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

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

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

I. Structural, biophysical and biochemical characteristics of secreted Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. It has been traditionally 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 the situation. In accordance with this line of thinking, various models have been proposed for Wnt transport between producing and receiving cells (Figure 1). Thus, to better understand the spatial regulation of tissue morphogenesis, the molecular mechanism underlying the spreading of Wnt proteins should be revealed.

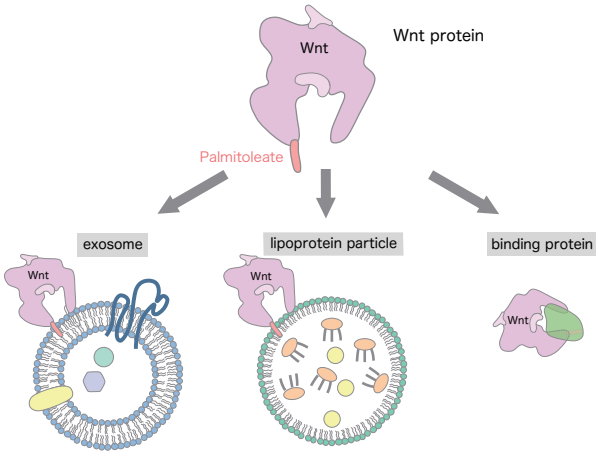

Figure 1. Various extracellular deliverers associating with Wnt involved in Wnt trafficking; extracellular membranous deliverers, including exosomes and lipoprotein particles, are known to associate with Wnts. In addition, specific Wnt-binding proteins, including Swim in Drosophila and sFRPs in vertebrates, were reported to be involved in extracellular Wnt trafficking. These various machineries appear to modulate the Wnt signaling range in differing ways.

To attain a better understanding of the spreading of Wnt proteins, we started to examine the characteristics of Wnt proteins both *in vitro* and *in vivo*. Since Wnt proteins are easily assembled during conventional biochemical analysis, we are trying to utilize non-invasive methods for this characterization. We established an L cell line, in which mouse Wnt3a tagged with monomeric GFP was efficiently produced, and analyzed the size of GFP-tagged Wnt3a secreted in the conditioned medium. The average size of GFP-Wnt3a, which was estimated by measuring the diffusion constant of fluorescent particles using Fluorescence Correlation Spectrometry (FCS), was larger than that predicted for the monomer.

To further examine Wnt proteins in the presence of serum, we used analytical ultracentrifugation in combination with a fluorescence detection system (AUC-FDS), which allowed us to monitor sedimentation of GFP-tagged proteins. While

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2018. The formertitle is indicated by an asterisk (*).

no peak corresponding to the monomeric form of Wnt3a was detected, a major peak with the apparent molecular mass of ~150 kDa was observed. Biochemical analysis including immune-depletion revealed that this peak corresponded to a complex with a serum protein called afamin, which was recently reported to bind with Wnt proteins in the culture medium.

In addition, AUC-FDS analysis revealed that secreted Wnt3a form high-molecular weight (HMW) complexes at their smallest peak with an apparent molecular mass of ~200 kDa. We further examined the HMW complexes more precisely by using FLAG-tagged Wnt3a, which can reduce the steric effect of tagged peptides than GFP-tagged ones. After affinity purification with anti-FLAG antibodies, purified Wnt3a proteins were separated by gel filtration. Western blotting of each fraction using Blue Native PAGE, which can separate protein complexes maintaining native conformation, indicated that the size of Wnt3a protein complex is gradually distributed between ~150 kDa and 1200 kDa.

Interestingly, the smallest fraction of Wnt3a proteins exhibited a distinct band in Blue Native PAGE analysis. Since Wnt3a is the dominant protein in this fraction, this result strongly suggested that Wnt3a forms a discrete homo-oligomer. Judging from the size of this distinct band, this oligomer corresponds with a trimer. Cross-linking analysis with this fraction confirmed as much. These findings are in agreement with the AUC-FDS results, where the apparent molecular mass of the smallest HMW complex was similar to that of GFP-Wnt3a homo-trimer. All of this evidence indicates that homo-trimers are the smallest form of the HMW complexes. Furthermore, crosslinking analyses with other Wnt3a containing fractions suggested that the homo-trimer is the smallest structural unit of larger HMW complexes recovered from other fractions.

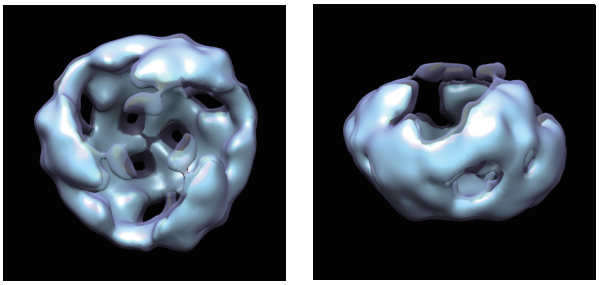

Figure 2. Image of Wnt3a reconstructed by single particle analysis. Top view (right) and side view (left) are shown.

Electron microscopic images of protein particles in each fraction revealed that the size and shape of most particles in the smallest fraction looked relatively uniform. We attempted to reconstruct a 3D structure of Wnt trimer by single particle analysis (Figure 2). As expected, the reconstructed image shows a three-fold rotational symmetry structure, indicating that three Wnt3a molecules were arranged on the same circumference of the circle in order to overlap each other. Given that the palmitoleoylate adduct should be shielded from aquatic environments, the partial overlapping would be beneficial in this case.

II. Regulation of spatial distribution of Wnt proteins in vertebrate embryos

To examine the mobility of Wnt3a in the extracellular milieu, we performed FCS analysis of GFP-Wnt3a in *Xenopus* embryos. This examination revealed that the dynamic behavior of Wnt3a could be divided into two distinct states. The fast population appears to reflect quickly and freely diffusing molecules, and the slow population reflects slowly moving molecules probably by interacting with extracellular matrices. Further precise analyses revealed that HMW complexes were less mobile than relatively smaller ones.

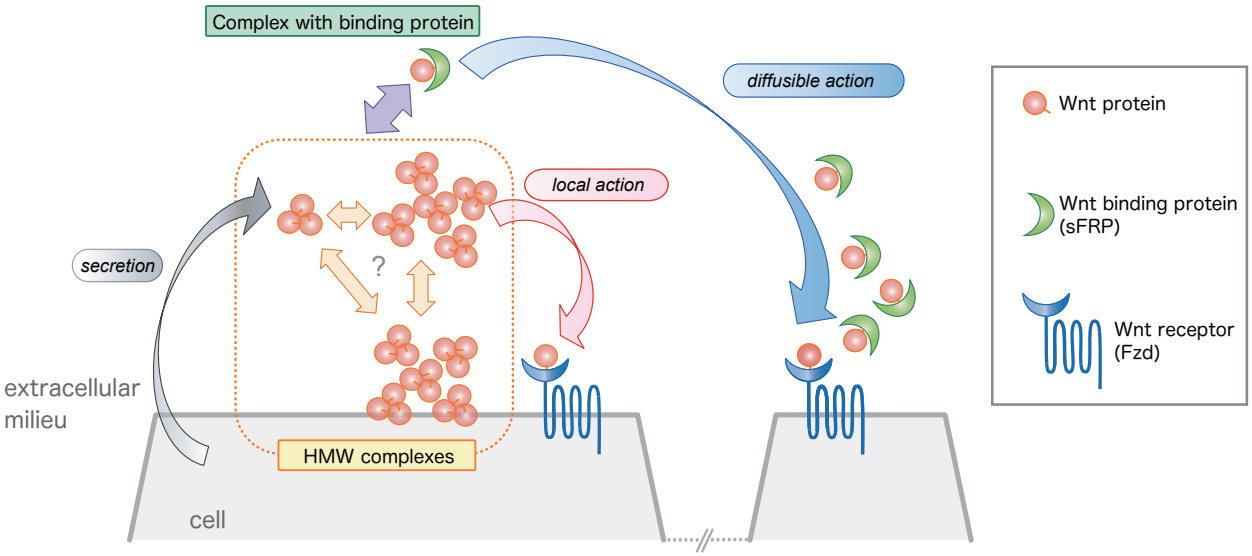

Figure 3. Model of Wnt protein diffusion: Wnt trimers are the smallest unit of the HMW complex. Both the trimer and the HMW complex appear to exist in the extracellular milieu although it is uncertain when the assembly to the HMW complex occurs during the process of Wnt secretion. The HMW complex is probably less mobile when interacting with the plasma membrane, resulting in the restriction of Wnt diffusion range. Some Wnt molecules can be dissociated by local interaction with Frizzled receptor (Fzd), resulting in a short-range signal (local action). In contrast, the HMW complex, probably as well as the trimer itself, can also be dissociated by interaction with soluble Wnt binding protein (partner protein), including sFRP. By this dissociation, Wnt turns to be more mobile and its diffusion range is expanded (diffusible action).

We showed that the trimer and larger HMW complexes of Wnt3a proteins could be dissociated by interaction with their receptor Frizzled8 and with secreted Wnt binding protein, sFRP2, in vitro by utilizing AUC-FDS. Similarly, this dissociation was detected in vivo by FCS. Importantly, the results of FCS suggested that dissociation of the large assembly of Wnt3a proteins by interaction with sFRP can make Wnt more mobile, probably resulting in longer diffusion distance in the embryo. These ideas are supported by a finding that states that the distribution range of Wnt3a was expanded by the co-expression of sFRP2 in *Xenopus* embryos. These results showed that large assemblies of Wnt3a are less mobile and sFRP2 can expand the diffusion range of Wnt proteins in *Xenopus* embryos. Based on these results, we propose a model that contends that assembly and dissociation of the dissociable oligomers modulate Wnt signaling range (Figure 3).

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 at the posterior of 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 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 them. This spatial pattern is established in the anterior PSM by converting the oscillatory gene expression into spatially restricted patterns of gene expression. Several intracellular molecules, as well as FGF and Wnt signaling, are involved in this conversion. It has been generally considered that Mesp of bHLH transcriptional regulator plays a critical role 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. In contrast, recent studies by this and other groups strongly suggest that Mesp2 does not directly define the position of the segmentation boundary, rather that other genes called *Ripply1* and *Ripply2* play more essential roles in this conversion in the mouse and zebrafish. *Ripply* genes encode ~100 amino acid proteins, which commonly possess 2 distinct amino acid sequences: a highly conserved WRPW stretch and a conserved ~50-amino acid stretch, called the Ripply homology (RH) domain, that interacts with the T-box proteins, including Tbx6, which is involved in the positioning of the segmentation boundary. Currently, we are examining the mechanism of this conversion by focusing on the regulation of Ripply's function in zebrafish.

Publication List:

〔**Original papers**〕

- Kinoshita, H., Ohgane, N., Fujino, Y., Yabe, T., Ovara, H., Yokota, D., Izuka, A., Kage, D., Yamasu, K., Takada, S., and Kawamura, A. (2018). Functional roles of the Ripply-mediated suppression of segmentation gene expression at the anterior presomitic mesoderm in zebrafish. Mech. Dev. *152*, 21-31.
- Takada, R., Mii, Y., Krayukhina, E., Maruyama, Y., Mio, K., Sasaki, Y., Shinkawa, T., Pack, C.-G., Sako, Y., Sato, C., Uchiyama, S., and Takada, S. (2018). Assembly of protein complexes restricts diffusion of Wnt3a proteins Commun. Biol. *1*, 165.
- Tsuchiya, Y., Mii, Y., Okada, K., Furuse, M., Okubo, T., and Takada, S. (2018). Ripply3 is required for the maintenance of epithelial sheets in the morphogenesis of pharyngeal pouches Dev. Growth Diff. *60*, 87-96.