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During morphogenesis of vertebrates, the embryonic body is gradually divided into sub-regions that are specified to give rise to functional units. Most of the processes of regionalization and specification are regulated by cell signaling molecules. So far, spatial distribution of secreted signals, called morphogens, has been considered to regulate regionalization and specification during embryogenesis. However, the molecular basis to generate a variety of function of secreted signals, including specific interaction between secreted signals and their receptors and specific activation of their target genes in particular cells, has not yet been discovered. To better understand this molecular basis, we are currently examining biochemical characteristics of Wnt proteins and the molecular mechanism to activate specific targets.

In contrast, some regionalization processes have also been shown to be regulated by different manners. One of the typical examples is the segmentation of somites. Somites are the morphologically distinct segmental units that are transiently formed during early vertebrate development and subsequently give rise to metameric and fundamental structures such as the vertebrae of the axial skeleton, their associated muscles, and tendons. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the “segmentation clock”. To gain insight into molecular mechanism underlying this specific regionalization, we are also characterizing genes involved in somite segmentation.

I. Specificity between Wnt ligands and their receptors

In vertebrates, Wnt proteins constitute a large family of cysteine-rich secreted glycoproteins. Secreted Wnt proteins exert their effects on neighboring cells by binding to 7-transmembrane Frizzled (Fz) family receptors, as well as to LDL receptor-related family members, LRP5 and 6, which act as co-receptors. The Fz receptor family also consists of many members, each of which shows a distinct expression pattern during embryogenesis. However, the receptor specificity of each Wnt ligand has been poorly understood.

To investigate systematically the combinatorial effects of Wnts and various Fzs on canonical Wnt/β-catenin signaling, we analyzed the ability of these Wnt proteins to increase stability of armadillo/β-catenin proteins in Drosophila S2 cells expressing vertebrate Fzs (Takada *et al.* 2005). Wnt-3a increases the amount of armadillo proteins in cells expressing Fzs 4, 5, and 8, but not Fzs 3 and 6; whereas Wnt-5a does not increase it in any cell line. In contrast, both Wnt-3a and Wnt-5a increase the phosphorylation of Dsh in combination with most of the Fzs. This Dsh phosphorylation is abrogated by decreasing the levels of casein kinase I α by double-stranded RNA-mediated translational interference. These observations indicate that both Wnt proteins can interact with the majority of Fz receptors and elicit signaling reactions exemplified by Dsh phosphorylation but that the stabilization of β-catenin/armadillo proteins in the Wnt/β-catenin signaling occurs only when specific combinations of Wnt and Fz meet.

II. Identification and functional characteristics of Wnt target genes during embryogenesis

To identify Wnt-target genes during mouse development, we used the induction gene-trap approach (Yamaguchi *et al.* 2005). We screened 794 trapped ES lines and recovered 2 ES cell lines that contained trapped genes responsive to WNT-3A protein. One gene was mainly expressed in the ductal epithelium of several developing organs, including the kidney and the salivary glands, and the other gene was expressed in neural crest cells and the telencephalic flexure. The spatial and temporal expression of these two genes coincided well with that of several *Wnt* genes. Furthermore, the expression of these two genes was significantly decreased in embryos deficient for *Wnts* or in cultures of embryonic tissues treated with a Wnt signal inhibitor. These results indicate that the gene trap is an effective method for systematic identification of Wnt-responsive genes during embryogenesis.

We have further analyzed the developmental role of the former gene by generating mutant embryos defective in its function from the gene-trapped ES cell line. Our results indicated that this gene plays essential roles in differentiation of ductal epithelial cells in the kidney and salivary glands.

III. Identification and characterization of genes required for somite segmentation

3-1 Hairy/Enhancer of split protein, Her13.2, links FGF signaling to cyclic gene expression in the periodic segmentation of somites

The somites are subdivided from the anterior end of the

unsegmented paraxial mesoderm, called the presomitic mesoderm (PSM), and sequentially generated in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals. 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 PSM.

Most genes that exhibit a cyclic expression pattern in the PSM are involved in the Notch signaling pathway. For instance, various *hairy/Enhancer of split* (*Espl*)-related bHLH genes, including *her1* and *her7* in the zebrafish, are transcriptional targets of the Notch signaling and are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. These cyclic genes, as well as other components of the Notch signaling pathway, were shown to be required for the proper somite segmentation in mice and zebrafish. Furthermore, the cyclic expression of *her1*, and *her7* requires their own activity, suggesting that a negative feedback loop involving these genes is a critical component of the oscillation machinery.

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. The activity gradient of fibroblast growth factor (FGF) is proposed to regulate the differentiation of PSM cells along the anteroposterior axis from a state permitting the oscillating gene expression to a state driving the segmentation program. However, the molecular mechanism by which FGF signaling permits the cyclic gene expression in the posterior PSM has been totally unknown.

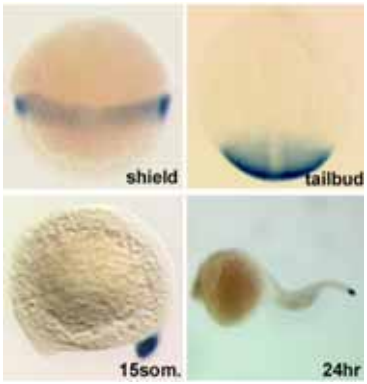

Figure 1. Expression of *her13.2* in zebrafish development. Expression of zebrafish *her13.2* is first observed at shield stage. During the stages when somites are generated, its expression is localized in the posterior PSM and the tailbud (15som. and 24hr). This expression is induced by FGF signaling to allow the cyclic gene expression in the posterior PSM (see Kawamura *et al.* 2005a).

To gain insight into the molecular mechanism underlying the segmentation process, we sought to identify genes specifically expressed in the PSM and tailbud by performing an *in situ* hybridization screening using zebrafish embryos (Figure 1). We show that a gene identified by this screening, *Hes6*-related *hairy/Enhancer of split*-related gene, *her13.2*, links FGF signaling to the Notch-regulated oscillation machinery in zebrafish (Kawamura *et al.* 2005a). Expression of *her13.2* is induced by FGF-soaked beads and decreased by an FGF signaling inhibitor. *her13.2* is required for periodic repression of the Notch-regulated genes, *her1* and *her7*, and for proper somite segmentation. Furthermore, Her13.2 augments auto-repression of *her1* in association with Her1 protein. Therefore, FGF signaling appears to maintain the oscillation machinery by supplying a binding partner, Her13.2, for Her1.

This result also provides a novel molecular mechanism whereby Notch signaling, which activates the expression of *her1* and *her7*, cooperates with FGF signaling. This molecular mechanism may be widely employed in regulation of developmental process where Notch signaling cooperates with FGF signaling.

3-2 Groucho-associated transcriptional repressor Ripply1 is required for proper transition from the presomitic mesoderm to somites

Prior to morphological segmentation, which is a process including inter-somitic boundary formation and mesenchymal-epithelial transition, a segmental pre-pattern, characterized by segmental gene expression, is established in the anterior PSM. The establishment of the segmental pre-pattern in the anterior PSM has been revealed to require a number of processes regulated by many transcription factors and signaling molecules. Concomitant with the transition from the anterior PSM to somites, the characteristic gene expression in the PSM is translated into the segmental structure. However, most of the events accompanying the transition from the anterior PSM to somites have remained obscure.

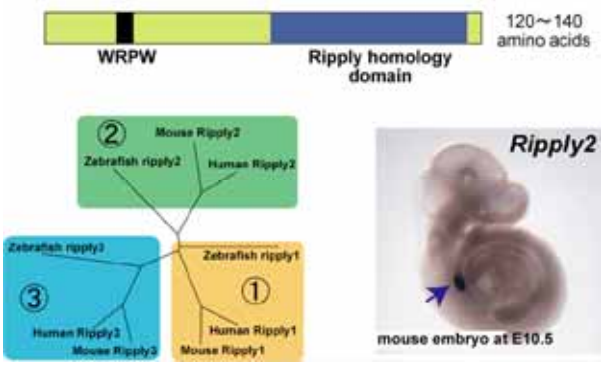

Figure 2. The structure and expression of *ripply* family proteins. Three *ripply* genes exist commonly in zebrafish, human and mouse genomes. All Ripply proteins possess the WRPW motifs and conserved sequences at the carboxyl terminus (Ripply homology domain). A typical example of *ripply* gene (mouse *ripply2*) expression is shown. *Ripply1* and *ripply2* are specifically expressed in the PSM and/or somites, suggesting that they function during somitogenesis (see Kawamura *et al.* 2005b).

We show that another gene identified by our *in situ* hybridization screening, *ripply1*, encoding a nuclear protein associated with the transcriptional co-repressor Groucho, is required for this transition (Figure 2,

Kawamura *et al*. 2005b). Zebrafish *ripply1* is expressed in the anterior PSM and in several newly formed somites. Ripply1 represses specific gene expression in the PSM through a Groucho-interacting motif. In *ripply1*-deficient embryos, somite boundaries do not form, the characteristic gene expression in the PSM is not properly terminated, and the initially established rostrocaudal polarity in the segmental unit is not maintained, whereas paraxial mesoderm cells become differentiated. Thus, *ripply1* plays two key roles in the transition from the PSM to somites: termination of the segmentation program in the PSM and maintenance of the rostrocaudal polarity.

3-3 Integrinα5-dependent Fibronectin assembly is required for maintenance of somite boundaries.

The segmental pre-pattern established in the anterior PSM lead to morphological segmentation. Boundary formation and epithelialization are crucial processes in the morphological segmentation of vertebrate somites, but the molecular mechanisms underlying these processes are not yet clearly understood.

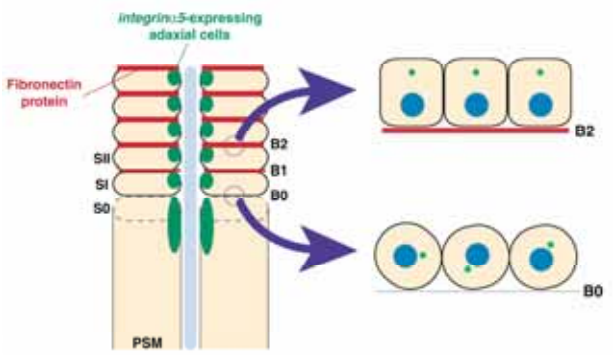

Figure 3. The role of Fibronectin assembly in somite boundary formation. Gradual accumulation of Fibronecin proteins (red) at somite boundaries is required for maintenance of the boundaries and for polarization of epithelial cells. Integrinα5 (green) is required for this accumulation (see Koshida *et al.* 2005).

To gain insight into the mechanism underlying somite development, we performed an ENU mutagenesis screening of zebrafish, in addition to *in situ* hybridization screening described above. We found that *integrinα5* and *fibronectin* were mutated in embryos showing defective boundary formation in their anterior somites (Koshida *et al.* 2005). Fibronectin proteins accumulate at somite boundaries in accordance with epithelialization of the somites. Both Fibronectin accumulation and the epithelialization are dependent on *itga5*, which is expressed in the most medial part of somites. While somite boundaries are initially formed, but not maintained, in the anterior trunk of the mutant embryos deficient in either gene, their maintenance is defective at all axial levels of embryos deficient for both of these genes. Therefore, Integrinα5-directed assembly of Fibronectin appears critical for epithelialization and boundary maintenance of somites (Figure 3). Furthermore, with an additional deficiency in *ephrin-B2a*, the segmental defect in *itga5* or *fn* mutant embryos is expanded posteriorly, indicating that both Integrin-Fibronectin and Eph-Ephrin systems function cooperatively in maintaining somite boundaries.

The results shown in “3-1” to “3-3” indicate that our strategies are effective for identification of genes involved in the somite segmentation process. We are further searching for other genes involved in this process by both the expression screening and the mutagenesis screening methods. This systematic screening should reveal another interesting mechanism underlying somite segmentation.

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