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The morphology of the body and tissues is established in spatio-temporarily regulated manners. A number of genes involved in morphogenesis have been identified, but it is still uncertain how the spatial and temporal information are established and converted to morphology during embryogenesis. Therefore, we are challenging to understand the mechanism by which the spatial information is established and that by which the temporal, or periodical, information is converted into morphology by several different approaches.

In the development of many tissues, secreted signal molecules are important for the formation of spatial information. These molecules are secreted from producing cells and transported to surrounding cells, resulting in the formation of concentration gradients. Given that their concentration decreases according to the distance from the source, the gradient of the signals defines relative positions of receiving cells in developing tissues. Many genetic studies revealed that a limited number of secreted signal proteins, including Wnt, BMP, and Hedgehog, function in the morphogenesis of tissues and embryos. In spite of the accumulation of genetic evidence, however, the molecular mechanism that regulates their spread in particular developing tissues remains to be elucidated. To this end, we started to visualize signal proteins and monitor their movement in tissues. In addition, we are examining structural and biochemical characteristics of these molecules, which appear to affect how they spread.

The segmental sub-regions of several specific tissues, including the paraxial mesoderm (or somites) and the pharyngeal pouches, by contrast, appear not to be simply directed by the gradient of the signal molecules, but by a unique mechanism proceeding periodically. For instance, somites are sequentially generated in an anterior-to-posterior order by converting the temporal periodicity created by a molecular clock into periodical structures. The molecular mechanism underlying this conversion and morphological segmentation, however, is not yet fully understood. Thus, another goal of our current studies is to reveal the molecular mechanism of this other and unique mode of patterning that underlies the periodical and sequential sub-division in the development of somites and pharyngeal arches.

I. Spatial regulation of secreted Wnt proteins in vertebrate development

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. In a classical view, it has been proposed that secreted signal proteins, like Wnt proteins, spread and function over a distance of several cells in many aspects of morphogenesis. In contrast, accumulating evidence also implies that Wnt proteins appear to transmit their signals locally in some particular cases. Therefore, their secretion and transport might be differently controlled depending on situation. Thus, for understanding the spatial regulation of tissue morphogenesis, the molecular mechanism underlying the spreading of Wnt proteins should be revealed.

For better understanding the spreading of Wnt proteins, we started to visualize Wnt proteins in the extracellular space by several different approaches. Our preliminary study indicated that Wnt proteins are not simply diffused during embryogenesis of the mouse. We precisely examined regulatory mechanisms and biological significance of Wnt protein distribution in mouse embryos. These analyses revealed a novel view of spatial regulation of Wnt signaling.

Extracellular molecules are known to regulate special distribution of Wnt proteins. Once Wnt proteins are secreted from the producing cell, their spread is regulated through interaction with these molecules. The heparan sulfate proteoglycan (HSPG), which is composed of a core protein with several chains of HS glycosaminoglycans, is a major component involved in this interaction. In collaboration with Prof. Taira at the University of Tokyo, we found that HSPGs with different sugar chain modifications form distinct extracellular structures, which are called heparan sulfate nanostructures (HSNSs), in the early *Xenopus* embryo.

II. Heterogeneity of secreted Wnt proteins secreted from culture cells

Although the structure of Wnt protein has already been revealed by X-ray crystallography, its higher order structure in extracellular space has not yet been fully understood. One of the proposed forms of Wnt proteins in extracellular space is binding to lipoprotein particles. On the other hand, recent studies indicated that Wnt proteins are secreted on another lipid-based carrier, called the exosome, which is an MVB (multivesicular body)-derived membrane vesicle (Figure 1). However, it remains unclear whether Wnt proteins are secreted only in these two forms, or also in some other forms from the same cells, or if different forms of Wnt proteins are secreted in a cell type specific manner.

To address this question, we systematically examined characteristics of Wnt proteins secreted from polarized MDCK (Madin-Darby Canine Kidney) cells. Although some Wnt3a proteins from mouse L cells were secreted together with lipoprotein particles, most of the Wnt3a proteins from either apical or basolateral side of MDCK cells did not. In contrast, secretion of a small amount of Wnt3a proteins via exosomes was detected. We found that different populations

of exosomes or exosome-like vesicles were released from MDCK cells depending on the cell polarity. Wnt3a associated with these vesicles was detectable in culture media collected from both apical and basolateral sides of the cells. Basolaterally secreted Wnt3a existed with typical exosomes. In contrast, most of apically secreted Wnt3a, as well as Wnt11, existed with non typical exosomes, whose density was higher than that of typical exosomes. The lipidation of Wnt3a was required for its basolateral secretion in exosomes but was dispensable for the apical secretion. These results indicated that epithelial cells release Wnt via distinct populations of vesicles differing in secretion polarity and lipidation dependency.

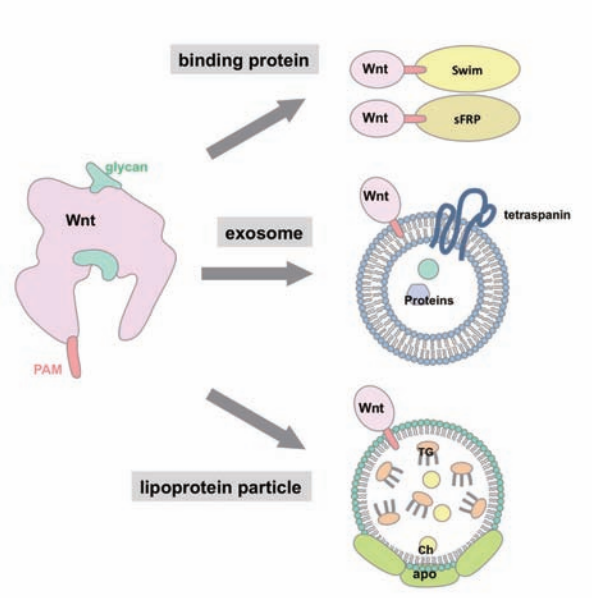

Figure 1. Heterogeneity of Wnt transport system. Secreted Wnt proteins are transported by several different ways, including binding with carrier proteins, associating with exosomes, or loading on lipoprotein particles.

III. Molecular mechanism of somite development

Somites are segmental epithelial blocks located symmetrically on either side of the neural tube. Somites are periodically generated in an anterior to posterior manner from their precursor, the presomitic mesoderm (PSM), which is located posterior to newly formed somites. This periodic generation is achieved by a complex and dynamic mechanism in the PSM. First, the molecular clock, the so-called segmentation clock, creates oscillatory expression of particular genes, *hairy* and some *notch*-related genes, in the posterior PSM. Because the phase of oscillation is gradually shifted along the posterior-to-anterior axis, a wave of the oscillation appears to move in a posterior-to-anterior fashion. This oscillatory gene expression subsequently resulted in periodical generation of morphologically segmented somites.

The spatial pattern of somites is defined by positioning of intersomitic boundaries and rostro-caudal patterning within a somite. This spatial pattern is established in the anterior PSM by converting the oscillatory gene expression into spatially restricted patterns of gene expression. Several intra-cellular molecules, as well as FGF and Wnt signaling, are involved in this conversion. In the mouse, the prospective segmentation boundary is periodically established in the anterior PSM at the rostral border of the *Mesp2* expression domain. Mesp2, one of the key regulators in this conversion, is initially expressed at the most anterior region of the Tbx6 protein domain. This expression is not anteriorly extended beyond the anterior border of the Tbx6 protein domain because *Mesp2* expression requires Tbx6 proteins. Thus, the anterior border formation of the Tbx6 protein domain is a more fundamental process in the positioning of the segmentation boundary.

Importantly, this border is not consistent with the anterior border of *Tbx6* mRNA, rather it is regulated by a proteasome-mediated mechanism. Although the molecules directly executing this proteolysis are still unclear, *Mesp2*, as well as *Ripply1* and *Ripply2*, have shown to be required for the down-regulation of Tbx6 proteins by analysis of mouse embryos defective in these genes. Since expressions of *Ripply1* and *2* are eliminated in *Mesp2* deficient mouse embryos, we previously proposed the following model; *Mesp2,* whose expression is activated in the most anterior part of the Tbx6 domain, causes retreat of the Tbx6 protein domain through activation of *Ripply1* and *2* expression, and

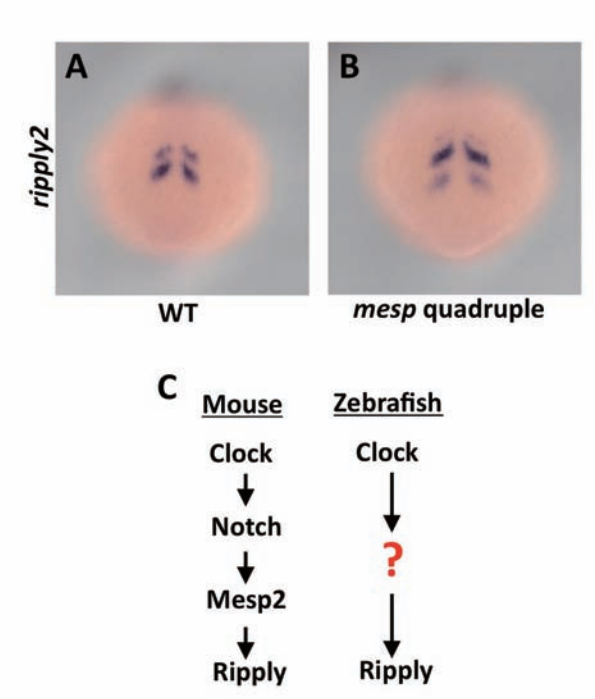

Figure 2. (A, B) Expression of *ripply2* in WT embryo (A) and *mesp* quadruple mutant embryo (B). In contrast to mice, the function of mesp is not required for the *ripply* expression in zebrafish. (C) Scheme of the regulation of *ripply* expression by the segmentation clock. In the mouse, temporal information generated by segmentation clock is converted to the expression pattern of *ripply* by the functions of Notch and Mesp2. However, this molecular machinery is not conserved between mouse and zebrafish and molecular mechanism regulating *ripply* expression still remains to be elucidated.

the retreated Tbx6 subsequently defines the next segmentation border and *Mesp2* expression. Recently, we showed that Ripply is a direct regulator of the Tbx6 protein level for the establishment of intersomitic boundaries. However, it is still to be elucidated whether *Mesp* is actually required for the formation of the boundaries in zebrafish.

To answer this question, we generated zebrafish mutants lacking all 4 zebrafish *mesp* genes by a genome-editing approach. In contrast to the case for the mouse *Mesp2* mutant, the positions of somite boundaries were normal in the zebrafish *mesp* quadruple mutant embryos. On the other hand, each somite was caudalized similarly to the mouse *Mesp2* mutant. These results clarify the conserved and species-specific roles of Mesp in the connection of the molecular clock to somite morphogenesis (Figure 2).

IV. Molecular mechanism of pharyngeal pouches

In addition to somites, metameric structures are observed in the pharyngeal region of vertebrates. Typical examples of such structures are skeletal elements of jaws, gills and cranial nerve projections. This metamerism is brought about by the segmental development of the pharyngeal pouches, which are generated by outpocketing of the pharyngeal endoderm. However, the molecular mechanisms underlying the segmentation of the pharyngeal pouches and the morphogenesis of the pharyngeal pouches still remained to be elucidated.

To understand these mechanisms, we examined the development of pharyngeal pouches in medaka embryos in collaboration with Prof. Wada at Tsukuba University. We found that the expression of *pax1* in the endoderm prefigured the location of the next pouch before the cells bud from the epithelium. Embryos deficient for *pax1* did not form the pharyngeal pouches posterior to the second arch. Precise analysis of gene expression suggests that *pax1* has a critical role in generating a primary pattern for segmentation in the pharyngeal endoderm (Figure 3).

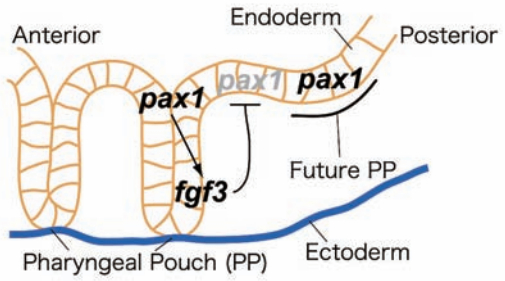

Figure 3. Model of molecular mechanism of the segmentation of pharyngeal pouches in the medaka embryo. Our results suggest that interaction between pax1 and fgf3 is a key process for generation of the segmental pattern of pharyngeal pouches.

Publication List:

〔Original papers〕

- Chen, Q., Takada, R., Noda, C., Kobayashi, S., and Takada, S. (2016). Different populations of Wnt-containing vesicles are individually released from polarized epithelial cells. Sci. Rep. *6*, 35562.
- Kawamura, A., Ovara, H, Ooka, Y., Kinoshita, H., Hoshikawa, M., Nakajo, K., Yokota, D., Fujino, Y., Higashijima, S.I., Takada, S., and Yamasu, K. (2016). Posterior-anterior gradient of zebrafish hes6 expression in the presomitic mesoderm is established by the combinatorial functions of the downstream enhancer and 3'UTR. Dev. Biol. *409*, 543-554.
- Ohta, Y., Kamagata, T., Mukai, A., Takada, S., Nagai, T., and Horikawa, K. (2016). Nontrivial effect of the color-exchange of a donor/acceptor pair in the engineering of Förster resonance energy transfer (FRET)-based indicators. ACS Chem. Biol. *11*, 1816-1822.
- Okada, K.*, Inohaya, K., Mise, T., Kudo, A., Takada, S.*, and Wada, H.* (2016). Reiterative expression of pax1 directs pharyngeal pouch segmentation in medaka. Development *143*, 1800-1810. (*: Co-corresponding authors)
- Takemoto, T., Abe, T., Kiyonari, H., Nakao, K., Furuta, Y., Suzuki, H., Takada, S., Fujimori, T., and Kondoh, H. (2016). R26-WntVis reporter mice showing graded response to Wnt signal levels. Genes Cells *21*, 661-669.
- Yabe, T., Hoshijima, K., Yamamoto, T., and Takada, S. (2016). *Mesp* quadruple zebrafish mutant reveals different roles of *mesp* genes in somite segmentation between mouse and zebrafish. Development *143*, 2842-2852.

〔Review articles〕

- Kondoh, H., Takada, S., and Takemoto, T. (2016). Axial level-dependent molecular and cellular mechanisms underlying the genesis of the embryonic neural plate. Dev. Growth Differ. *58*, 427-436.
- Yabe, T., and Takada, S. (2016). Molecular mechanism for cyclic generation of somites: Lessons from mice and zebrafish. Dev. Growth Differ. *58*, 31-42.