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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. Genetic analyses with Drosophila mutants defective in the core proteins or GAG biosynthesis indicate the requirement of HSPG in Wnt signaling and gradients. 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. Interestingly, *Xenopus* Wnt8 proteins were preferentially retained by HSNSs with N-sulfo modification (N-sulfo HSNSs). To understand the mechanism by which distinct distributions of HSNSs, especially N-sulfo HSNSs are formed, we focused on NDST1, an enzyme catalyzing N-sulfation of heparan sulfate. Gain and loss of function studies of NDST1 indicated that NDST1 is necessary and sufficient for the conversion of N-acetyl HSNSs into N-sulfo HSNSs. Extracellular accumulation of Wnt proteins and Wnt signaling were also affected by perturbation of NDST1 expression. These results indicated that N-sulfo modification of heparan sulfates are important for extracellular distribution of Wnt proteins and Wnt signaling (Figure 1).

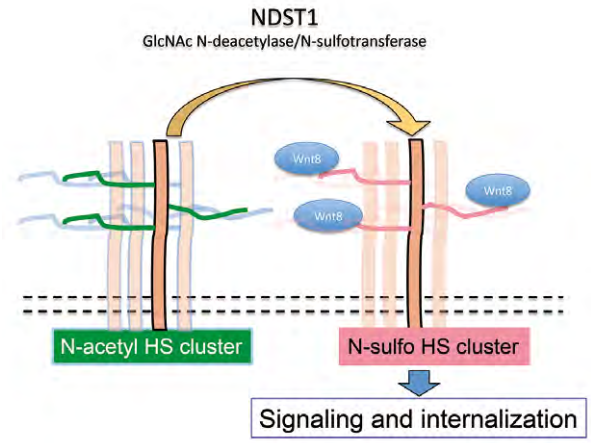

Figure 1. Schematic representation of the 2 different HSPG clusters of Wnt signaling.

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. 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 detectable from the basolateral side of MDCK cells in a lipidation dependent manner. Interestingly, some apically secreted Wnt3a was secreted with an exosome marker, CD63, but its density was higher than typical exosomes. Signaling activity of Wnt proteins on basolaterally secreted exosomes and apically secreted exosome-like vesicles were lower than that of Wnt3a proteins in conditioned media. We also found that Wnt11, which is preferentially secreted from the apical side of MDCK cells, did not associate with exosomes, but with CD63-containing exosome-like vesicles. Our results indicate that Wnt are secreted with multiple carriers from polarized epithelia cells and suggest that Wnts are differently packaged depending on Wnt subtype and cellular context.

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 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 *mesp*-deficient zebrafish. Since four *mesp* genes have been identified in the zebrafish genome, we generated mutant fish carrying the frame shift mutation in all of the *mesp* genes using TALEN mediated mutagenesis and analyzed its phenotype (Figure 2). Unlike the mouse *Mesp2* mutant, zebrafish *mesps* quadruple embryo exhibited normally segmented somites and a normal boundary of the Tbx6 protein domain, although segmented expression of *epha4a* was disturbed in the anterior PSM, suggesting *mesps* and *mesps* dependent expression of *epha4a* are dispensable for somite positioning and segment boundary formation (Figure 3). Further analysis revealed zebrafish *mesp* genes are required for establishment of caudal identity

of somites similar to mouse *Mesp2*. *mesps* quadruple mutant embryos also showed disrupted superficial horizontal myoseptum formation probably resulting in the mis-migration of pigment cells and lateral line primordia in later development.

In contrast to *mesp*, double mutant for *ripply1* and *ripply2* revealed that these two genes are essential for segmental boundary formation. We found that *ripply1* and *2* expression is induced by a manner independent of *mesp*. Rather, striped expression of *ripply1* and *2* at the anterior PSM were severely disrupted in double mutant for *her1* and *7*, which are zebrafish clock genes encoding Hairy-related transcriptional repressors, suggesting proper regulation of *ripply1* and *2* expression by Hairy is essential for somite boundary formation. Furthermore, in *ripply1* and *2* double embryos, oscillatory expression of *her1* was expanded to the anterior paraxial mesoderm, indicating that *ripply1* and *2* are also required for termination of oscillation associated with mature somite formation.

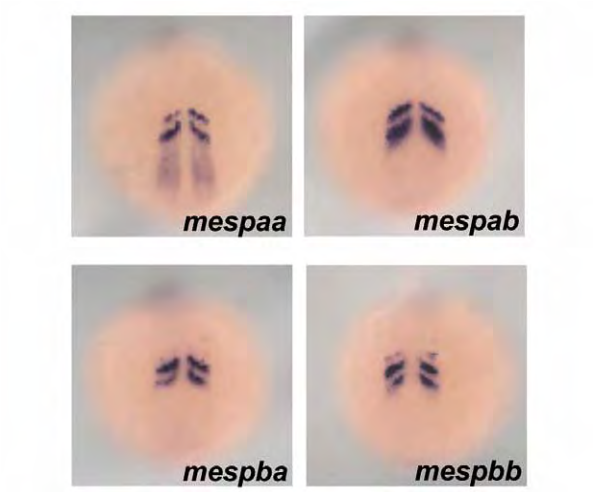

Figure 2. Expression of four *mesp* genes in zebrafish embryos. The zebrafish four *mesp* genes, *mespaa, maspab, mespba,* and *mespbb*, are similarly expressed in the anterior presomitic mesoderm during somitogenesis.

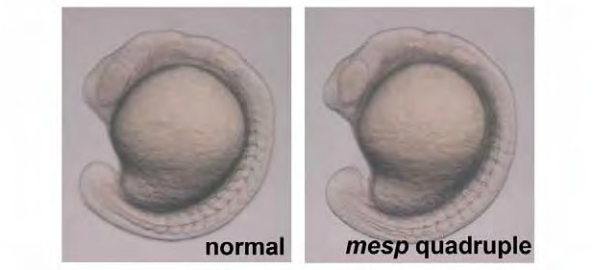

Figure 3. Phenotype of *mesp* quadruple mutant embryo. In contrast to the mouse, zebrafish *mesp* genes are dispensable for the formation of segmentation boundaries

IV. Molecular mechanism of the development 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 are currently examining the function of several genes essential for the segmentation and morphogenesis of pharyngeal pouches using fish and mice as model systems.

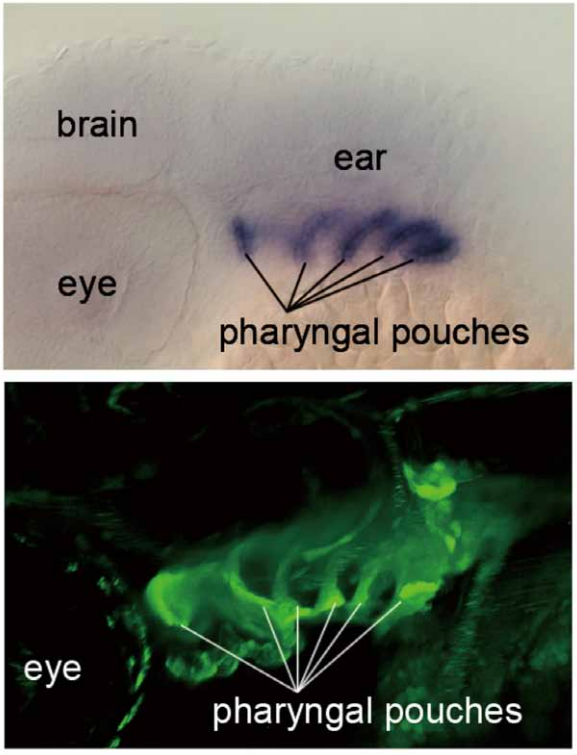

Figure 4. Segmental structure of the pharyngeal pouches in zebrafish embryos. Endodermal gene expression of *pax1* mRNA (Upper) and *sox17-egfp* (Lower) show the formation of segmental structures of the pharyngeal pouches.

Publication List:

〔Original papers〕

- Kametani, Y., Chi, N.C., Stainier, D.Y.R., and Takada, S. (2015). Notch signaling regulates venous arterialization during fin regeneration. Genes Cells *20*, 427-438.
- Okubo, T., and Takada, S. (2015). Pharyngeal arch deficiencies affect taste bud development in the circumvallate papilla with aberrant glossopharyngeal nerve formation. Dev. Dyn. *244*, 874-887.