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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. 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). Thus, the Wnt protein appears to require 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.

The palmitoleoylation requires an enzyme, Porcupine (Porcn), a member of the family of membrane-bound O-acyltransferases (MBOAT). The *Porcn* gene was first identified as a segment polarity gene, like *wingless*, in *Drosophila* and is evolutionally conserved from worms to mammals. Porcn proteins localize to the endoplasmic reticulum (ER) and are required for Wnt trafficking from the ER in mammalian cells and probably in *Drosophila* embryos. Porcn overexpression promotes lipid-modification of Wnt1 and Wnt3a and causes a steepened Wnt gradient in the chick neural tube, suggesting its important function in vertebrate embryogenesis.

To reveal the biological significance of palmitoleoylation by Porcn, we used zebrafish as a model system and examined the effects of defects in Porcn function on Wnt signals mainly in early embryonic stages because the roles of Wnt signals have been precisely characterized in early zebrafish embryos. We identified two zebrafish homologs of *porcupine, porcn* and *porcupine-like (porcn-l)*. Zebrafish *porcn*, but not *porcn-l*, restores secretion of Wnt proteins in

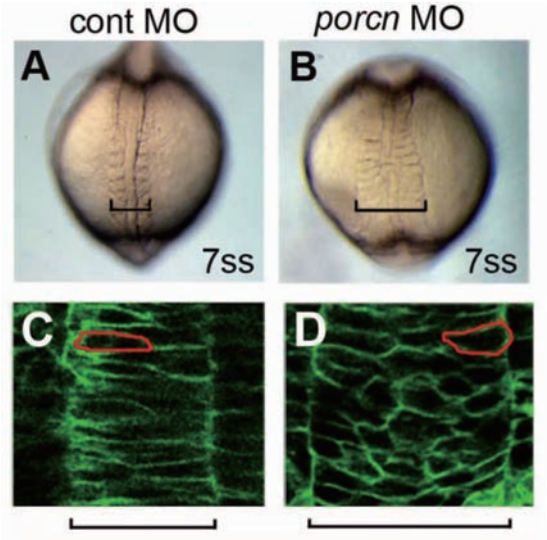

Figure 1. Porcn deficiency causes abnormalities in the convergence and extension. Zebrafish embryos injected with control (A, C) and Porcn-specific (B, D) MOs were shown, respectively. The notochord was expanded and shortened due to defect in the convergence and extension movement during gastrulation. The brackets indicate the notochords.

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2012. The former title is indicated by an asterisk (*).

porcn-deficient mouse L cells. Morpholino-mediated knockdown of *porcn* in zebrafish embryos impairs convergence and extension (CE) during gastrulation without changing embryonic patterning (Figure 1). Moreover, *porcn* interacts genetically with *wnt5b* and *wnt11* in regulating CE. In contrast, *porcn*-deficient embryos do not exhibit phenotypes caused by failure in canonical Wnt signaling, which is activated by several Wnt ligands, including Wnt3a. Furthermore, expression of genes regulated by the canonical Wnt signaling pathway is not perturbed in knockdown embryos relative to that in the controls. While the trafficking and lipidation of ectopically expressed zebrafish Wnt5b and mouse Wnt5a are impaired in *porcn*-deficient embryos, those of ectopically expressed Wnt3a are less or not affected (Figure 2). In addition, the secretion of Wnt5a is inhibited by less porcn inhibitor than that of Wnt3a in 293 cells. Thus, decrease of Porcn activity does not equivalently affect trafficking and lipidation of different Wnt proteins in zebrafish embryos and in mammalian culture cells. These results suggest that 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 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 two Ripply genes, Ripply1 and Ripply2 play essential roles in this patterning. To gain insight into the mechanism of somite segmentation, we are examining the segmental patterning in zebrafish embryos by focusing on Ripply and related molecules.

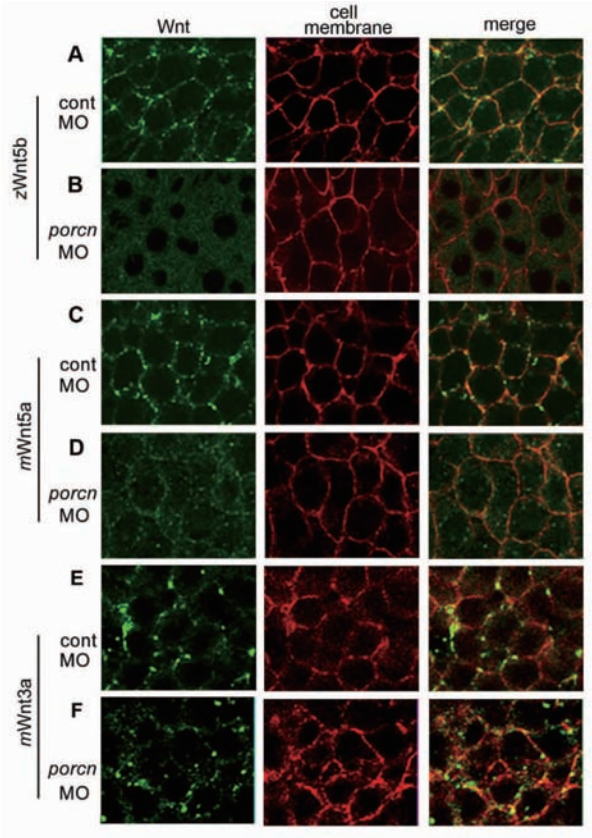

Figure 2. Secretion of Wnt proteins in zebrafish embryos. Confocal images of localization of EGFP-tagged zebrafish Wnt5b (A, B), mouse Wnt5a (C, D), and mouse Wnt3a (E, F) with membrane markers, in epiblast cells of embryos injected with 5mis control MO (A, C, E,) or *porcn* MO (B, D, F) are shown. Merged images of Wnts and membrane markers are also presented. Secretion of Wnt5a, but not Wnt3a, is defective in Porcn-deficient embryos.

III. Molecular mechanism of mesoderm development from stem cell-like progenitors.

The posterior body of the vertebrate is generated from progenitor cells residing in the tailbud. The mesodermal progenitor cells (MPCs), which appear to have stem cell-like characteristics, continuously produce the presomitic mesoderm (PSM) cells, which further differentiate into somites. Accumulating evidence has revealed the molecular mechanism underlying the maintenance of the undifferentiated state of the MPCs. For instance, in zebrafish embryos, *wnt* and zebrafish orthologues of brachyury, *no tail (ntl)* and *bra*, mutually activate their expressions in the MPCs and this autoregulatory loop is essential for maintenance of the undifferentiated state of the MPC.

On the other hand, the molecular mechanism promoting MPC differentiation into PSM cells should also be elucidated for a better understanding of the MPC-based development of the paraxial mesoderm. Of note, genetic studies with the zebrafish have indicated that the molecular mechanisms underlying the development of somites are not the same between trunk and tail. For instance, *spadetail (spt)/tbx16*

mutant embryos are impaired in the development of their trunk somites, but generate relatively normal tail ones. However, in contrast to this phenotype restricted to the trunk somites, other evidence suggests that *spt* is required for PSM differentiation in both trunk and tail somites and that some additional factors compensate the loss of Spt function during tail development. Therefore, for understanding the molecular mechanism that controls the maintenance and subsequent differentiation of the MPCs, it is important to reveal the function of Spt and these additional factors during the development of tail somites, especially in terms of their interaction with the Wnt/Brachyury autoregulatory loop.

One candidate as an additional factor seems to be Mesogenin1 (Msgn1), which is a bHLH transcription factor expressed in the PSM. Interestingly, mouse embryos deficient in functional Msgn1 have impaired development of their posterior somites, in spite of having normal formation of the first 7 somites, as well as show an abnormal accumulation of an undifferentiated cell mass at the tip of their tail. Thus, Msgn1 seems to be involved in PSM differentiation during the development of posterior, or tail, somites. However, it is still uncertain as to how the differentiation from the MPCs to PSM cells is controlled by *msgn1* during somite development. Furthermore, it has remained to be elucidated whether zebrafish *msgn1* interacts with *spt* during PSM differentiation during tail development.

We assessed the functions of *msgn1* in zebrafish development by injecting *msgn1* specific MO into wild-type and *spt* mutant eggs. Zebrafish embryos defective in *msgn1* and *spt* failed to differentiate into PSM cells in tail development and show increased expression of *wnt8* and *ntl* (Figure 3). Msgn1 acted in a cell-autonomous manner and as a transcriptional activator in PSM differentiation. The expression of *msgn1* initially overlapped with that of *ntl* in the ventral tail bud, as previously reported; and its mis-expression caused ectopic expression of *tbx24,* a PSM marker gene, only in the tail bud and posterior notochord, both of which expressed *ntl* in zebrafish embryos. Furthermore, the PSM-inducing activity of misexpressed *msgn1* was enhanced by co-expression with *ntl*. Thus, Msgn1 exercised its PSM-inducing activity in cells expressing *ntl*. Based on these results, we speculate that *msgn1* expression in association with that of *ntl* may allow the differentiation of progenitor cells to proceed during development of somites in the tail.

Publication List

〔Original papers〕

- Chen, Q., Takada, R., and Takada, S. (2012). Loss of Porcupine impairs convergent extension during gastrulation in zebrafish. J. Cell Sci. *125*, 2224-2234.
- Chiu, C.H., Chou, C.W., Takada, S., and Liu, Y.W. (2012). Development and fibronectin signaling requirements of the zebrafish interrenal vessel. PLoS ONE *7*, e43040.
- Yabe, T., and Takada, S. (2012). Mesogenin causes embryonic mesoderm progenitors to differentiate during development of zebrafish tail somites. Dev. Biol. *370*, 213-222.

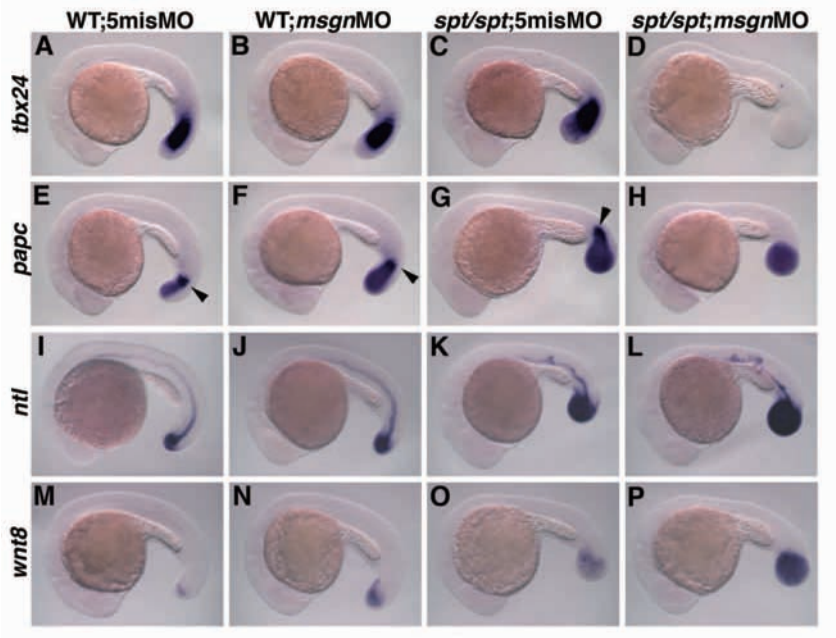

Figure 3. *msgn1* and *spt* are required for the differentiation of posterior mesoderm cells in zebrafish. Wild-type embryos injected with control (5mis) MO (A, E, I, and M) or *msgn1* MO (B, F, J, and N) and *spt* homozygous embryos injected with 5mis MO (C, G, K, and O) or *msgn1* MO (D, H, L, and P) at the 24-somite stage were hybridized with *tbx24* (A-D), *papc* (E-H), *ntl* (I-L) or *wnt8* (M-P) probes. The expression of paraxial mesoderm markers, *tbx24* and *papc* in the PSM is severely reduced in the *msgn1* MO-injected *spt* homozygous embryo (D, H). Injection of *msgn1* MO into wild-type embryos increased the expression area of *ntl* and *wnt8,* both of which are expressed in the mesoderm progenitor cells in the tailbud, in the tailbud (J, N) compared with that for the control (5mis) MO (I, M). In addition, *ntl* and *wnt8*-expressing cells are also increased by *msgn1* MO in *spt* mutant embryos (K, L, O, and P).