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The body and tissues of the developing embryo are repeatedly divided into sub-regions specified by characteristic gene expression and morphology. The process that gives rise to these sub-regions, according to a defined pattern, is called “pattern formation” or “patterning.” Our laboratories aim to understand the molecular mechanisms underlying pattern formation by several different approaches.

The most popular model to explain the patterning process is the “morphogen gradient and threshold” theory. Many genetic results indicate that secreted signal proteins such as Wnt, BMP, and Hedgehog function as morphogens in many aspects of the patterning process. In spite of the accumulation of genetic evidence, however, the biochemical characteristics of morphogens, including modification and higher order structure, remain to be elucidated. Thus, one of our major goals is to reveal *the real image* of morphogens and the molecular mechanisms underlying the formation of morphogen gradients, including the secretion and extracellular transport of these morphogens.

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 morphogen gradient and threshold, but by a unique mechanism proceeding periodically. For instance, somites are sequentially generated in an anterior-to-posterior order by converting oscillatory gene expression into repeated 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 mechanisms 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 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, presumably since their secretion and transport are under tight control. To understand the molecular mechanisms underlying morphogenesis mediated by extracellular transmission of Wnt signals, it will be required to reveal manners of diffusion and/or accumulation of Wnt proteins in extracellular space.

For a better understanding of the extracellular transport of Wnt proteins, we started to visualize Wnt proteins in the extracellular space by a two different approaches. One is a live imaging approach with EGFP-tagged Wnt proteins and the other is immnohistochemistry. Our preliminary study indicated that Wnt proteins are not simply diffused during embryogenesis of the mouse. We precisely examined regulatory mechanisms and the biological significance of Wnt protein distribution in mouse embryos. These analyses reveal a novel view of spatial regulation of Wnt signaling.

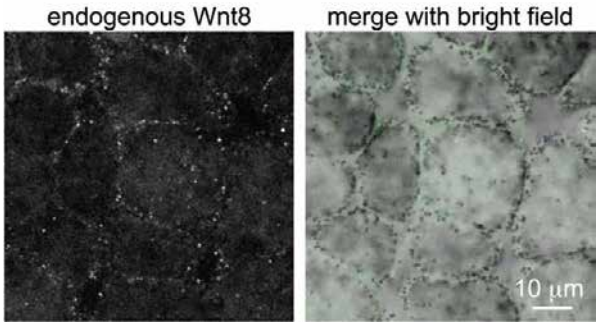

Figure 1. Accumulation of Wnt proteins between cells. Wnt proteins are acculmulated in a punctate pattern between epithelial cells in a *Xenopus* embryo. (Left) Immunostaining of endogenous Wnt8 proteins (Right) The left image is merged with a bright field one.

Extracellular molecules are known to regulate special distribution of Wnt proteins. Heparan sulfate sugar chains with different 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 an enzyme catalyzing N-sulfation of heparan sulfate. Gain and loss of function studies of this enzyme indicated that it is necessary and sufficient for the conversion of N-acetyl HSNSs into N-sulfo HSNSs. Extracellular accumulation of Wnt proteins and the convergent extension movement, which is regulated by Wnt non-canonical signaling, were also affected by perturbation of expression of this enzyme. These results indicated that N-sulfo modification of heparan sulfates are important for extracellular distribution of Wnt proteins and Wnt signaling.

II. Heterogeneity of secreted Wnt proteins secreted from cultured cells

Although the structure of Wnt protein has already been revealed, its higher order structure in extracellular space has not yet been fully understood. One of the proposed forms of Wnt protein in extracellular space binds 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 in these two forms from the same cells.

To address these issues, we systematically examined characteristics of Wnt proteins secreted from several different cell lines. First, we fractionated conditioned media of Wnt3a-expressing cells by density gradient centrifugation with Sodium Bromide. To examine whether Wnt proteins are attached to exosomes, we then collected exsosome-like molecules by ultracentrifugation and fractionated the pellet by sucrose density gradient ultracentrifugation. Our results indicate that secreted forms of Wnt proteins differ among different Wnt subtypes and this diversity is dependent on 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 results 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 mice, the prospective segmentation boundary is periodically established in the anterior PSM at the rostral border of 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 *2*, have shown to be required for the down-regulation of Tbx6 proteins by analysis of 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. However, it is still to be elucidated whether Mesp2 causes the retreat of the Tbx6 protein border through the activation of Ripplys' expression or that Mesp2 and Ripplys regulate this retreat in parallel.

To answer this question, we precisely examined function of mwsp and ripply in the regulation of Tbx6 proteins in zebrafish embryos. By utilizing an antibody against zebrafish Tbx6/Fss, we found that the anterior border of Tbx6 domain coincided with the presumptive intersomitic boundary also in the zebrafish and it shifted dynamically during 1 cycle of segmentation. Consistent with the findings in mice, the *tbx6* mRNA domain was located far anterior to its protein domain, indicating the possibility of posttranscriptional regulation. When both *ripply1/2* were knockeddown, the Tbx6 domain was anteriorly expanded. We further directly demonstrated

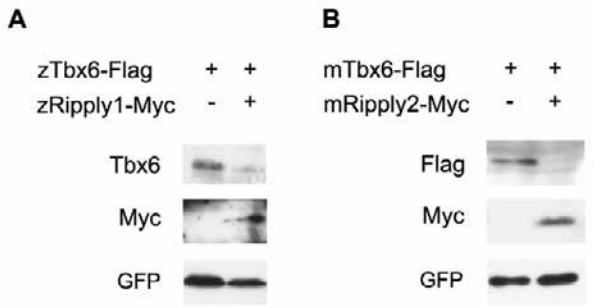

Figure 2. Ripply reduces Tbx6 protein levels: Western blotting with proteins recovered from embryos injected with Flag-tagged zebrafish *tbx6*mRNA and Myc-tagged zebrafish *ripply1* (A) or mouse Ripply2 mRNAs (B). Both zebrafish ripply1 and mouse Ripply2 reduceTbx6 protein levels.

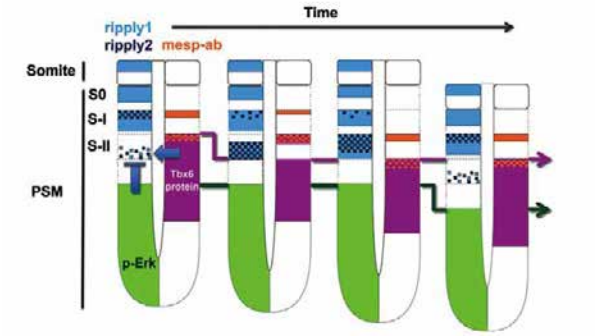

Figure 3. Schematic representation of spatial patterns of Tbx6 and p-Erk domains with *ripply1*, *ripply2*, and *mesp-ab* expressions during a single segmentation cycle. Expression of *ripply1* and *ripply2* is initially activated in the high Tbx6 / low FGF signaling region. These activated Ripplys appear to suppress Tbx6 protein resulting in formation of a new anterior border of the Tbx6 core domain and the upper band.

that Ripply could reduce the expression level of Tbx6 protein depending on physical interaction between Ripply and Tbx6 (Figure 2). Moreover, the onset of *ripply1* and *ripply2* expression occurred after reduction of FGF signaling at the anterior PSM, but this expression initiated much earlier on treatment with SU5402, a chemical inhibitor of FGF signaling. These results strongly suggest that Ripply is a direct regulator of the Tbx6 protein level for the establishment of intersomitic boundaries and mediates a reduction in FGF signaling for the positioning of the presumptive intersomitic boundary in the PSM (Figure 3).

IV. Establishment of gene knock out methodology based on genome editing technologies in the zebrafish

Zebrafish are one of the most suitable model animals for genetic analysis. Until now, chemical mutagenesis-mediated screening provided us a great number of mutant strains, which contribute to studies of morphogenesis of vertebrates. In addition to this well-established genetic approach, recent advances in genome editing technologies have made it possible to create any mutation of a gene of interest.

Previous studies revealed mouse *Mesp2* has essential roles for multiple processes of somitogenesis including determination of position of segment boundaries, generation of somite boundary structure and establishment of rostro-caudal polarity in each somite. Although four *mesp* genes have been identified in the zebrafish genome, their roles in somitogenesis still remained unclear. To address this question we generated mutant fish carrying the frame shift mutation in all *mesp* genes using TALEN mediated mutagenesis and analyzed its phenotype. Unlike the mouse *Mesp2* mutant, zebrafish mesps quadruple knockout embryo exhibited normally segmented somites. We are currently examining several mutants generated by TALEN mediated mutagenesis.

Publication List

〔Original papers〕

- Kimura, T., Nagao, Y., Hashimoto, H., Yamamoto-Shiraishi, Y., Yamamoto, S., Yabe, T., Takada, S., Kinoshita, M., Kuroiwa, A., and Naruse, K. (2014). Leucophores are similar to xanthophores in their specification and differentiation processes in medaka. Proc. Natl. Acd. Sci. USA *111*, 7343-7348.
- Osipovich, A.B., Long, Q., Manduchi, E., Gangula, R., Hipkens, S.B., Schneider, J., Okubo, T., Stoeckert, C.J. Jr., Takada, S., and Magnuson, M.A. (2014). Insm1 promotes endocrine cell differentiation by modulating the expression of a network of genes that includes Neurog3 and Ripply3. Development *141*, 2939-2949.
- Wanglar, C., Takahashi, J., Yabe, T., and Takada, S. (2014). Tbx protein level critical for clock-mediated somite positioning is regulated through interaction between Tbx and Ripply. PLoS One *9*, e107928.