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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 t his 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

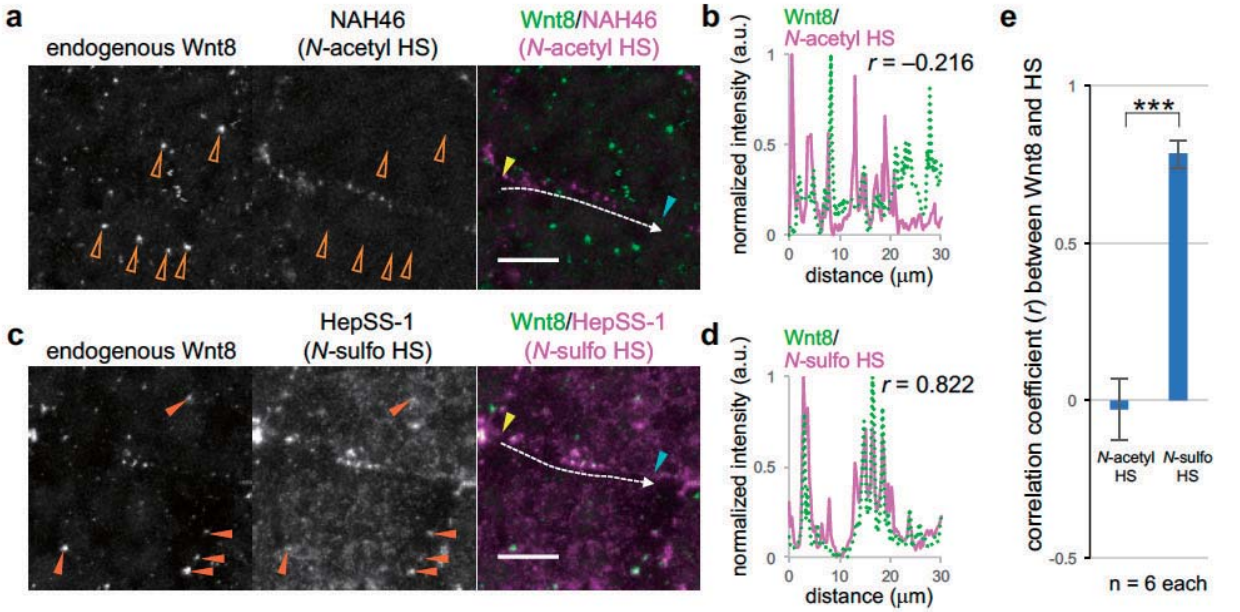

Figure 1. Colocalization of Wnt8 and N-sulfo-rich HS clusters. **a**-**d**, The colocalization of endogenous Wnt8 and a subpopulation of HS. Gastrula embryos (st. 10.5) were coimmunostained for Wnt8 and NAH46 epitopes (*N*acetyl HS) or HepSS1 epitopes (*N*sulfo HS) at the ventral marginal zone (**a**, **c**). Signal intensities along white arrows were plotted (**b**, **d**), starting and ending points as indicated by yellow and cyan arrowheads, respectively, in **a** and **c**. Correlation coefficients (*r*) for the plots were as indicated. Of note, Wnt8 puncta inside cells were overlapped with *N*sulforich HS clusters (orange arrowheads in **b**) but not with *N*acetyl-rich HS clusters (open arrowheads in **a**). Scale bars, 10 μm. **e**, A high correlation between distributions of Wnt8 and *N*-sulfo HS. Correlation coefficients of distributions between Wnt8 and *N*-sulfo HS were significantly higher than that between Wnt8 and *N*-acetyl HS (mean +/– s.d. ***$p < 0.001$, t-test).

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2017. The former title is indicated by an asterisk (*).

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 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 some non-invasive methods for this characterization. Our preliminary study indicated that Wnt proteins are secreted by forming specific complexes. In addition, analysis of the mobility of Wnt complexes in the extracellular space suggested that Wnt proteins are not simply diffused during embryogenesis. 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.

We first examined the distribution of the endogenous Wnt8 protein in *Xenopus* embryos. Wnt8 shows a gradient in the embryo, but at the same time, it is accumulated on the cell surface in a punctate manner. Accumulated Wnt8 is colocalized with a specific state HS, the sulfonated state of HS (*N*-sulfo HS; Figure 1). Furthermore, we found that the default or non/less-sulfonated state (*N*-acetyl HS; Figure 1a, b) and the sulfonated state of HS (*N*-sulfo HS; Figure 1c, d) are separately clustered. *N*-sulfo HS clusters with accumulated Wnt8 recruit the receptor Frizzled (Fzd) and the transducer Dishevelled (Dvl), possibly forming a signaling complex called the signalosome or multivesicular body, suggesting a direct connection between the distribution of Wnt and signalling. In contrast *N*-acetyl HS cluster can accumulate the secreted Wnt inhibitor Frzb. With overexpressed Frzb, Wnt8 is trapped by *N*-acetyl HS cluster, which is suggested to contribute to antagonism of Wnt signaling. We further demonstrate that Gpc4, a Dlp-ortholog forms both *N*-sulfo and *N*-acetyl HS while Gpc5, a Dally-ortholog forms *N*-sulfo HS. Thus, our finding of discrete clusters of HS provide a regulatory basis for the distribution and signaling of Wnt, discussed above. We further demonstrate that internalization of *N*-sulfo HS is more frequent than that of *N*-acetyl HS, which could explain the differences between the short- and long-range distributions of Wnt and Frzb, respectively and also range-expansion of Wnt8 by Frzb (Figure 2) .

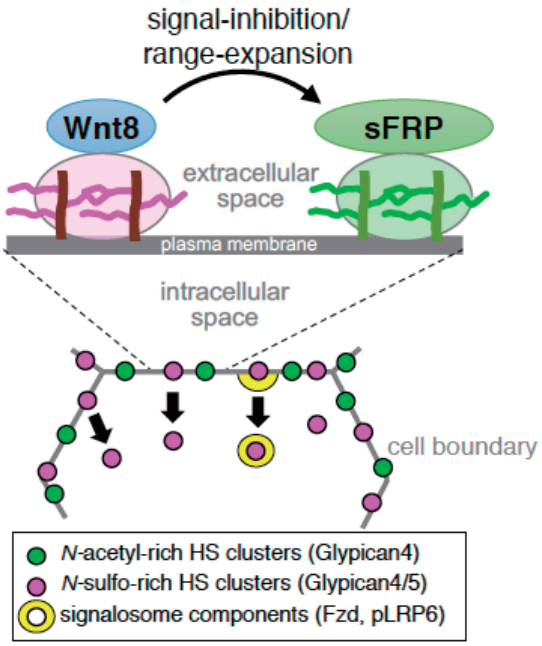

Figure 2. Model of the *N*sulforich and *N*acetylrich HS cluster system for the regulation of the extracellular distribution and signal reception of the Wnt morphogen. The two types of HS clusters, *N*-sulfo-rich and *N*-acetyl-rich HS clusters differ in several aspects including core proteins, tendency of internalization and capability of signaling. Together with the specificity of Wnt8 to the *N*-sulfo-rich HS clusters and that of Frzb to the *N*-acetyl-rich HS clusters, the two types of HS clusters could serve as a system to regulate the extracellular distribution and signal reception of the Wnt morphogen.

II. 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. 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 us and other groups strongly suggested that Mesp2 does not directly define the position of the segmentation boundary, rather 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.

III. 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 the mechanism of the development of pharyngeal pouches, we sought to examine mouse embryos defective for *Ripply3*, a member of the Ripply family. In mouse embryos, *Ripply3* is specifically expressed in the pharyngeal endoderm and ectoderm. We previously showed that *Ripply3*-deficient embryos fail to develop the pharyngeal arches posterior to the 2nd pharyngeal pouch. However, the molecular and cellular mechanisms by which Ripply3 regulates the morphogenesis of pharyngeal pouches have still been unclear.

To better understand the cellular process mediated by *Ripply3*, we sought to identify the primary defect observable in *Ripply3*-deficient mouse embryos. First, by following *Ripply3* expression during the formation of the 3rd pharyngeal pouch, we found that *Ripply3* expression was correlated with the position where epithelial bending took place. In addition, precise histological analysis at short-term intervals revealed that *Ripply3* was required for the maintenance of a continuous monolayer of pharyngeal epithelial sheets during their bending. Further analyses using *Ripply3*-deficient embryos and Ripply3-expressing culture cells supported an idea that Ripply3 promotes maturation of adhesive contacts transmitting mechanical force between the extracellular matrix and the interacting cells. Based on these results, we propose a model in which Ripply3 plays a role for the resistance against the mechanical stress in epithelial bending probably by enhancing the attachment of the pharyngeal endoderm cells to the extracellular matrix.

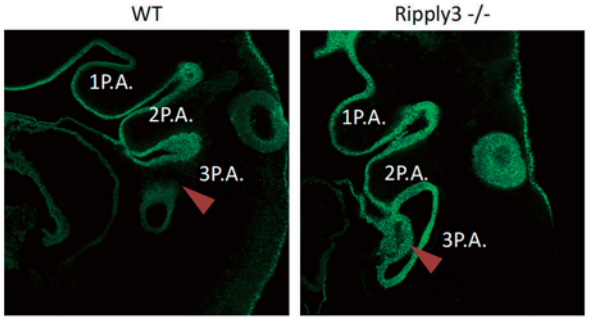

Figure 3. Ripply3 is required for the proper morphogenesis of the posterior pharyngeal pouches in the mouse embryo. The formation of the 3rd pharyngeal arch (red arrowhead) was impaired in Ripply3 deficient embryos.

Publication List:

〔Original papers〕

- Mii, Y., Yamamoto, T., Takada, R., Mizumoto, S., Matsuyama, M., Yamada, S., *Takada, S., and *Taira, M. (*Co-corresponding authors) (2017). Roles of two types of heparan sulphate clusters in Wnt8 distribution and signalling in Xenopus. Nat. Commun. *8,* 1973.
- Tokue, M., Ikami, K., Mizuno, S., Takagi, C., Miyagi, A., Takada, R., Noda, C., Kitadate, Y., Hara, K., Mizuguchi, H., Sato, T., Taketo, M.M., Sugiyama, F., Ogawa, T., Kobayashi, S., Ueno, N., Takahashi, S., Takada, S., and Yoshida, S. (2017). SHISA6 confers resistance to differentiation-promoting Wnt/β-catenin signaling in mouse spermatogenic stem cells. Stem Cell Rep. *8,* 561-575.

〔Review article〕

- Takada, S., Fujimori, S., Shinozuka, T., Takada, R., and Mii, Y. (2017), Differences in the secretion and transport of Wnt proteins. J. Biochem. *161*, 1-7.