New twists on embryonic patterning

EMBO workshop: Embryonic organizer signaling: the next frontiers

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The EMBO workshop 'Embryonic organizer signaling: the next frontiers' was held at the European Molecular Biology Laboratory in Heidelberg, Germany, April 28–30, 2001.

asymmetry. Intercellular signaling is effected through a surprisingly small number of cell surface receptor-mediated signaling pathways, in particular Hedgehog, Wnt, FGF, Delta/Notch and TGF-β family members. Molecular and genetic studies on the various organizing centers have revealed that these signaling pathways are highly modulated, by agonist and antagonist signals and a variety of post-translational modifications, to generate the intricate patterns observed in developing embryos. The complexity, mechanisms of action and the conceptual convergence/divergence of distinct embryonic organizer centers was discussed at the EMBO workshop on 'Embryonic organizer signaling: the next frontiers' held at the EMBL on April 28–30, 2001 in Heidelberg. In this review, we highlight some of the salient themes that emerged.

## Secreted antagonists, inhibitory modulation of growth factor activity

A major conclusion to emerge in recent years is that embryonic patterning is controlled to a large extent by the modulatory action of secreted antagonists or agonists (Figure 1). For example, Chordin and Noggin are antagonists secreted by the gastrula organizer, which both act by binding to members of the bone morphogenetic protein (BMP) family, thus preventing them from interacting with their cognate membrane receptors. Many other molecules that act by binding directly to ligands have been reported. These include the secreted Frizzled Related proteins (Frzb-1, FrzbA, Crescent, Sizzled and others) and the structurally unrelated WIFs (Wnt inhibitory factors), all of which antagonize Wnt glycoproteins, as well as the multifunctional antagonist Cerberus which, besides inhibiting Wnts, also inactivates BMPs and Nodal-related TGF-β factors.

Dickkopf-1 (Dkk-1) is probably the most potent secreted Wnt inhibitor. It belongs to a new protein family, which comprises three family members in mammals. Dkk-1 is required for head development in *Xenopus*, a finding that has led to the proposal

Introduction

The term 'organizer' was coined by Hans Spemann and Hilde Mangold in 1924, based on the seminal observation that a small region of the amphibian gastrula, the dorsal lip, when transplanted to an ectopic location, had the ability to induce, or organize, the formation of a twinned embryo, recruiting neighboring cells from the host into a variety of cell differentiation pathways. The gastrula organizer has been reviewed *in extenso* recently (special issue of *Int. J. Dev. Biol.*, **45**). It has become apparent that organizing centers, which secrete signals that pattern adjacent regions, are a recurring theme in developmental biology. Organizing centers function during neural tube formation in vertebrates, compartment boundary formation in *Drosophila*, limb development and the establishment of left–right
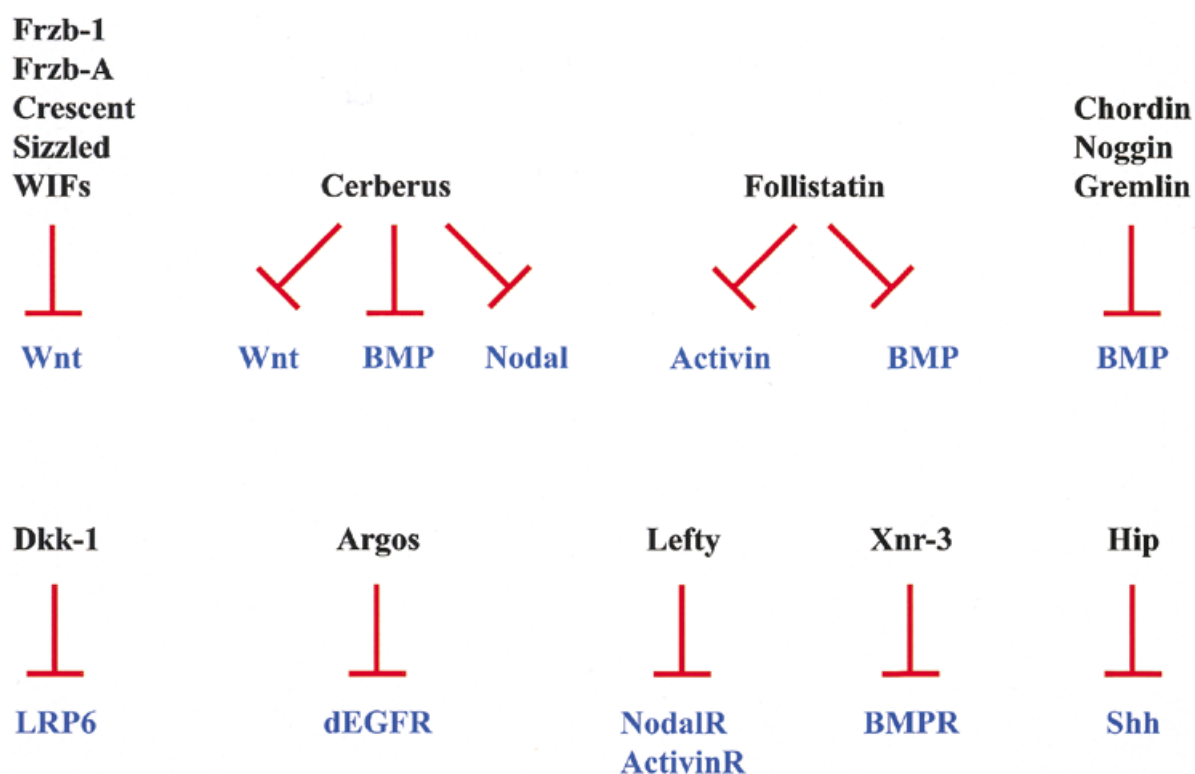
Fig. 1. A large number of extracellular antagonists function as modulators of cell–cell signaling. Some act by sequestering growth factors, others bind to receptors but do not activate signaling. Xnr-3 is a potent BMP antagonist in Xenopus, but a direct interaction with BMPR has not been demonstrated.

that head induction results from the mutual inhibition of Wnt and BMP signaling, whereas trunk organizer activity results principally from BMP inhibition. Christof Niehrs (Heidelberg, Germany) unveiled the inhibitory mechanism of Dkk-1. Wnt/Wg glycoproteins signal through a heteromeric receptor complex that consists of a serpentine seven transmembrane protein, Frizzled and a single-pass transmembrane protein, called low-density lipoprotein related protein (LRP) in vertebrates and Arrow in *Drosophila*. The extracellular domain of LRP6 binds to the Frizzled CRD in a Wnt-dependent manner (Tamai *et al.*, 2000). The intracellular domain of LRP5 binds to Axin, an inhibitory component of the Wnt pathway Mao *et al.* (2001a) thus providing a direct physical link to intracellular protein complexes that mediate canonical Wnt signaling through the β-catenin pathway. Dkk-1 does not act as a direct Wnt antagonist, but rather exerts its inhibitory effect by binding to the co-receptor LRP6 with very high affinity (K_D of 340 pM) (Mao *et al.*, 2001b). Dkk-2 also binds, although with lower affinity. This seems a bit puzzling because Dkk-2 promotes Wnt signaling instead of inhibiting it. Perhaps Dkk-2 binds preferentially to other proteins, such as LRP5, which also participate in Wnt signaling. Clearly these important studies mark the beginning of a detailed understanding of Wnt/Wg signal transduction.

Andy McMahon (Cambridge, MA) discussed the role of Hip-1, a negative feedback inhibitor of Hedgehog (Hh) signaling (Chuang and McMahon, 1999). Hip-1 is a glycoprotein containing two EGF domains and a C-terminal membrane anchor. It is induced adjacent to all cells expressing Hh proteins. Although three Hip genes exist in mammals, no ortholog is present in *Drosophila* or *Caenorhabditis elegans*. Roel Nusse (Stanford, CA) also stressed the remarkable fact that of four distinct families of extracellular Wnt inhibitors (Frzbs, WIFs, Dkks and Cerberus) no orthologs are present in the fly or worm genome. The same applies to Noggin, Lefty, Xnr-3 and Gremlin (Figure 1). This suggests that extracellular inhibitory modulation of growth factor activity was expanded in the vertebrate lineage. However, the reciprocal also appears to be true, with *Drosophila* Argos not having been found in the human genome. As described by Benny Shilo (Rehovot, Israel), *Drosophila* EGF receptor (DER) signaling is triggered by the proteolytic cleavage of a transmembrane EGF ligand, Spitz, which acts at short range to signal to neighboring cells. This, in turn, activates Argos, a long-range diffusing EGF domain-containing secreted molecule that binds to DER and blocks signaling.

Much discussion was triggered by the apparent lack of evolutionary conservation of secreted antagonists. One possible explanation for this is that an entire signaling pathway from extracellular ligand to nuclear transcription factor is very difficult to evolve and that, once achieved, it may be utilized in many developmental contexts. It may be that generation of an antagonist, or a 'spanner in the works', is less demanding from a structural point of view. In this way, novel regulatory steps might be intercalated to modulate ancient signaling pathways.

meeting report

Evo-devo conservations

The lack of conservation does not apply to all extracellular antagonists. In *Drosophila* Short-gastrulation (Sog) is the ortholog of Chordin, an inhibitor containing four cysteine-rich (CR) domains that can bind BMP. Sog is required in the ventral epidermis to inhibit BMP signaling, but paradoxically it is also required for peak BMP signaling in the amnioserosa, the dorsal-most tissue. Matthias Hammerschmidt (Freiburg, Germany) presented genetic evidence that in zebrafish, as in flies, embryonic dorsal–ventral patterning is mediated by the BMP pathway. Most dorsalizing mutations have been cloned and encode BMP-2b (*swirl*), BMP-7 (*snailhouse*), the Alk-8 receptor (*lost-a-fin*), Smad-5 (*somitabun*) and Tolloid (*minifin*). Tolloid is a metalloprotease that cleaves Chordin/Sog at specific sites. The ventralized *chordino* mutation encodes Chordin, and behaves as a mutant with reduced Spemann organizer activity. However, in certain compound mutants (*chordino⁻/⁻; swirl⁺/⁻*) the ventral tail fin is also lost. The ventral fin is the ventral-most ectoderm of the fish and requires the highest BMP signaling. Thus, as in flies, Chordin/Sog inhibits BMP signaling at its site of synthesis on the dorsal side of the embryo, but promotes BMP signaling at the opposite side.

Another conserved protein is Twisted-gastrulation (Tsg), a small BMP-binding protein that has a pattern of expression similar to that of BMP-4/Dpp (reviewed in De Robertis *et al.*, 2000). Tsg has limited sequence homology to the Chordin CR repeats and functions as a co-factor of Chordin in an intricate biochemical cycle. Tsg has at least three functions (E. De Robertis, Los Angeles, CA). First, it forms ternary complexes with Chordin and BMP, making Chordin a much better BMP antagonist. Secondly, it facilitates the cleavage of Chordin by Tolloid. Thirdly, after cleavage has taken place Tsg dislodges BMPs bound to the Chordin CR repeats. Since Tsg does not interfere with binding of BMP to the BMP receptor (BMPR), Tsg provides a permissive signal that allows peak levels of signaling by BMPs transported bound to Chordin.

One of the surprising findings reported at the meeting was obtained by molecular analysis of the diploblastic organism, Hydra. In evolutionary terms, Hydra is at the base of metazoan evolution and its divergence long predates the Urbilateria, i.e. the last common ancestor of the arthropod and vertebrate lineages. T. Holstein (Darmstadt, Germany) explained that the first organizer transplantation experiment had actually been carried out in Hydra in 1909 by Ethel Browne, who transplanted a small graft of hypostome (mouth tissue) to the flank of Hydra. This resulted in induction of a complete ectopic head, including tentacles recruited from the host. Intriguingly, Holstein's studies demonstrate that signaling by the Hydra organizer shows remarkable conservation to the signaling pathways used by the vertebrate gastrula organizer. During head regeneration HyWnt is found as a tight spot at the terminus of the regenerating body axis. β-catenin and TCF are also upregulated, but over a wider region of the head (Hobmayer *et al.*, 2000). In addition, a Chordin-like molecule is also expressed over a wide but progressively refined region of the developing head region. These observations are particularly intriguing because they suggest that the oral–aboral axis of Hydra uses a robust agonist/antagonist patterning system involving Wnt and Chordin/BMP signals. The relationship between the oral–aboral axis of Hydra

and the antero-posterior and dorso-ventral axes of bilaterians poses an interesting evolutionary question.

The advantage of using diverse model organisms was illustrated further by analyzing the development of the rabbit, a species at the other end of the phylogenetic tree. Martin Blum (Karlsruhe, Germany) explained how the rabbit gastrula, like those of humans and chicken, forms a flat blastodisc, which offers great advantages for embryological manipulations. Rabbit embryos can be easily detached from their uterine implantation sites at the gastrula stage and cultured through somitogenesis in the presence of various signaling molecules to test their effect on development. For example, by placing a BMP-4-soaked bead on the right side, it was found that the normally leftward-restricted Nodal expression became bilateral in the lateral plate. This observation is in line with the fact that left–right situs becomes randomized in *Xenopus* embryos treated with low doses of UV light or in zebrafish embryos mutant for Chordin; in both cases BMP signaling is increased. In the future we can expect that molecular studies on a wide range of embryos will be increasingly possible and valuable, in that such comparative studies will shed light on the evolution of developmental control mechanisms.

Limb buds: progress or allocation?

Vertebrate limb development involves some of the classical organizing centers. In posterior mesoderm, the zone of polarizing activity (ZPA) produces SHH, which acts in a feedback loop with fibroblast growth factors (FGFs) in the apical ectodermal ridge (AER). This, in turn, promotes outgrowth of the apical mesoderm, generating what is called the progress zone (PZ). J.C. Izpisúa-Belmonte (San Diego, CA) described an early requirement for Wnt signaling before outgrowth of the limb bud starts. Wnt-2b is expressed in mesoderm at the level of the forelimb, where it turns on FGF-10, which eventually activates the limb outgrowth programme (Kawakami *et al.*, 2001). Over-expression of Wnt-2b, β-catenin or FGF in the flank will induce ectopic limbs, and overexpression of the Wnt signaling inhibitor Axin represses FGF-10 and formation of endogenous limbs. Once SHH is activated in the ZPA, the secreted BMP antagonist Gremlin is maintained in a fraction of responding mesenchymal cells and acts to maintain the production of FGFs by the AER (Zuniga *et al.*, 1999). Thereby, the SHH/FGF feedback loop is established and propagates the ZPA distally during limb bud outgrowth (Rolf Zeller, Utrecht, The Netherlands).

The real treat came from two keynote lectures by Cliff Tabin (Cambridge, MA) and Gail Martin (San Francisco, CA). Using entirely different experimental approaches they conceptually converged on the conclusion that the progress zone at the tip mesoderm might not be a zone in which proximo-distal identity is continuously specified as limb bud outgrowth progresses. Summerbell and Wolpert's progress zone model was based on their observations that when the AER is removed early, the resulting limb lacks humerus, radius and ulna and digits, but that if the same manipulation is done late, only distal digits are missing. The Martin laboratory, in collaboration with the Tickle laboratory, and the Fallon laboratory independently, had shown earlier that an FGF bead could replace the need for the AER for limb outgrowth. In the new experiments, AER specific loss-of-function

mutations for both FGF-4 and FGF-8 were generated in the mouse. These embryos never expressed FGF-4 and FGF-8 in the presumptive hindlimb and, as a result, the hindlimb was entirely absent at later stages. In the forelimb bud, however, there was a brief period of expression of FGF-4 and FGF-8 before the genes were deleted (via CRE recombinase). Despite lacking these two crucial AER FGFs, the forelimbs developed rather well. The 'progress zone model' would have predicted that early exposure to FGFs would result in the development of only proximal limb skeletal elements. Cliff Tabin found that when the AER is removed in chick limb buds, a region of about 200 μm of the underlying distal mesoderm undergoes apoptosis. Early on this death involved precursors of all regions of the limb, but later on, just those of the distal elements. In other words, the tip mesoderm may already contain separate precursor cells specified to become stylopod, zeugopod and autopod. In contrast to predictions based on the 'progress zone model', these experiments suggested that limb cells would not progressively change their proximo-distal fate during limb outgrowth. The new model has been tentatively dubbed the 'early allocation and progenitor expansion model'. Vivid discussion was enjoyed by all participants and most people agreed that the main value of this model is that it provides a new way of looking at limb development. Cell fate would not depend on the number of cell divisions or the time spent in the progress zone.

Signal trafficking, modification and interpretation

The intricate meshwork of the extracellular matrix is not really compatible with passive diffusion of growth factors throughout embryonic fields. In fact, ligands such as Wg and FGF tightly bind to heparin and other heparin sulfate proteoglycans (HSPGs). A. Schier (New York, NY) presented evidence that Squint, a Nodal TGF-β growth factor, can act as a bona fide long-range morphogen in zebrafish (Chen and Schier, 2001), in line with seminal observations by Gurdon and Smith on Activin in *Xenopus*. The elucidation of the precise trafficking mechanism of Squint, Activin and other ligands through an embryonic field still poses a scientific challenge in vertebrates. J.-P. Vincent (London, UK) presented data that endocytic trafficking might regulate the range of Wg activity in the embryonic epidermis of *Drosophila* and that selective EGFR-mediated degradation of Wg posterior to the Wg source contributes to the unidirectional action of Wg (Dubois *et al.*, 2001). An intriguing, alternative way of distributing ligands was described by N. Hirokawa (Tokyo, Japan). Studies on several mouse mutants suggest that left–right asymmetry in mice may be initiated by the clockwise rotation of monocilia, which facilitate the unidirectional leftward flow of a yet unidentified node-derived ligand (Nonaka *et al.*, 1998). Alternatively, active ciliar movement could act as a physical boundary preventing rightward diffusion of a 'left-sidedness determinant'. However, so far, no rotating cilia have been identified in gastrula organizers of other vertebrates, suggesting that this might be a specific adaptation of murine embryos. Other means of active ligand transport that could facilitate long-range cell–cell communication are being entertained as well. S. Eaton (Dresden, Germany) showed extensive intercellular vesicular trafficking of basolateral membrane

particles, called argosomes, throughout the *Drosophila* wing disc using live-imaging of a GPI-anchored GFP chimera. Alternatively, actin-based extensions, called cytonemes might facilitate active anterograde transport (Ramirez-Weber and Kornberg, 1999). However, there is not yet direct evidence that either of these intriguing organelles do play an active role in long-range transport of extracellular ligands.

The strength of further cell biological dissection was elegantly demonstrated by findings that intrinsic affinity differences between cell populations might generate local organizing centers. A. Lumsden (London, UK) provided evidence that the Zona Limitans Intrathalamica (Zli), a presumptive prosencephalic local organizer, might be generated by a cell sorting mechanism depending on the Fringe glycosyltransferase. This cellular restriction appears similar to the establishment of the dorso/ventral lineage restriction boundary in the *Drosophila* imaginal disc. The latter affinity boundary is controlled/maintained by Apterous-dependent expression of two cell adhesion molecules, which prevent the sorting of cells committed to a dorsal fate into the ventral compartment (M. Milan and S. Cohen, Heidelberg, Germany).

Finally, one talk highlighted the intrinsic complexity of particular signal transduction pathways. K. Basler (Zürich, Switzerland) presented the identification and characterization of a putative membrane-bound *O*-acyl transferase, termed *skinny hedgehog* (*Ski*), adding another spine to the Hedgehog saga. *Ski* is a novel segment polarity gene that is essential for the complex maturation process of Hh, which is a fatty acid and cholesterol-modified growth factor that controls many developmental processes. Intriguingly, Ski has homology to Porcupine, a polytopic ER-resident transmembrane protein involved in maturation/secretion of Wg, suggesting that Wg biosynthesis might too include lipid modification.

Conclusion

This workshop nicely illustrated the strength of multidisciplinary approaches in invertebrate and vertebrate model organisms to dissect the molecular and cellular basis of organizer signaling centers. In particular, the in-depth analysis on both the cellular and subcellular levels will, in future, make possible the precise unraveling of the mechanisms of intercellular trafficking of morphogens, compartment boundary formation and cell–cell communication. More refined genetic modifier screens, functional expression screens and a number of systematic genomic and proteomic approaches will undoubtedly add new gene products to the limited number of signaling modules that are currently known to control pattern formation in the developing embryo.

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