Distinct WNT Pathways Regulating AER Formation and Dorsoventral Polarity in the Chick Limb Bud

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The apical ectodermal ridge (AER) is an essential structure for vertebrate limb development. Wnt3a is expressed during the induction of the chick AER, and misexpression of Wnt3a induces ectopic expression of AER-specific genes in the limb ectoderm. The genes β-catenin and Lef1 can mimic the effect of Wnt3a, and blocking the intrinsic Lef1 activity disrupts AER formation. Hence, Wnt3a functions in AER formation through the β-catenin/LEF1 pathway. In contrast, neither β-catenin nor Lef1 affects the Wnt7a-regulated dorsoventral polarity of the limb. Thus, two related Wnt genes elicit distinct responses in the same tissues by using different intracellular pathways.

The Wnt gene family encodes a group of signaling molecules that are implicated in numerous aspects of morphogenesis in both vertebrates and invertebrates. Several chick Wnt genes are expressed in a specialized epithelial structure running along the distal margin of the limb bud, called the apical ectodermal ridge (AER), which is essential for limb morphogenesis (1, 2). Wnt3a is the first of these genes to be expressed in the limb. We therefore examined the spatiotemporal pattern of expression of Wnt3a in developing limb buds with respect to that of Fgf8, the earliest known AER marker during chick development (3, 4) (Fig. 1, A through D) (5).

Wnt3a transcripts are detected before Fgf8 transcripts in the limb field ectoderm but not in the flank outside the limb fields. Subsequently, Wnt3a expression is up-regulated in the ectoderm cells near the dorsoventral (DV) border. Fgf8 expression is initiated and then up-regulated within the region of high Wnt3a expression during AER formation. From stage 20 on, Wnt3a and Fgf8 expression are confined primarily to the mature AER. Thus, Wnt3a expression appears to presage Fgf8 expression and AER formation.

To verify the epistatic relationship between Wnt3a and Fgf8 that is suggested by the expression data, we ectopically delivered each factor to developing limb buds. We misexpressed Wnt3a in the limb ectoderm using a replication-competent retroviral vector and assayed for the expression patterns of the various AER markers (6). Misexpression of Wnt3a induced ectopic expression of AER-specific genes, including Bmp2, Fgf4, and Fgf8, in broad patchy domains in the ectoderm of nearly 100% of infected limbs (Fig. 1E) (5). However, Wnt3a expression was not induced in the ectoderm by either fibroblast growth factor 4 (FGF4) protein or Fgf8-virus (5). This suggests that Wnt3a acts upstream of FGFs in establishing AER gene expression.

In addition to its effect on AER gene expression, Wnt3a misexpression occasionally led to disruption of the AER or to formation of an ectopic AER extending ventrally, or both (Fig. 1, E and F). These morphological effects on the AER are reminiscent of those seen after misexpression of Radical fringe (7). We therefore examined Radical fringe expression and found that it was ectopically expressed in Wnt3a-infected limbs (8). Disruption of the AER morphology was only seen in a subset of Wnt3a-infected limb buds, which is consistent with the finding that Radical fringe only affects AER formation when it is misexpressed at the earliest stages of limb development (7).

The FGFs produced in the AER are responsible for maintaining the proliferative state of the undifferentiated mesoderm at the distal tip of the limb bud, the progress zone (PZ) (1). To verify that the FGFs induced in the limb bud ectoderm by Wnt3a are functional signals, we examined the expression of several PZ markers: Fgf10 (9), Mx1 (10), Nmyc (11), and Slug (12). Equivalent results were obtained with each of these markers (Fig. 2) (8). When the AERs were removed from experimental limb buds, expression of the PZ markers was rapidly lost (Fig. 2, C and D). Application of Wnt3a-expressing cells to the AER-deprived limbs induced Fgf8 expression and restored expression of the PZ markers (Fig. 2, E and F). To show that this response was due to the ectopic expression of FGFs and not to a direct action of Wnt3a itself, we removed the adjacent distal ectoderm as well as the AER so that the Wnt3a cells could not induce ectodermal Fgf8 expression (Fig. 2G). Under these conditions, Wnt3a induced little or no expression of the PZ genes (Fig. 2H). The maintenance of the PZ is critical for outgrowth of the limb bud. The long-term effects of virally mediated Wnt3a misexpression, and consequent ectopic FGF production by the ectoderm, included some cases in which extra outgrowth formed digitlike structures (5). Wnt3a thus appears to influence both morphological AER formation and induction of AER-specific genes in the early limb bud.

The members of the vertebrate Wnt gene family have been categorized by their relative ability to transform murine mammary epithelial cells (13). A similar classification can be made on the basis of the ability to
promote axis duplications in early *Xenopus* embryos (14). Yet even Wnt genes categorized into the same functional group can play distinct roles during limb development. During the period of AER formation, Wnt7a, which is placed in the same highly transforming axis-duplicating class as Wnt3a, is exclusively expressed in the dorsal limb ectoderm (15, 16) and functions as the signal for DV patterning in the distal limb (17–19). In early stages of limb development, expression domains of Wnt3a and Wnt7a overlap in the dorsal ectoderm. To assess whether the two Wnt family genes are functionally redundant, we compared their abilities to induce expression of AER markers and affect DV patterning. Early limb buds were infected with either Wnt3a or Wnt7a virus (5) and examined for Fgf8 expression (Fig. 3, A, C, and E). Unlike misexpression of Wnt3a, misexpression of Wnt7a never affected the expression pattern of Fgf8 or the morphology of the AER, which suggests that Wnt7a is not involved in AER formation. The effects of Wnt3a and Wnt7a on DV patterning were assessed by monitoring expression of the dorsal mesenchymal marker Lmx1 (Fig. 3B). Misexpression of Wnt7a induced strong ectopic expression of Lmx1 in the distal half of ventral mesenchyme (Fig. 3F), and produced a biconvex-shaped limb bud, in contrast to the normal ventrally curving limb bud morphology (Fig. 3, B and F) (17, 18). Wnt3a misexpression did not produce the morphological bidental phenotype and had little or no effect on Lmx1 expression (Fig. 3D).

To understand how these two Wnt genes elicit distinct responses, we investigated whether Wnt3a and Wnt7a act through the same signaling pathway. In other systems, signaling by the highly transforming Wnt genes, including Wnt3a, has been shown to be transduced by preventing degradation of cytoplasmic β-catenin, a protein that is ubiquitously expressed in vertebrate embryos (20–22). We misexpressed an activated mutant form of β-catenin (20, 23, 24) in limb buds (5) and found that β-catenin activity simulated the effect of Wnt3a but not of Wnt7a. Misexpression of the activated form of β-catenin induced ectopic expression of Fgf8 (Fig. 3G) and expression of other AER markers, including Fgf4 and Bmp2, in the ectoderm and up-regulated the downstream PZ markers Msx1 and Nmyc in the mesoderm (8). In contrast, misexpression of β-catenin did not induce the strong mesenchymal expression of Lmx1 seen in response to Wnt7a (Fig. 3H). Moreover, like Wnt3a-infected limb buds, activated β-catenin–infected limbs retain a concave ventral morphology in contrast to the biconvex morphology of Wnt7a-infected ones (Fig. 3, D, F, and H). Thus, Wnt3a and Wnt7a function through β-catenin–dependent and –independent pathways, respectively. β-catenin forms a complex with members of the LEF/XTCF family to activate
To explore the possible involvement of LEF in WNT3a signaling, we cloned a chick homolog of Lef1. Lef1 is strongly expressed in the AER and distal mesenchyme of the chick limb primordia (Fig. 4A). In addition to the limb buds, intense expression is observed in the developing medial somites and in the tail bud (8), both of which are regions believed to be targets of Wnt3a signaling (29, 30).

To examine whether Wnt3a regulates Lef1 expression during chick development, embryos were injected with Wnt3a virus at stage 10 and assayed for Lef1 expression at stages 22 to 24. Misexpression of Wnt3a markedly enhanced Lef1 expression in both the ectoderm and mesoderm of the limb bud and also in the tail bud (Fig. 4B) (8). Misexpression of Wnt7a had no effect on Lef1 expression (Fig. 4C). Thus, Lef1 is a specific target of Wnt3a signaling.

The ventral mesenchyme by viral spread in the two tissues. (A and B) Normal expression of Fgf8 and Lmx1. Fgf8 expression is ectopically induced in the limb ectoderm by Wnt3a (C) in nearly 100% of infections, but never by Wnt7a (E). In contrast, Lmx1 expression is strongly induced throughout the ventral mesenchyme by Wnt7a in nearly 100% of infections (F) but not by Wnt3a (D). In 20 to 30% of infected limbs, Wnt3a induces patchy weak expression in the ventral mesenchyme and shifts the distal border of Lmx1 expression slightly ventral to the AER (5), which appeared to be correlated with the formation of widened and ectopic AERs. Activated β-catenin is able to mimic Wnt3a in inducing ectopic Fgf8 expression in the dorsal and the ventral ectoderm (G) and both the punctate ventral expression and the ventral shift in the distal border of Lmx1 expression (H) at a comparable frequency. However, activated β-catenin does not induce strong ventral expression of the Wnt7a target Lmx1 in the mesenchyme.
ingly, Wnt3a is neither expressed in the AER nor implicated in its formation during mouse limb development (15, 30). Several other Wnt genes have also been shown to be expressed differentially between mouse and chick embryos, both in the developing central nervous system and in limb buds, including some in the murine AER (15, 32). Different Wnt genes could substitute for one another as long as they activate the same intracellular signaling pathway mediated by β-catenin/LEF1. It is therefore probable that another species of Wnt that is expressed in the mouse AER plays the same role as Wnt3a in the chick.

Both WNT3a and WNT7a proteins act, at least in part, on the mesoderm, where they activate distinct targets; WNT3a induces Lef1 whereas WNT7a induces Lmx1, which implies that receptors for both factors must be present on the surface of mesenchymal cells. In spite of previous data suggesting that all members of the highly transforming class of Wnt genes act through β-catenin, our results indicate that the induction of Lmx1 expression by WNT7a signaling is not mediated by β-catenin and LEF1. Precedent exists for more divergent Wnt genes, such as Wnt5a, to act through distinct signaling cascades (33). Transcriptional activation of downstream genes by distinct WNT genes allows for their different inductive roles in the same tissue during development.

REFERENCES AND NOTES

5. See supplementary figures at www.sciencemag.org/feature/data/0764400.sht.
6. A mouse Wnt3a cDNA encoding the entire open reading frame and a deleted mutant of β-catenin containing the internal Armadillo repeats that acts as a stable constitutively activated variant (23) were individually cloned into the shuttle vector SLAX-13 and then subcloned into retroviral vector RCAS/BPA (34). Retrovirus was produced using a line 0 chick embryo fibroblast and harvested as described (35). Embryos at stage 11 to 11 were injected in the fore- or hindlimb primordia. This protocol results in widespread infection of the limb ectoderm by the time the embryos are harvested at stage 22 to 24 for whole-mount in situ hybridization (7, 8, 17). A significant number of samples (>10) were analyzed in each case. To generate embryos primarily infected in the limb ectoderm, injection was targeted onto the embryo fibroblast and harvested as described (7).
8. M. Kengaku et al., unpublished data.
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Activation and covalent attachment of complement component C3 to pathogens is the key step in complement-mediated host defense. Additionally, the antigen-bound C3 fragment interacts with complement receptor 2 (CR2; also known as CD21) on B cells and thereby contributes to the initiation of an acquired humoral response. The x-ray crystal structure of human C3d solved at 2.0 angstroms resolution reveals an aαα backbone with the residues responsible for thioester formation and covalent attachment at one end and an acidic pocket at the other. The structure supports a model whereby the transition of native C3 to its functionally active state involves the disruption of a complement domain interface and provides insight into the basis for the interaction between C3d and CR2.

Serum complement protein C3 is a central component of host defense because its proteolytic activation is the point of convergence of the classical, alternative, and lectin pathways of complement activation. C3 cleavage products mediate many of the effector functions of humoral immunity, including inflammation, opsonization, and cytolysis. Proteolytic cleavage of C3a into C3a and C3b exposes an internal thioester bond that through transacylation mediates covalent attachment of C3b to the surface of foreign pathogens (1). Although surface-bound C3b is itself a ligand for complement receptor 1 (CR1; also known as CD35), it can subsequently be degraded into the successively smaller fragments iC3b and C3dg, tagging the pathogen for recognition by other receptors, including the B cell complement receptor CR2 (CD21) (1). The interaction between B cell CR2 and antigen-bound iC3b or C3dg is an essential component of a normal antibody response (2), making an important link between the innate and adaptive arms of the immune system (3). The C3d fragment (a CR2-
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