Building limb buds

Workshop on limb development

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A recent workshop (April 8–10, 2002) on limb development, co-sponsored by the Juan March Foundation and EMBO, gathered 50 people together in Madrid to present and discuss their latest results.

Introduction

This workshop on limb development was a highly enjoyable meeting that covered a wide range of topics related to appendage development and evolution. The discussions were vivid, open and stimulating for all participants. It was particularly interesting to see how broadly accepted concepts, such as the morphogen models in the fly and the progress zone model in vertebrates, were re-evaluated on the basis of new evidence presented. Important contributions included intense analyses of the roles of factors important in limb development, such as Wingless (Wg) in the fly and Sonic Hedgehog (Shh) in vertebrates. In addition, the overall view of appendage evolution was significantly expanded by data arising from new animal models such as dogfish and cricket and from the analysis of regulatory regions of genes involved in appendage patterning. Within the limits of this report, we have tried to condense the essentials of the advances presented at this meeting.

Limb initiation and identity

Limb initiation in vertebrates is coupled to the general embryonic patterning system controlled by Homeobox (Hox) genes. Although the Hox-based mechanisms that pattern the embryo and determine limb-type identity are similar in insects and vertebrates, the molecular readout of these early processes is increasingly showing them to be distinct. The trend towards differences between the systems was also reflected at this meeting, since unrelated mechanisms that trigger these processes in vertebrates and insects were reported.

The current model for limb specification in vertebrates postulates a complicated interplay between Wnt (orthologous to Wg in the fly) and FGF (fibroblast growth factor) signaling pathways in this process (Kawakami *et al.*, 2001). Two new elements were added to this model at the meeting. J.C. Izpisua-Belmonte (San Diego, CA) reported on the role that the mitogen-activated protein (MAP) kinase phosphatase MKP3 plays in limb initiation. MKP3 dephosphorylates ERK2 (extracellular-signal-regulated MAP kinase), preventing its translocation to the nucleus. It is expressed in the prospective limb mesoderm before limb bud initiation, becoming confined to the distal-most mesoderm at later stages. A variety of misexpression experiments presented by Izpisua-Belmonte showed that MKP3 expression is highly dependent on FGF signaling, but that once activated it inhibits FGF signaling, thereby providing negative feedback. Izpisua-Belmonte, and also M. Logan (London, UK), reported that *Tbx5*, encoding a member of the T-box family of transcription factors and previously shown to determine forelimb identity, is also absolutely required specifically for initiation of the limb. Indeed, the conditional limb-specific *Tbx5* knockout presented by Logan completely lacks forelimbs and any sign of limb initiation, failing even to express detectable levels of limb development markers. The data presented are compatible with *Tbx5* mediating the interaction between Wnt and FGF signaling and/or between different FGF signals during limb initiation and apical

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ectodermal ridge (AER) induction, and indicate that there is probably an intimate link between limb initiation and the acquisition of limb-type identity. Interestingly, *Tbx5* expression is also detected in the pectoral fin of the cartilaginous dogfish, showing the broad conservation of these mechanisms at least among vertebrates (C. Tickle, Dundee, UK).

New insights into mechanisms that determine limb identity in the fly came from the studies presented by G. Morata (Madrid, Spain), who addressed dorso-ventral (DV) differences in appendages. *Buttonhead* (*Btd*), a gene known to be involved in the early determination of fly head segments, was found to be expressed in the primordia of ventral *Drosophila* appendages, such as legs. *Btd* overexpression in dorsal regions of the fly, for example in wings, induced their transformation to legs. *Btd*, and possibly its paralog *dSP1*, are therefore involved in specifying ventral character in *Drosophila* appendages. Two other genes involved in this process were identified by enhancer trapping (a method whereby genes are tagged with reporters via transposon insertion) and *Drosophila* genome sequence annotation. The products of these genes select antennal, as opposed to leg, identity (E. Sánchez-Herrero, Madrid, Spain).

Short- versus long-range signaling: patterning insect limbs

One of the main themes of the meeting was the reassessment of patterning models in terms of either long-range morphogens or a series of successive shorter-range interactions. Long-range morphogens provide a general, elegant explanation for processes such as the development of whole limbs. However, these processes increasingly seem to be quite complex, involving multiple independent, and often mechanistically unrelated, patterning events. One alternative for the integration of such events invokes morphogens only at early stages of limb development, to divide limb primordia into a few rough domains by activating key target genes. This would be followed by patterning processes mediated by local signals and interactions between the products of such target genes. Because of this cascade effect, morphogens would produce wide-ranging yet precise patterning effects (Figure 1B). According to this model, a morphogen's range need not be as long as first had been thought (Figure 1A), since early limb primordia are only tens of cells across, rather than hundreds or thousands as they are later on. Support for this kind of step-patterning model came from several of the *Drosophila* talks: a clear distinction was made between early morphogen activity and later local interactions in the patterning of the fly leg. Moreover, even the early morphogen-mediated signaling events were questioned in the case of the wing.

Proximo-distal limb development in insects. In *Drosophila* legs, development and growth along the proximo-distal (PD) axis is thought to be driven by a combination of Wg and Dpp signaling. Different doses of the Wg and Dpp proteins, expressed from ventral and dorsal cells respectively, activate the expression of genes such as *Distalless* and *dachshund* in their specific PD domains. Interestingly, surgical manipulation of the cricket leg demonstrates local ectopic expression of wg and dpp in regenerating cricket legs (S. Noji, Tokushima, Japan), suggesting that the early steps of molecular patterning during regeneration are similar to those of normal leg development. Nonetheless, in the

Fig. 1. Long- versus short-range patterning. Patterning can be achieved over broad areas by (A) a long-range morphogen (m) instructing cell fates directly, many cell diameters away from the source, or (B) a sequence of short-range patterning signals (m1–m3) as the cells proliferate. Note that in either case, alteration of the initial signal will have both long-range and widespread effects, and also the early (t1) and late (t2) functions of m1 will be difficult to disentangle.

case of normal *Drosophila* legs, PD interactions between *Distalless*, *dachshund* and a short-range distal signaling gradient mediated by the EGF receptor, rather than a continued, direct long-range effect mediated by Wg and Dpp, were shown to control subsequent leg development (J.P. Couso, Brighton, UK). These PD interactions led to the determination of tarsal segments by activating the expression of the *rotund* and *bric-a-brac* genes and of the pretarsus by *aristalless* and *dlim1* expression.

Dpp and Wg signaling and their function as morphogens. In *Drosophila*, Dpp is postulated to drive wing growth along the anterior–posterior (AP) axis by acting as a long-range morphogen; it is thought to form a gradient emanating from its source of expression along the AP compartment boundary (reviewed in Serrano and O'Farrell, 1997). A. García-Bellido (Madrid, Spain) presented an alternative model based on the pattern of mitosis in the wing. He proposed that the confrontation of disparate positional values causes local proliferation to create intermediate values and to smooth out the disparity. However, the audience concurred that the model of Dpp as a mitogenic morphogen is more in keeping with the available data. While clones in which Dpp is ectopically expressed trigger extra growth and proliferation,

clones expressing an activated form of the Dpp receptor Thick veins (Tkv) do not, although they do trigger patterning abnormalities. The latter experiment shows that the extra growth at the edges of the clone that would be predicted by the 'local proliferation model' does not occur. Nevertheless, this finding is also difficult to reconcile with the 'Dpp mitotic morphogen' model, which can accommodate this finding only if receptors other than Tkv are postulated for Dpp-driven growth. Regardless of which model is correct, what is not disputed is that the Dpp effect on patterning is largely mediated by the precise regulation of the target transcription factor Spalt, whose complex regulatory region includes binding sites for the transcription factors Mad and Brinker, as well as for the wing-identity protein Scalloped, as was shown by J. de Celis (Madrid, Spain).

New components of the signaling pathway mediated by Wg, at both the extracellular and intracellular levels, were presented. The secreted Wg protein forms a gradient that is not wholly based on free diffusion, but is mediated by its interaction with elements of the extracellular matrix, in particular the Dally and Dally-like proteins. S. Cohen (Heidelberg, Germany) presented an analysis of the *Notum* gene, which encodes a secreted inhibitor of Dally and Dally-like that is positively regulated by Wg. Thus, via Notum, Wg shapes its own gradient. K. Basler (Zürich, Switzerland) introduced two new components on the Wg intracellular pathway: Legless and Pygopus. These two proteins interact directly with Armadillo (Arm)/ β -catenin, but do not compete with Arm for binding to the effector transcription factor TCF (T-cell factor). Basler's model favors a role for *Pygopus* and *Legless* as adaptors for members of an extended transcriptional complex.

Wg has been proposed to drive wing growth along the DV axis (the main growth axis for the *Drosophila* wing) by acting as a secreted long-range mitogenic morphogen from its narrow domain of expression along the DV compartment boundary (reviewed in Serrano and O'Farrell, 1997). This model was questioned (A. Martínez-Arias, Cambridge, UK) on the basis of (i) the relatively late expression of Wg along the DV boundary, which seems to follow rather than precede key events in DV wing signaling such as activation of the transcription factor Vestigial, and (ii) the paucity of extra growth phenotypes produced by ectopic Wg, which appears to require the prior presence of Vestigial. In Martínez-Arias' view, Wg potentiates the expression and mitogenic action of genes such as *vestigial*, rather than generating and organizing new expression patterns (Klein and Martínez-Arias, 1999).

Patterning vertebrate limbs

Old and new models for PD patterning. The progress zone (PZ) model (Summerbell *et al.*, 1973) has been used in the last three decades to explain PD specification in the vertebrate limb. The PZ is the mesodermal area underneath the AER (Figure 2) in which cells are thought to undergo progressive distalization, under the influence of the AER. When cells leave the PZ, a specific PD fate that correlates with the time they have spent in the PZ is determined. This model implies a clock mechanism for distalization that would be intrinsic to the mesenchyme but powered by an external 'battery': the AER signals (Figure 2A). The meeting hosted a stimulating debate on the mechanisms

Fig. 2. The progress zone (PZ) model versus the new early specification model. According to the PZ model (A), the cells are progressively specified as a result of the time they spend under the apical ectodermal ridge (AER). In the early specification model (B), the cells are already specified early on, and progressive expansion occurs with growth.

involved in PD specification in the limb, with the presentation by C. Tabin (Boston, MA) of the 'early specification' model (Dudley *et al.*, 2002). Tabin proposed that the different PD limb segments are indeed specified from early stages of limb development, but that they expand in a PD sequence as the limb elongates (Figure 2B). Tabin indicated that the cell death and the reduction in proliferation that occur in the distal mesenchyme after AER removal are sufficient to explain the ensuing truncation phenotype. Furthermore, after AER removal, the distal-most PZ cells do not contribute to more proximal skeletal elements, as would be predicted by the PZ model. Tabin also presented labeling experiments showing that the descendants of cells located at a certain distance from the AER are usually found exclusively in one limb segment corresponding to the initial position of the marked cells. L. Wolpert (London, UK), 'father' of the PZ model, reminded the audience of the main features of the PZ model and its predictions, and explained how it still provides a valuable explanation for limb development. Overall, the conclusion was that further experimentation is required to test the validity of both models.

For the last decade, FGFs have been considered the AER signals that permit distalization in the PZ model (Martin, 1998). However, the phenotype of the limb-specific double knockout for *Fgf4* and *Fgf8* presented by G. Martin (San Francisco, CA) is difficult to reconcile with the PZ model. The analysis of this double mutant reveals an early FGF8 activity influencing initial limb bud size, a parameter which appears to be related to the number of skeletal elements that will form later in limb development. FGFs from the AER are later required for cell survival, but surprisingly, the abnormal cell death in the double mutant occurs proximal to, and at a considerable distance from, the endogenous FGF source. Moreover, the skeletal defects observed in the mutant limbs include hypoplasia of large domains along the PD limb axis and do not support the idea that

FGFs are the driving force behind the PZ model clock (Sun *et al.*, 2002). Other AER molecules such as Wnts, alone or in combination with FGFs, might thus contribute to the complete set of AER functions.

A novel view of the specification of the PD axis was presented by M. Torres (Madrid, Spain). He proposed a two-signal model involving both a proximalizing and a distalizing signal. The proximalizing signal is probably retinoic acid (RA), which is known to promote proximal fates by activating the Meis transcription factors. Among the distal signals are the FGFs from the AER, which promote distalization by antagonizing RA effects.

Evolution of PD limb axis patterning. Further insights into the mechanisms relevant to limb PD patterning came from the evolution studies of M. Tanaka (presented by C. Tickle) on the comparative molecular analysis between the dogfish and tetrapods. The metapterygial axis, thought to represent the precursor of the PD tetrapod limb axis, runs parallel to the main body axis in these animals (Figure 3). Interestingly, *Shh* is not expressed in dogfish limb buds. In contrast, dHAND, a transcription factor upstream of *Shh* in the limb-specific signaling in other vertebrates, is expressed in and confined to the posterior limb bud as it is in the other vertebrates. These results identify the dogfish as an important intermediate in tetrapod limb evolution and suggest that *Shh* acquisition is related to the rotation of the main limb axis to generate 'freed' extremities that orient perpendicular to the main body axis and to the generation of distal limb parts (Figure 3). These results are consistent with the phenotype of the *Shh* null mice, whose limbs appear to develop in the body wall, with only the distal parts protruding.

An interesting question then arises: at what point in evolution was limb *Shh* expression acquired and what is the molecular basis for this acquisition? To address this, new data showing the existence of a limb-specific regulatory element for *Shh* in chick, mouse and man were presented. One report covered a chick mutant called *Oligozeugodactyly* (OZD) (M. Ros, Santander, Spain), which lacks *Shh* signaling specifically in the limbs. All of the morphological, experimental and molecular data are compatible with the mutation affecting a *Shh* limb-specific regulatory element. Similarly, the study of human preaxial (anterior border) polydactyly and the mouse polydactylous *Sasquatch* mutation led R. Hill (Edinburgh, UK) to propose that the *Shh* limb-specific regulatory element resides in the *Lmbr1* gene, at a distance of 1 Mb from the *Shh* locus. This long-range regulatory element appears to be highly conserved and provides an additional control for *Shh* expression. The acquisition of this level of regulation probably represents an important advance in tetrapod evolution.

How does *Shh* pattern the AP axis? This was a recurring question during the meeting. *Shh* has been a protagonist in vertebrate limb development since its discovery in the early 1990s. It was shown to elicit all the effects of the zone of polarizing activity (ZPA) and was thus considered to be the morphogen emanating from the ZPA. Recent analysis of *Shh* distribution shows that it is indeed able to diffuse away from the ZPA, but its mechanism of action in the limb is still under investigation and probably involves different short- and long-range functions. The recent detailed study of the *Shh* knockout limb clearly showed that *Shh* only becomes necessary from the elbow/knee joint outward, and that the complete PD axis can be generated in its absence

Fig. 3. Evolution of the proximo-distal (PD) axis. In the dogfish limb (A), the PD axis (yellow arrow) remains parallel to the main body axis and *Shh* is never activated. In tetrapods (B), the recently acquired expression of *Shh* results in rotation of the axis, contributing to the protrusion of the limbs. *Shh*'s function in limb patterning requires Gli3.

(Chiang *et al.*, 2001; Kraus *et al.*, 2001). The presentation of the limb phenotype of the compound mutation for *Shh* and *Gli3* (J. Fallon, Madison, WI, and R. Zeller, Utrecht, The Netherlands) once more raised the question of *Shh*'s role in limb development. Gli3 is one of the vertebrate homologs of *Drosophila* Ci and is considered to be a negative regulator of *Shh* expression. Surprisingly, the phenotype of the double knockout is indistinguishable from that of the *Gli3* null mice: the limb is polydactylous, but the digits that form are iterative and generic.

As for Ci, proteolytic cleavage of Gli3 generates a short form (83 kDa) that acts as a repressor of *Shh* targets. In the presence of *Shh*, Gli3 is not processed (Wang *et al.*, 2000). The preaxial polydactyly in the *Gli3* mutation was previously interpreted to be a consequence of the ectopic *Shh* activation that occurs at the anterior bud. This now requires a new interpretation, since the double *Shh/Gli3* mutation indicates that anterior ectopic *Shh* expression in the *Gli3* null limb is completely irrelevant to the phenotype. The double *Shh/Gli3* mutant demonstrates that limb skeletal formation and limb patterning are separable developmental processes and that patterning effects of *Shh* require Gli3.

Patterning the digits. The digits, an evolutionary acquisition of the tetrapod limb, present specific morphologies according to their positions along the AP axis. Fallon discussed whether the identity of each digit is a fixed property of the digital primordium. A variety of barrier experiments, in which digit primordia are isolated from interdigital tissue, led him to propose that the digit primordia do not have fixed AP values but rather are naive and able to respond to local signals. These signals arise from the interdigits and are probably mediated by BMP (bone morphogenetic proteins) signaling. Whether interdigital signaling acts directly upon the digital primordia or indirectly through the AER is an open question. Tickle presented data from the work of J.J. Sanz-Ezquerro, showing that if AER action is prolonged, the digital primordia grow for an extended period and the penultimate phalanx duplicates, forming an extra joint, whereas the tip of the digit is normal. There are a number of other

situations in which the distal phalanx also forms normally even though the rest of the digit is absent or malformed. This may indicate that the digit tip is unique and raises the question as to whether it is laid down earlier than the rest of the digit, as noted above.

D. Duboule (Geneva, Switzerland) reported work from his laboratory on the identification and functional analysis of the genomic element responsible for the last phase of the expression of the *Hoxd* genes in mouse limb buds, termed phase III, which is related to generation of the autopod (the distal segment of the limb that contains the digits and is unique to tetrapods). Interestingly, exhaustive genetic analysis identified a remote digit enhancer that, upon interaction with the set of *Hoxd* promoters, controls the graded co-linear expression of these genes in the limb. To some extent, the more 5' *Hoxd* genes were found to be functionally equivalent, and the differences in their natural roles depend mainly on their location along the chromosome and levels of expression. Accordingly, S. Mackem (Bethesda, MD) showed that the misexpression of either *Hoxd12* or *Hoxd13* at later stages of chondrogenic condensation (the first step in the formation of the skeletal elements) gave a virtually identical phenotype, which was characterized by the absence of proximal and anterior skeletal elements. All of these results highlight the importance of the regulatory system versus specific *Hoxd* protein function. In evolutionary terms, phase III *Hoxd* expression was acquired at some point between the divergence of the teleost lineage and the appearance of the tetrapods. These studies suggest that, at some stage, the *Hoxd* cluster fell under the influence of the long-range enhancer discussed by Duboule, implementing phase III *Hoxd* expression and perhaps seeding the autopod for the first time in evolution. Remarkably, the same regulatory region also controls *Hoxd* expression in the penis, and further similarities between limb and genital development, with regard to Shh and FGF signaling, were presented by M. Cohn (Reading, UK).

Regression of the interdigital tissue to free the digits, a process in which BMP and FGF signaling cooperate, also received considerable attention. L. Niswander (New York, NY) delighted the audience using the bat limb as a model, presenting data showing that there is a strong correlation between FGF signaling and interdigital webbing in this species. J. Hurlé (Santander, Spain) has used the interdigital tissue to assess the assignment of chondrogenic versus apoptotic fates in the autopod. At this meeting, he reported that members of the Sox transcription factor family are the most precocious markers of developing cartilage. His experiments also suggested an additional role for these genes in establishing the AP identity of limb cartilage. In summary, forming a digit appears to be a complex process that requires successive steps from early patterning events in the limb bud to late local interactions between digital and interdigital tissues and is governed throughout by the permissive action of the AER.

Perspectives

This meeting provided many cues for pondering the way appendage development is currently understood. Challenging

well-established models and concepts was a remarkable trend at this meeting. Many of the participants were left with the feeling that even established concepts can be questioned as new tools and ideas become available. To be productive, however, a move away from established models requires the proposal of more plausible alternatives. We believe that all participants took some 'homework' away with them and that the re-evaluation of current models will be fruitful in the near future.

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