolutionary biology. To elucidate how chordate ancestors gained the new expression domains, we used Amphioxus, the most ancestral chordates. We functionally analyzed the Amphioxus *Brachyury* promoter regions by reporter assay, using *Ciona intestinalis* embryos.

As results, we found that some fragments promoted the reporter expression in multiple regions such as blastopore, muscle and notochord, respectively, in ascidians. This result indicates that those Amphioxus enhancers have capacities

of driving *Brachyury* expression in the lateral mesoderm and notochord, in addition to the blastopore of ascidians. Furthermore, we found some other regions drive the expression specifically in the notochord, suggesting that Amphioxus notochord *Brachyury* expression is regulated by the combination of multiple enhancers.

VI. The *Ciona intestinalis* cleavage clock is independent of DNA methylation

DNA methylation at cytosine residues is an important epigenetic modification found in eukaryotes ranging from plants to humans. Invertebrates offer an interesting model for studying evolutionary changes in the targets and the function of DNA methylation. A marine invertebrate chordate *Ciona intestinalis* has a genome-wide mosaic methylation pattern comprising methylated and unmethylated genes. It has been observed that DNA methylation is targeted to the transcribed region of ubiquitously expressed genes, and a constant targeting of "gene body methylation" irrespective of cell types. To reveal the function of gene body methylation in gene transcription, we analyzed newly synthesized RNA from *C. intestinalis* embryos. By using 4sU labeling and sequencing methods, revealing global RNA processing kinetics at nucleotide resolution, we obtained snapshots of active transcription. Significant differences were seen in co-transcriptional splicing efficiency, in connection with methylation status of exons and introns. The splicing efficiency and DNA methylation status were also correlated to nucleosomal positions, suggesting that epigenetic states in the bodies of transcribed genes control the pre-mRNA processing through nucleosomal positioning.

VII. Cnidarian-symbiodinium Symbiosis

Corals are declining globally due to a number of stressors. Such stresses lead to a breakdown of the essential symbiotic relationship between coral and *Symbiodinium*, a process known as coral bleaching. Although the environmental stresses cause this breakdown, the molecular and cellular mechanisms of symbiosis are still unclear. Corals are not very suitable as laboratory systems due to their slow growth, long generation times, and calcareous skeletons. To overcome these limitations, we focused on the small sea anemone *Aiptasia* as a novel experimentally tractable cnidarian model organism. *Aiptasia*, just as reef-building corals, establishes a stable but temperature-sensitive symbiosis with *Symbiodinium*. *Aiptasia* can be repeatedly bleached and repopulated with *Symbiodinium*, grows rapidly, and lacks a calcareous skeleton, allowing microscopic and cell biological analyses.

As the innate immune system recognizes the self and others, it might distinguish *Symbiodinium* as well as pathogens from the host. In fact, the genomic information of *Acropore digitifera,* one of the reef-building corals, revealed that the coral innate immune repertoire is far more complex than that of non-symbiotic cnidarians, *Nematostella* and *hydra*. We thus speculate that the complex innate immune repertoire may reflect adaptation to the symbiotic state. We used chemical inhibitors for the inanate immune signaling, and found that the symbiotic state of coral and *Aiptasia* is influenced by the inhibitors .

Publication List:

〔Original papers〕

- Inoue, Y., Suzuki, M., Watanabe, T., Yasue, N., Takeo, I., Adachi, T., and Ueno, N. (2016). Mechanical roles of apical constriction, cell elongation, and cell migration during neural tube formation in *Xenopus*. Biomech. Model Mechanobiol. *15*, 1733-1746.
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- Negishi, T., Miyazaki, N., Murata, K., Yasuo, H., and Ueno, N. (2016). Physical association between a novel plasma-membrane structure and centrosome orients cell division. eLife *5*, e16550.
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- Session, A.M., Yamamoto, T.S., Takagi, C., Ueno, N., *et al*. (2016). Genome evolution in the allotetraploid frog *Xenopus laevis*. Nature *538*, 336–343.
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- Suzuki, M.M., Mori, T., and Satoh, N. (2016). The *Ciona intestinalis* cleavage clock is independent of DNA methylation. Genomics *108*, 168-176.