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

I. Lipid modification and extracellular trafficking of Wnt proteins

The Wnt family of secreted signal proteins plays a key role in numerous aspects of embryogenesis. Most Wnt proteins transmit their signals locally in most of the cases where they function, presumably since their secretion and transport are under tight control. One important step regulating the extracellular transport of various secreted signal proteins involves post-translational modification with lipid moieties. We found that murine Wnt-3a is modified with a mono-unsaturated fatty acid, palmitoleic acid, at a conserved Ser residue (Figure 1). Wnt-3a defective in this modification is not secreted from cells in culture or in Xenopus embryos, but is retained in the endoplasmic reticulum (ER). Furthermore, Porcupine (Porcn), a protein with structural similarities to membrane-bound O-acyltransferases, is required for this Ser-dependent modification, as well as for Wnt-3a transport from the ER for secretion. These results strongly suggest that the Wnt protein requires a particular lipid modification for proper intracellular transport during the secretory process. We expect that the discovery of this unexpected lipid modification might provide a clue regarding the higher-order structure of secreted Wnt proteins and their gradient formation.

Figure 1. Post-translational modification of Wnt proteins: Several secreted signal proteins are specifically modified. These modifications are required for proper secretion, extracellular trafficking, and binding to their receptors. Wnt proteins are lipidated, glycosylated, and sulfated. We have shown that one type of lipidation, palmitoleoylation, is required for proper secerion of several Wnt proteins and is dependent on an acyl-transferase, Porcupine. In this figure, a schematic representation of mouse Wnt3a, which is lipidated at Cys77 and Ser209 and glycosylated at Asn87/298, is shown. In addition, the positions of Tyrosine residues sulfated in Wnt5a/11 are also indicated. The conserved cysteines are shown with vertical bars.

On the other hand, recent evidence suggest that this Ser-dependent lipidation may not always be required for secretion of Wnt proteins. In the case of Wingless, the *Drosophila* ortholog of Wnt1, this Ser residue is not required for secretion from *Drosophila* S2 cells, but for the function of this protein, suggesting that the role of the Ser-dependent lipidation may differ for different Wnt proteins or in different cellular contexts. To reveal the role of the Ser-dependent lipidation in different contexts, we examined the role of Porcn in zebrafish embryos. *Porcn*-deficient zebrafish embryos only exhibited defects in convergent-extension (CE) movement, which requires non-canonical Wnt signaling. Surprisingly, these embryos showed no sign of reduction in canonical Wnt signaling. Consistent with this array of phenotypes, intracellular trafficking of zebrafish Wnt5b and mouse Wnt5a, but not zebrafish and mouse Wnt3a, was specifically impaired in *Porcn*-deficient embryos. These results indicate that *Porcn* is required for proper function and

trafficking of at least one Wnt protein during early developmental stages of zebrafish, but decrease of Porcn does not equivalently affect the trafficking and lipidation of different Wnt proteins in these embryos. Together, the mechanism of trafficking and modification of Wnt proteins appear to be inconsistent between different types of Wnts. We are currently examining the molecular mechanism underlying the variability of Wnt modification.

In addition to the study of the secretory process of Wnt proteins, we are also examining the extracellular transport of Wnt proteins during embryogenesis using frog and mouse embryos.

II. Molecular mechanism of somite development.

The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the "segmentation clock," which has been evidenced by the cyclic expression of genes in the presomitic mesoderm (PSM). For example, *hairy/Enhancer of split* (*Espl*)-related bHLH genes, including *her1* and *her7* in zebrafish, are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. Prior to morphological segmentation, a segmental pre-pattern, characterized by the periodical borders between neighboring somites and by the rostro-caudal within a somite, is established in the anterior PSM. We have already shown that a gene identified by our *in situ* hybridization screening, *ripply1,* is required for the maintenance of the rostro-caudal patterning in zebrafish embryos. Ripply proteins have been shown to associate with the transcriptional co-repressor Groucho/TLE and the T-box transcription factors through two distinct amino acid sequences, one termed the WRPW motif, which is a highly conserved 4-amino acid stretch in the N-terminal half, and the other, the Ripply homology (RH) domain, a conserved ~50-amino acid stretch that interacts with the T-domain of the T-box proteins. Therefore, Ripply is able to recruit the Groucho/TLE co-repressor to T-box proteins and control their intrinsic transcriptional properties.

Recently, we also showed that Ripply1 and another structurally related-protein, Ripply2, are required for the positioning of intersomitic boundaries in mice. The positions of somite boundaries are defined by the anterior limit of the Tbx6 protein domain, which is regulated by degradation of Tbx6 proteins. In addition, a transcription factor, Mesp2, is required for this degradation although it is uncertain how Mesp2 induces this degradation. We showed that expression of mouse *Ripply1* and *Ripply2* is dependent on Mesp2 and *Ripply1/2*-deficient mouse embryos exhibit anterior expansion of the Tbx6 protein domain, suggesting that *Ripply1/2* expression by *Mesp2* is important in the establishment of somite boundaries by regulating Tbx6 degradation. To compare the mechanism of somite segmentation between different species, we are also examining the mechanism of the formation of intersomitic boundaries in zebrafish.

III. The role of Ripply3 in the development of pharyngeal arches.

The pharyngeal apparatus is another example of segmental structure in the vertebrate embryo. The pharyngeal apparatus is a transient structure formed ventrolateral to the hindbrain in vertebrate embryos. This structure consists of bilaterally segmented arches, and ectodermal grooves and endodermal pouches, both of which are formed between the arches. Components of the pharyngeal apparatus give rise to distinct tissues in later stages of development. For instance, the pharyngeal arteries and neural crest cells in the caudal pharyngeal arches contribute to cardiovascular development, while the endodermal cells located in the caudal pouches give rise to several organs, including the thymus and parathyroid gland. Thus, pharyngeal development is a key process in the generation of these organs.

Chromosome 22q11 deletion syndrome (22q11DS), which includes the DiGeorge syndrome (DGS), is characterized by the abnormal development of the pharyngeal apparatus in the form of thymic hypoplasia or aplasia, hypocalcemia arising from parathyroid hypoplasia, and defective cardiac outflow. A number of mouse genetic studies and mutational analyses in human patients have indicated that *Tbx1*, which encodes a member of the T-box family of transcription factors, is the most likely gene responsible for the phenotype of 22q11DS. During murine pharyngeal development, *Tbx1* is first expressed in the mesoderm at E7.5. Between E8.5 to 11.5, *Tbx1* also becomes located in the pharyngeal endoderm, ectoderm, and core mesoderm, but not in the neural crest cells. Cell type-specific inactivation and analysis of downstream targets of *Tbx1* in mice indicate that *Tbx1* plays multiple roles in endoderm, mesoderm, and ectoderm cells during pharyngeal development. However, the molecular mechanisms underlying the cell type-specific roles of Tbx1 have not been fully elucidated.

Interestingly, another member of the Ripply family, Ripply3, is expressed in the pharyngeal endoderm cells. Because we found that both *Ripply3* and *Tbx1* were strongly expressed in the pharyngeal endoderm and endoderm, we then examined the role of Ripply3 by generating *Ripply3*-deficient mice, as well as its relationship with Tbx1. We show that Ripply3 can modulate Tbx1 activity in *in vitro* luciferase reporter assays. Furthermore, *Ripply3*-knock out mouse showed hypotrophy of the caudal pharyngeal apparatus (Figure 2). Ripply3 represses Tbx1-induced expression of *Pax9* in *in vitro* luciferase assays, and *Ripply3*-deficient embryos exhibit up-regulated *Pax9* expression. Thus, our results showed that *Ripply3* most likely plays its role in pharyngeal development by regulating Tbx1 activity.

More precise analysis revealed that the development of pharyngeal derivatives was impaired in *Ripply3* mutant mice, including ectopic formation of the thymus and the parathyroid gland, as well as cardiovascular malformation. For instance, the disrupted development of the caudal pharyngeal arches seems to have resulted in two distinct cardiovascular defects in the *Ripply3*$^{-/-}$ embryos. One involved an abnormality in heart development. The severe

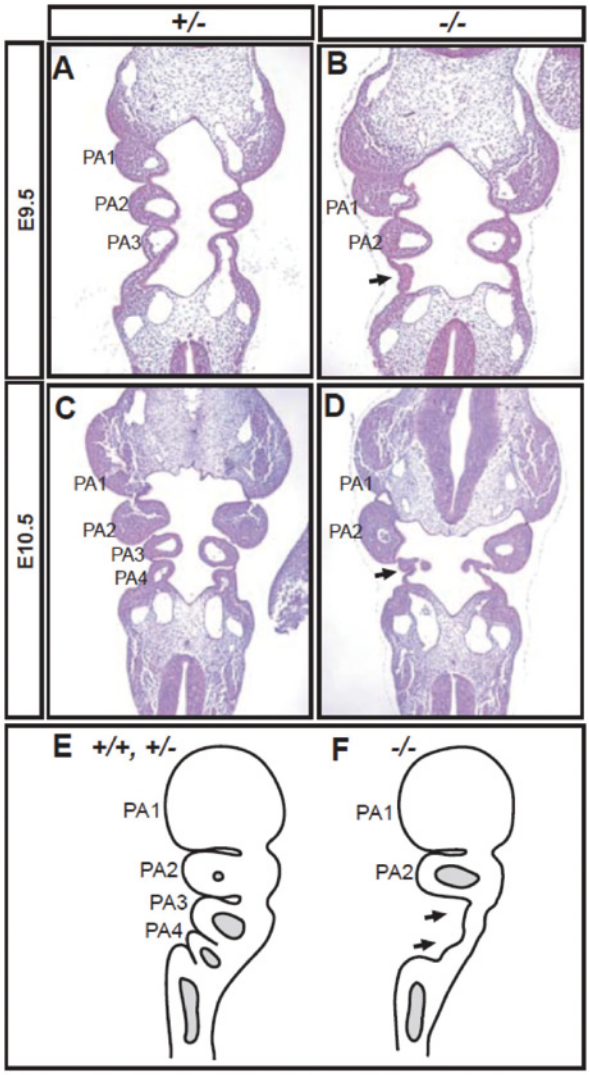

Figure 2. *Ripply3* is required for proper development of the third and fourth pharyngeal arches: (A-D) Hematoxylin-eosin-stained coronal sections of *Ripply3* $^{+/-}$ (A, C) and *Ripply3* $^{-/-}$ (B, D) embryos at E9.5 (A,B) and E10.5 (C,D). (E, F) Schematic images illustrating phenotypes of *Ripply3* $^{+/-}$ (E) and *Ripply3* $^{-/-}$ (F) embryos.

reduction in neural crest cell number in the caudal pharyngeal arches appears to have led to the abnormal development of the outflow tract, including hypotrophy of the aorta and incomplete formation of the ventricular septum. The other defect involved the loss of the 3rd and 4th PAAs. This defect appeared to result in abnormal development of the vascular system including deletion of the aortic arch and misshapen major blood vessels. Interestingly, the second PAAs, which normally disappear after E10.5, persisted until birth in the *Ripply3*$^{-/-}$ embryos (Figure 3). Persistence of the 1st and 2nd PAAs has also been reported in *Endothelin-1*-deficient mouse embryos, where the 4th PAAs are also poorly developed, suggesting that the formation of PAA proximal to the heart may be a pre-requisite for the regression of the more distal ones. We speculate that development of the proximal PAAs resulted in a decrease in blood flow running through the existing distal PAAs, which in turn decreased the mechanical stress caused by blood flow, making it virtually impossible for the distal PAAs to be maintained.

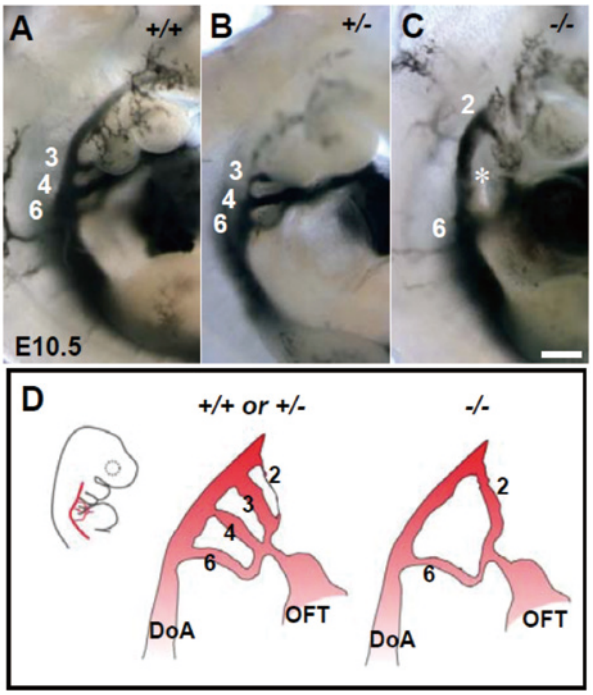

Figure 3. Cardiovascular defects in the Ripply3-deficient mice: (A-C) Typical morphology of pharyngeal arch arteries (PAAs) in the wild-type (A), heterozygous (B), and homozygous mutant (C) mice at E10.5 as visualized by intracardiac ink injection. The asterisk indicates the absence of the 3rd and 4th PAAs. (D) Schematic representation of PAA pattern.

Publication List

〔Original paper〕

- Okubo, T., Kawamura, A., Takahashi, J., Yagi, H., Morishima, M., Matsuoka, R., and Takada, S. (2011). Ripply3, a Tbx1 repressor, is required for development of the pharyngeal apparatus and its derivatives in mice. Development *138*, 339-348.