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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 mechanism 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 mechanism 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 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 underlie the periodical and sequential sub-division in the development of somites and pharyngeal arches.

I. Structural features of Wnt proteins secreted from polarized epithelial cells

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 mechanism 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 the extracellular space. Of note, Wnt proteins are specifically modified with fatty acids during their secretion, as shown by several groups including us, suggesting that some specific structural feature caused by this modification may affect movement of Wnt proteins in the extracellular space.

Epithelial cells, as well as other type of cells, secret Wnt proteins during morphogenesis. Wnt proteins are secreted both from the apical and baso-lateral side of epithelial cells, but evidence suggested that the action range of Wnt proteins may be different according to side of secretion. On the other hand, structural features of secreted Wnt proteins appear to be heterogeneous. For instance, in Drosophila imaginal disc epithelium, Wg, the ortholog of vertebrate Wnt1, could be loaded onto lipoprotein particles and transported over large distances. Recent reports showed that Wnts were also secreted on exosome-like vesicles from cultured mouse L cells or Drosophila S2 cells. However, what is the possible contribution of lipoprotein particles or exosome in polarized secretion of Wnts is still unknown.

To better understand the molecular basis of generating a variety of signaling ranges, we examined structural features of Wnt proteins secreted from either the apical or the baso-lateral side of epithelial cells by using the MDCK (Madin-Darby Canine Kidney) cell as a model system. MDCK cells that stably express mouse Wnt3a were grown on a polarized monolayer on tissue culture inserts, which separate an upper apical compartment from the lower compartment. We found that polarized MDCK cells preferentially secreted Wnt3a from the basolateral side. To determine if the protein is present in the culture medium as freely soluble protein or associated with exosomes or with lipoprotein partiles, we fractionated conditioned medium by centrifugation. We found most Wnt3a proteins were present in the medium, indicating that they were secreted as a freely soluble form. Wnt3a proteins were also found to be packaged onto exosome-like vesicles and were exclusively secreted basolaterally. In addition, we found that the conserved serine residue, S209, which is required for lipid modification, was essential for proper secretion of Wnt3a from MDCK cells. These results indicated that Wnt3a secretion via exosomes specifically occurred on the basolateral side and required lipid modification (Figure 1).

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2013. The former title is indicated by an asterisk (*).

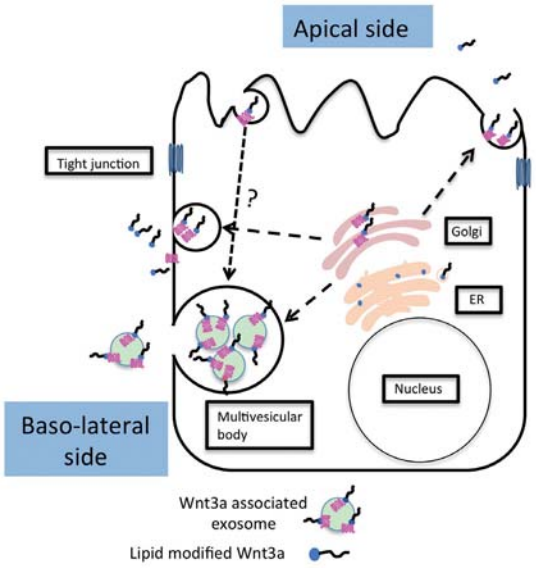

Figure 1. Model of polarized secretion of Wnt proteins form epithelial cells. Most Wnt3a proteins are secreted into both apical and basolateral sides. In addition, some Wnt3a proteins were also found to be packaged onto exosome-like vesicles and were exclusively secreted basolaterally. It is unclear how these exosome-like vesicles are formed during their secretion process..

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. In the mouse, 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 Tbx6 protein domain because Mesp2 expression requires Tbx6 proteins. Thus, the anterior border formation of Tbx6 protein domain is a more fundamental process in the positioning of the segmentation boundary (Figure 2).

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. In addition, contrasting to the analysis with mouse mutants, Ripplys have also been implicated in acting in transcriptional repression in association with co-repressor Groucho/TLE and some T-box proteins, including zebrafish and Xenopus Tbx6. Therefore, it is also unclear whether Ripplys regulate Tbx6 proteins at the protein level in other species besides mouse.

For better understanding of somite patterning, we have examined zebrafish embryos, in which the gene functions involved in this process seem to be relatively precisely understood. In this study, by generating an antibody specific for zebrafish Tbx6/Fss, previously referred to as Tbx24, we examined the relationship between spatial distribution of

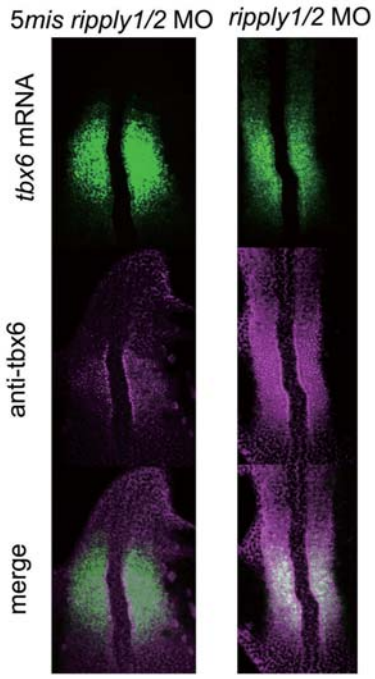

Figure 2. The zebrafish tbx6 mRNA expression expands from the PSM anteriorly to several newly formed somites. In contrast, the anterior border of the protein is restricted in the PSM to define the segmentation boundary. This restriction is regulated by ripply1/2 as observed in ripply1/2 defective embryos where the tbx6 protein domain is expanded anteriorly.

Tbx6 proteins and positioning of intersomitic boundaries in zebrafish embryos. We found that the anterior border of Tbx6 defines the segmentation boundary in zebrafish as previously shown in mouse. However, the dynamics of Tbx6 proteins are partly different from that in the mouse, suggesting that the spatial dynamics of the Tbx6 protein domain is also involved in the rostro-caudal patterning. Furthermore, we directly examined the ability of Ripply or Mesp to reduce the level of Tbx6 proteins in zebrafish eggs. Ripply, but not Mesp, down-regulated Tbx6 protein level when these mRNAs were co-injected into zebrafish eggs. Importantly, this down-regulation is dependent on physical interaction between Ripply and Tbx6. Consistent with these results, *ripply1* and *2* morphants showed expansion of Tbx6 protein domain in zebrafish embryos (Figure 2). These results strongly suggest that Ripply, but not Mesp, is a direct regulator of Tbx6 protein level in the establishment of intersomitic boundaries.

III. Establishment of gene knock out methodology based on TALEN-mediated gene editing 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 the 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.

One of these technologies is based on Transcription Activator-Like Effector Nuclease (TALEN). To establish efficient conditions for TALEN-mediated gene disruption in zebrafish, we designed 48 TALENs for targeting 14 genes and confirmed that 31 of the 48 TALENs could induce mutations in zebrafish. As a result, we succeeded in efficiently generating gene disrupted lines for all of the genes tested, indicating that TALEN-mediated gene disruption is a convenient technology for generating mutation of a gene of interest in a laboratory setting. By using this technology, we generated mutants for many genes specifically expressed during early embryogenesis (Figure 3).

Publication List

〔Original papers〕

- Hisano, Y., Ota, S., Takada, S., and Kawahara, A. (2013). Functional cooperation of spns2 and fibronectin in cardiac and lower jaw development. Biol. Open. *2*, 789-794.
- Takahashi, Y., Yasuhiko, Y., Takahashi, J., Takada, S., Johnson, R.L., Saga, Y., and Kanno, J. (2013). Metameric pattern of intervertebral disc/vertebral body is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somites in the mouse embryo. Dev. Biol. *380*, 172-184.

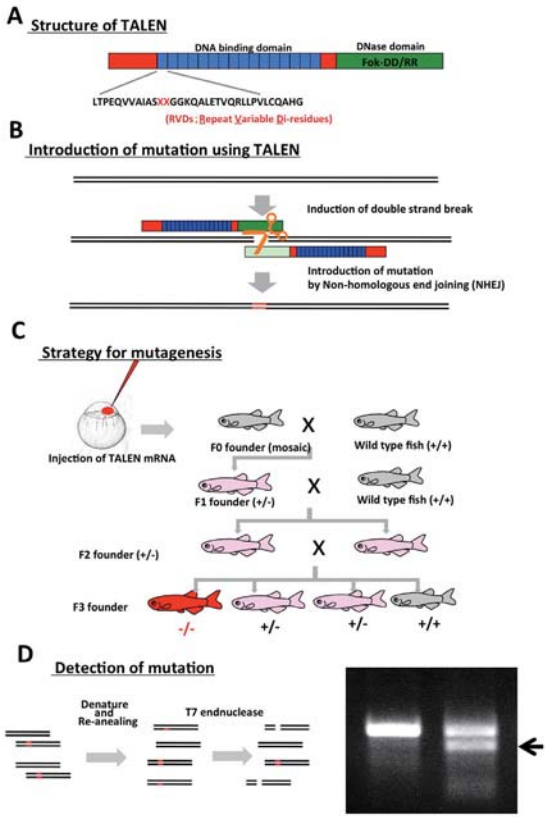

Figure 3. Example of TALEN-mediated gene disruption. A. Structure of TALEN construct. TALEN is an artificial nuclease containing Transcritional Activator Like Effector (TALE) of Xenthomonas and DNase domain. TALE domain is constituted from repeat units, each of which specifically recognize one particular nucleotide. The specificity of each unit is defined RDV sequence. B. Mutagenesis by TALEN. Two TALENs designed to recognize a particular site in the genome cause double strand break down, resulting in deletion and/or small insertion. C. Generation of homozygous mutant fish by crossing. D. Detection of mutation generated by TALEN. Genome DNA containing targeted site by TALEN was amplified by PCR. By heat denaturing and subsequent renaturation of the PCR products, DNA from embryos carrying mutations generate heteroduplex containing a mismatched sequence. Because this mismatched sequence can be digested by T7 endonuclease, heterozygous mutations can by detected. PCR products shown in the photo are example of T7 assay; Right lane: control embryo, Left lane: TALEN injected embryo