Instituto Juan March
de Estudios e Investigaciones

137 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

Limb Development

Organized by

D. Duboule and M. A. Ros

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Introduction

M. A. Ros and D. Duboule

Investigation in recent years has led to a tremendous advance in our knowledge of the mechanisms and molecular basis underlying development. Quite surprisingly, these studies have also shown that evolutionary distant organisms (e.g. insects and mammals) use similar molecules and genetic interactions to outline pattern. These findings have challenged classical concepts such as analogy and homology and have brought the disciplines of Development and Evolution closer than ever.

Traditionally, the development of the appendages has provided a fruitful system to analyze multiple paradigmatic questions of Developmental Biology. For example, the study of the wing and leg imaginal disk of *Drosophila* has come a long way in identifying the genetic cascades controlling patterning in each axis of the primordium. The studies in vertebrates have benefit from the knowledge obtained in *Drosophila* obtaining a rapid advancement and demonstrating a high degree of conservation not only of the genes but also of their genetic interactions.

This Workshop on Limb Development provided an excellent opportunity to bring together and compare different experimental, genetic and molecular approaches in vertebrates and insects. Emphasis was placed in analyzing the establishment of the dorsoventral axis in the developing *Drosophila* wing and whether it relays on a morphogen gradient from the organizer or involves modulatory short-range interactions. The available experimental data were interpreted and discussed accordingly to both models. Latest findings in wingless signaling, at both intra and extracellular levels, were also presented. Further talks presented recent advances in the regulation and function of different gene expressions implicated in *Drosophila* appendage development.

In vertebrates the "progress zone" model has officiated our understanding of the development of the proximodistal axis in the past thirty years. An alternative model is now put forward and all the support in favor and against each model was vividly exposed. Data from mouse knockouts and mutations in several species attracted much attention and challenged the common way in which signaling pathways such as Fgf and Shh, or factors such as Homeobox genes are presently interpreted.

The evolutionary implications of the different issues addressed in the workshop were treated throughout the whole meeting, particularly in the final session. The similarities and differences in developmental mechanisms and gene expressions in different organisms were explored and discussed.

In sum, this Workshop on Limb Development was remarkably timely. We believe that the insights gained will have great implications for the scientific community in the field and we look forward seen how the new thoughts and arguments will evolve in the near future.

Marian Ros and Denis Duboule

Session 1: Early steps in appendage development
Chair: John F. Fallon

Regulation of Wingless morphogen gradient formation by Torero, a novel secreted antagonist

Stephen Cohen

Secreted signaling proteins of the Hedgehog, Wingless/Wnt and Dpp/BMP/nodal families function as morphogens during animal development. In some cases these proteins have been shown to form extracellular gradients that instruct cells in developing tissues about their prospective fate (reviewed in Teleman et al., 2001). Cells distant from the source of the ligand may experience a low level of signaling and exhibit low threshold responses, whereas cells closer to the source may elicit high threshold responses (reviewed in Gurdon et al., 1998). A variety of factors can contribute to shaping morphogen gradients. These include extracellular and cell surface proteins that can bind the ligands as well as factors that modulate a cell's ability to respond to them. Heparan Sulfate Proteoglycans (HSPGs) influence many ligand-receptor interactions and have been shown to play a role in Wingless signaling (reviewed in Selleck, 2001). Wingless protein binds tightly to glycosaminoglycans and embryos defective in HSPG synthesis show reduced sensitivity to Wingless. Two specific HSPG core proteins have been implicated in Wingless signaling. The *Dally* and *Dally-like* genes encode GPI-anchored proteins of the glipican family that are required in the embryo for cells to have normal sensitivity to Wingless. Although it is required for normal Wingless activity, Dally has a limited capacity to increase the level of extracellular Wingless binding to cells when overexpressed in the wing disc. In contrast, cells overexpressing Dally-like protein, Dly, accumulate Wingless to considerably higher levels than surrounding cells. Together with the observation that Wingless binding is reduced in *sulfateless* mutant clones these observations have led to the proposal that binding to Dally and Dly may stabilize Wingless and help it interact productively with its receptor. An alternative view is that Dly may help increase the local concentration of Wg near the cell surface and provide a pool of Wg protein that can become available for receptor binding on release from the HSPG. Thus HSPGs may have a role in gradient formation. I will report on the identification of a novel secreted repressor of the Wingless pathway, called *Torero*. When overexpressed *Torero* blocks Wingless activity. Loss of *Torero* function leads to increased Wingless activity by altering the shape of the Wingless protein gradient. *Torero* encodes a member of the a/b hydrolase superfamily, with homology to pectin acetylesterases. We present evidence that Torero influences Wingless protein distribution by modifying the heparan sulfate proteoglycans Dally-like and Dally. *Torero* expression is elevated by high levels of Wingless signaling. Thus Wingless contributes to shaping its own gradient by regulating expression of a protein that modifies its interaction with cell surface proteoglycans.

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The role of *buttonhead* and *DSp1* in the formation of imaginal disc primordia in *Drosophila*

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The zinc finger genes *buttonhead (btd)* and *DSp1* (the *Drosophila* homologue of the human *Sp1* gene) play a role in the development of the antennal, intercalar and mandibular head segments of *Drosophila*. They are also expressed in the thoracic imaginal disc primordia during embryogenesis but it was not clear whether they serve a function there. We report recent results from our laboratory about the regulation and function of *btd* and *DSp1*. We find that the expression of *btd* and *DSp1* in the thorax requires *wingless* activity and also appears to be modulated by the epidermal growth factor *Spitz*. The activity of the BX-C genes prevents *btd* and *DSp1* expression in the abdominal segments. *btd* and *DSp1* are necessary for the activation of the genes *Distal-less (Dll)* and *headcase (hdc)* in the thoracic segments.

Moreover, *btd* is able to induce ectopic *Dll* activity during embryogenesis, suggesting that *btd* is a positive regulator of *Dll*. We propose that *btd* and *DSp1* are the genes responsible for the activation of the set of genes involved in the formation and development of the thoracic disc primordia. This conclusion is also supported by the results obtained from experiments inducing *btd* function during imaginal development. Groups of cells expressing *btd* in the eye form antennal structures, whereas in the wing and haltere they form mid- and hindleg patterns respectively. These transformations result from the suppression in *btd*-expressing cells of genes specifying dorsal identity such as *vestigial* or *eyeless*, and the concomitant activation of genes conferring ventral identity such as *homothorax*, *Dll*, *dachshund*, etc. The fact that the type of ventral transformation is congruent with the segment indicates that the cells expressing *btd* integrate input from the Hox genes.

WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo

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The establishment and consolidation of specific interactions between instructive signaling pathways is a key characteristic of embryonic development. During the development of any given structure or organ, several signaling mechanisms cooperate in providing positional information to cells in the corresponding developmental fields. In many cases, this "cross-talk" of signaling pathways involves interactions between different tissues of the developing embryo, with sequential (and often directional) transfer of positional information from one tissue to another in a very stereotyped way. If development is to proceed smoothly, the cross-talk between signals must be tightly regulated in space and time, so that the flux of positional information between tissues has the direction and intensity required at any specific time during development of each organ or structure.

One of the best examples of signaling molecules involved in the complex cross-talk mechanisms that pattern developing embryos is the Fibroblast Growth Factor (FGF) superfamily of secreted factors. FGFs play important roles in multiple aspects of embryonic development in a variety of organisms. One of their most interesting activities is related to their involvement in limb development in vertebrates. Specifically, several FGFs have been shown to play key roles in the control of limb initiation, the induction of the apical ectodermal ridge (AER), and the activity of the AER itself. Due to the importance of the vertebrate limb bud as a model system for the study of pattern formation, the analysis of the mechanisms of action of FGFs as well as the study of in this experimental system is of paramount importance if we are to understand the multiple instructive activities of FGFs during development.

The current models of limb initiation and AER induction in vertebrates stress the role of a regulatory loop between two members of the FGF superfamily: FGF-8 and FGF-10. Among the possible candidates for engaging in regulatory cross-talk with FGFs during limb initiation and AER induction, the WNT superfamily of secreted factors is particularly interesting. FGFs and WNTs have been shown to interact in a variety of developmental systems, including tracheal development in *Drosophila*, mesoderm induction and neural patterning in *Xenopus*, and brain, tooth and kidney development in other vertebrates. During vertebrate limb outgrowth, the *Wnt-3a* gene has been shown to act upstream of *Fgf-8* during AER induction in the chick limb. So far, however, no *Wnt* gene has been implicated in limb initiation, and the mechanisms by which *Wnt-3a* participate in AER induction and *Fgf-8* activation are not fully understood.

We will show that specific instances of cross-talk between WNTs and FGFs control limb initiation and AER induction in the chick embryo. The *Wnt* genes *Wnt-2b* (expressed in the IM and the LPM at the forelimb level), and *Wnt-8c* (expressed in the LPM at the hindlimb

level), in a ß-catenin-dependent process that results in activation of *Fgf-10,* are both capable of inducing ectopic limbs in the embryonic flanks. Moreover, a third *Wnt* gene, *Wnt-3a*, mediates the induction of *Fgf-8* in the limb ectoderm by FGF-10, in a process also mediated by ß-catenin. Thus, three *Wnt* genes that signal through ß-catenin mediate the FGF-8/FGF-10 loop that directs limb initiation and AER induction in the chick embryo.

The results to be presented provide a specific example of cross-talk between signaling pathways that results in a stereotyped flux of positional information between the tissues involved in the early control of limb development. This kind of specific interaction between FGF and WNT signals may be an efficient mechanism to control the timing and directionality of inductive signals in a variety of developmental processes. It will be interesting to investigate whether WNT pathways also interact with FGFs in other regions of the embryo where FGFs are also known to play an organizing role, such as the brain, lungs and other structures. Overall, our results underscore the importance of cross-regulation between signaling pathways, which ensures a fine-tuning of the activities of organizing factors that shape the developing embryo.

Digit patterning and morphogenesis

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Sonic hedgehog is expressed in the polarizing region of vertebrate limb buds. Analysis of the Shh-/- knockout showed that Shh is essential for proper establishment of the main limb axis and development of distal structures such as digits (1). Thus acquistion of Shh signalling must have been a key event in evolution of vertebrate appendages (2; also poster at this meeting). In chick wing buds, sonic hedgehog signalling regulates expression of Bmps, with *Bmp2* expression being induced and *Bmp4* expression repressed (3). Shh and Bmp signalling co-operate to specify digit number and identity and one of the target genes regulated by polarizing region signalling is *Tbx3* (3). Morphogenesis of digital primordia can be modulated by Shh application that prolongs outgrowth resulting in development of an additional segment (4; also poster at this meeting). There is evidence that the tip of the primordium is specified early and by a special mechanism (4).

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Session 2: Axes and signaling centers (I)
Chair: Cheryll Tickle

Regulation and function of the *spalt* gene during *Drosophila* wing formation

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The *Drosophila* wing imaginal disc, that forms the wing and thorax of the fly, has been extensively used as a model system to study the genetic and molecular bases of limb development. The wing disc is subdivided into lineage units called compartments, and the borders between compartments act as "organising centers" with determining roles on growth control and pattern formation (Lawrence and Struhl, 1996). The secreted protein Hedgehog activates the transcription of decapentaplegic (*dpp*), which in turn promotes growth and organises the pattern of wing veins (Sanicola et al., 1995). Dpp is required for the expression, in broad domains centered on the anterior/posterior boundary, of several genes encoding transcription factors, such as *sal, salr* (de Celis et al., 1996: Lecuit et al., 1996; Nellen et al., 1996), *vestigial* (Kim et al., 1997) and *optomotor blind* (Grimm and Pflugfelder, 1996; Lecuit et al., 1996; Nellen et al., 1996). Since most of the effects of *dpp* mutations on vein patterning are observed when the function of the *sal/salr* genes is reduced, it appears that, to a large extent, Dpp organises the pattern of veins through the regulation of *sal/salr* expression (de Celis et al., 1996). The regulation of *sal/salr* expression relies on the presence of discrete enhancers distributed both 5' and 3' of each gene (Barrio et al, 1999; de Celis et al., 1999). We are focussing on the *sal* regulatory sequences that mediate the response to Dpp signaling in the wing blade. In this territory the *sal* gene is activated by Dpp in the center of the disc, repressed by Brinker (Brk) in the lateral regions and repressed by Ultrabithorax (Ubx) in the haltere imaginal disc. All the sequences that mediate this regulation are confined within a 1.8 kb fragment located 5′ to the *sal* gene. We have assigned those regulatory functions to distinct seuences through the analysis in transgenic flies of several reporter constructs containing sub-fragments derived from the original 1.8 kb fragment. Furthermore we detected direct interaction of the enhancer with the regulatory molecules Ubx, Brk, Mad/Med and CREB by means of electrophoresis mobility shift assays (EMSA) using fragments of the 1.8 enhancer labeled radiactively.

The function of Sal and Salr is required for the correct positioning of veins, but very little is known about the mechanism of action of these proteins or the nature of their downstream genes. Our current hypothesis, based on the structure of the proteins, their nuclear localisation, and the ability of at least Salr to bind to AT rich DNA sequences (Barrio et al., 1996), is that Sal and Salr act as transcriptional regulators. So far, we have identified two gene complexes that are good candidates for Sal/Salr targets, the *iroquois* and *knirps* gene complexes (Barrio and de Celis 2000). The expression of *iro* is repressed by Sal/Salr. In contrast the expression of *kni* is activated by low levels of Sal/Salr and repressed by higher levels. The effects on *kni* and *iro* expression observed in *sal/salr* mutants are cell autonomous, suggesting that they could be direct. These observations imply that Sal and Salr have the ability to act both as transcriptional activators or repressors, depending on their concentration and the target gene.

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Wingless transduction by Legless and Pygopus

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Wnt/Wingless signaling controls many fundamental processes during limb development. Wnt transduction is mediated by the association of beta-catenin with nuclear TCF DNA-binding factors. We identified two novel segment polarity genes in Drosophila, *legless* (*lgs*) and *pygopus* (*pygo*), and found that their products are required for Wnt signal transduction at the level of nuclear beta-catenin. Lgs encodes the homolog of human BCL9. Genetic and molecular evidence indicates that these proteins permit beta-catenin to transcriptionally activate Wnt targets during development by physically linking Pygo to beta-catenin.

Wing development in *Drosophila*: molecular activities and functions of the DV axis

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Wing development in *Drosophila* results from a series of sequential and mutually interdependent regulatory steps involving a set of conserved signalling molecules: Hedgehog, Wingless, Dpp and Delta. The activities of these signalling molecules are integrated through the transcription factor Vestigial (1, 2). This integration relies on local inputs from signalling centers located along the dorsoventral (DV) and the anteriorposterior (AP) axes. The information from the AP axes has a constant source throughout development at the AP boundary, and is contained in a hierarchical set of gradients of signalling and transcription factor activities. Hedgehog and Dpp are at the top of this hierarchy (3, 4 and see review in 5). The information from the DV axis is more complex, It does not have a constant source and does not operate through graded signalling events, but rather relies on a series of sequential and mutually interdependent patterns of expression of the Notch ligands Delta and Serrrate and of the signalling molecule Wingless (2, 6, 7) which create and mediate local cell interactions and, in some instances, modulate some of the information provided from the AP axis.

A common model of the contribution of the DV axis to wing development surmises that early in wing development Notch signalling, at the interface between cells expressing and not expressing the Apterous transcription factor, creates a stripe of expression of Wingless which then acts as a "DV organizer" for wing development much as Hedgehog and Dpp do from the AP axis (8, 9). Therefore in this model Wingless is the effector of Notch signalling, much as Dpp is the primary effector of Hh signalling in the AP axis. However, overactivation of Wingless signalling cannot rescue the loss of wing caused by loss of Notch signalling (7) ruling out a simple hierarchical relationship like the ones that exist in the AP axis. Furthermore, in contrast to Hedgehog and Dpp which have dramatic effects on wing development when they are expressed from ectopic sources (e.g 3, 4), Wingless on its own is incapable of altering the main parameters of growth and pattern of the wing (2, 7). Wingless appears to operate as a modulator of the activity of other signalling molecules and transcription factors (2, 10). On the other hand, Notch signalling is involved in the growth of the wing in a manner that is independent of Wingless (see e.g 11 and 12) and which involves both canonical (Su(H) dependent) and non canonical (Su(H) independent) signalling events.These observations suggest a model in which the DV axes provides a series of modulatory rather than inductive influences in the development and patterning of the wing.

While the interface between Apterous expressing and not expressing cells is important for wing development early in larval development (1,2, 13), the DV compartment boundary and the associated stripe of Wingless expression do not seem to be necessary for the growth and large scale patterning of the wing (2, 11, 13). Instead Wingless in the DV striped pattern

and the DV compartment boundary are required for the development and patterning of chemo- and mechanosensory organs in the late stages of larval development (14).

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Analysis of a chick limb mutant [Oligozeugodactyly (Ozd)] that lacks Sonic Hedgehog function in the limb bud

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We have analyzed a new limb mutant recently described in the chicken (Smyth et al., 2000) and have renamed it *oligozeugodactyly* (*OZD*). The mutant limbs form a normal stylopod but in the zeugopod only the anterior elements form (radius, wing; tibia, leg) while the posterior elements (ulna and fibula) are never initiated and are absent. Additionally, the wing lacks digits but the leg invariably develops with a clearly identified digit 1. Classical recombination experiments indicated that the affected germ layer is the mesoderm and molecular analysis revealed that the *OZD* limbs develop in the complete absence of Shh expression and signaling. This loss of Shh activity is restricted to the limb buds as other tissues known to be dependent upon Shh signaling continue to express Shh and are morphologically and functionally normal. Neither *Ptc*, nor *Gli1* are detectable in mutant limb buds. However *Bmp2* and *dHAND* are expressed in the postaxial wing and leg bud mesoderm, although at reduced levels compared to normal. Expression of *Hoxd11-13* was also normal in the mutant limbs up to stage 23/24. *Fgf8* and *Fgf4* expression are initiated normally in the mutant AER but their expression was progressively downregulated in the anterior AER eventually becoming restricted to a discrete point in the posterior AER.

We conclude that Shh becomes necessary for limb patterning at the elbow and knee joints, similar to the Shh null mouse (Chiang et al. 2001). We further suggest that Shh, in conjunction with the AER, also plays a crucial role with the AER in maintaining limb bud mesoderm mass. Our data are consistent with a model of limb development that proposes that the radius/tibia and digit one are Shh independent, while the ulna/fibula and other digits are Shh dependent.

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Chiang

GLI3 and dHAND prepattern the limb bud mesenchyme prior to SHH signalling

Rolf Zeller

Others have shown that the bHLH transcription factor dHAND is required for establishment of SHH signalling by the limb bud organiser in posterior mesenchyme, a step crucial to development of vertebrate limb buds. Our most recent studies establish that the transcriptional repressor GLI3 restricts *dHAND* expression to posterior mesenchyme prior to activation of SHH signalling in mouse limb buds. dHAND in turn excludes "anterior" genes such as *Gli3* and *Alx4* from posterior mesenchyme. This mutually antagonistic genetic interaction directs establishment of the SHH/FGF signalling feedback loop by restricting the BMP antagonist *Gremlin* posteriorly. These and other results show that the nascent mesenchyme is prepatterned prior to activation of SHH signalling. The limb bud organiser is positioned at the posterior limb bud margin most likely as a direct consequence of differential mesenchymal responsiveness to SHH signalling. The SHH/FGF feedback loop is then established to propagate signalling by the limb bud organiser.

A new proximal-distal patterning mechanism in *Drosophila*

Juan Pablo Couso, Maximo-Ibo Galindo and Sarah Bishop

The signalling molecules wingless and decapentaplegic establish the proximal-distal (PD) axis of *Drosophila* legs by activating the expression of specific genes such as *distalless* and *dachshund* in broad PD domains during early leg development. However, here we show that Wingless and Decapentaplegic are not required for PD development after the first 84 hours of development, and are not directly required for the development of all the PD pattern of the leg. The tarsus, which has recently been proposed to be an ancestral structure developing without homeotic gene activity, is instead defined by the activity of Distalless, Dachshund and a Distal gradient of EGFR-Ras signalling. Thus, EGFR-Ras signalling triggered by the diffusible vn protein promotes distal leg development and restricts tarsal development. The tarsus is defined in those Dll-expressing cells not simultaneously exposed to either the dac protein or EGFR-Ras signalling.

In conclusion, regulatory relationships between genes expressed in PD domains (PD genes) mediate a patterning process that results in the development of new PD fates. We call this process PD gene patterning, and suspect that its influence might extend to further events of PD leg development. In vertebrates, the Dll, dac and al genes are conserved, and their expression in limbs is similar to insects. Thus, an interesting possibility is that a form of PD patterning similar to that described here is also present in vertebrate limbs.

Session 3: Axes and signaling centers (II)
Chair: Ginés Morata

Role and regulation of differential cell affinities in proximodistal limb development

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Vertebrate limbs develop in a proximodistal sequence: regions close to the trunk are specified and generated earlier than distal ones (Saunders, 1948). Limb outgrowth is triggered by interactions between a distal organizing center, the Apical Ectodermal Ridge (AER), and the underlying mesenchyme, called Progress Zone (PZ). Fibroblast Growth Factor (FGF) and WNT family members underlie such interactions (Cohn et al., 1995; Fallon et al., 1994; Kawakami et al., 2001; Niswander et al., 1993). During limb development, the PZ contributes increasingly distalized cells that incorporate to the limb axis (Summerbell et al., 1973).

The TALE homeobox genes *Meis1* and *Meis2* function as determinants of proximal limb compartments (Capdevila et al., 1999; Mercader et al., 1999). During limb development, *Meis1* and *Meis2* function is restricted to the proximal limb region up to the stylopodal-zeugopodal (S-Z) boundary. Ectopic overexpression of Meis genes during chicken limb development inhibits PZ cell distalization and distal limb differentiation. Retinoic acid (RA) is an upstream activator of *Meis1* and *Meis2* genes in the proximal chicken limb, thereby promoting the proximal character of limb cells (Mercader et al., 1999). FGF, Wnt, and BMP distal signals are responsible for restricting Meis genes expression to the proximal limb (Capdevila et al., 1999; Mercader et al., 2000). Currently, we are analysing the role of Meis genes in mouse and chicken limb development and urodele limb regeneration models.

One of the important aspects regulated by the RA-Meis pathway is the adhesive properties of limb cells. Vertebrate limb cells express homophilic adhesive properties typical of their position along the P-D limb axis. During normal development, dissociated limb cells reaggregate preferentially with cells of a matched P-D identity (Ide et al., 1994; Koibuchi and Tochinai, 1998; Koibuchi and Tochinai, 1999). During regeneration, experimental relocation of limb cells to ectopic positions stimulates their migration to match their level of origin along the P-D axis (Crawford and Stocum, 1988). Both, Meis overexpression and RA treatment, stimulate distal cells to express proximal cellular affinities and to become incorporated to proximal compartments during development (Crawford and Stocum, 1988; Mercader et al., 2000; Tamura et al., 1997). We will present and discuss data on the molecular basis of the adhesive properties regulated by RA/Meis relevant for proximodistal limb development.

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Limb development in the absence of Sonic hedgehog (Shh) and Gli3 function

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Current models propose *Sonic hedgehog* (*Shh*) is the primary determinant controlling anteroposterior (A/P) development of amniote limbs, directing digit formation and specifying digit identities through dose-dependent activation of target gene expression. *Gli3* is thought to negatively regulate *Shh* by restricting its expression to the posterior mesoderm. Using the *Shh*$^{-/-}$; *Gli3*$^{-/-}$ null mouse, we show that *Shh* and *Gli3* are not only dispensable for the formation of mouse limb skeletal elements, but normally act to constrain skeletogenic potential. *Shh*$^{-/-}$;*Gli3*$^{-/-}$ limbs are distally complete and polydactylous, but completely lack wildtype digit identities. Interestingly, *Shh*$^{-/-}$; *Gli3*$^{-/-}$ and *Gli3*$^{-/-}$ limbs are indistinguishable from one another, demonstrating that *Shh* exerts no effect on skeletal patterning in the absence of *Gli3*. We suggest that *Shh* signaling in the limb has a single primary output- regulating the relative balance of *Gli3* full length (or transcriptional activator) and *Gli3* repressor activities. We propose that the principal developmental function of *Shh* and *Gli3* in normal limb patterning is to refine autopodial morphology, concomitantly imposing pentadactyl constraint on the mesoderm's polydactyl potential and organizing the specification of discrete digit identities.

Patterning the proximodistal axis of the limb bud

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Classic experiments have provided important clues into the development of the vertebrate limb bud, including insights into the patterning of the three limb axes. For example, removal of a specialized epithelium found at the distal tip of the limb bud (the Apical Ectodermal Ridge, or "AER") provided the basis for models of progressive patterning of the proximodistal axis. When an AER is removed early in limb development it results in truncations of most of the distal limb skeleton. Later AER removals produce limbs with more limited truncations, still missing the distal tip. This has been interpreted as indicating that the AER is continually respecifying cells at the distal tip to make ever-more-distal structures. Subsequent demonstration that a bead soaked in Fgf protein can replace the ridge in supporting proximodistal outgrowth and patterning, and the observation that the AER produces several Fgf family members, has suggested that it is Fgf that is responsible for proximodistal patterning. However this model now needs to be reevaluated. Various single and double Fgf conditional mutants have now been constructed but they do not produce the expected distal truncations. Moreover clonal analysis of cells lying under the early AER does not support this model for proximodistal specification. A previous study reported that AER removal results in distal cell death. Further work now shows that this cell death can explain the pattern of truncations seen after AER removal. A new model of proximodistal patterning, and the role of Fgf's will be presented.

A new model for FGF function in limb development based on inactivating *Fgf4* and *Fgf8* at different stages

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By eliminating FGF4 and FGF8 signaling from the apical ectodermal ridge (AER) at different stages of mouse limb development we have discovered that FGF8 influences cell number in the nascent limb bud and that subsequently, FGF activity is required for survival of cells in the proximal limb bud mesenchyme. These conclusions, together with results of our analysis of the skeletal phenotypes of the mutant mice, have suggested a new model for FGF function in limb development. This model differs markedly from the hypothesis that FGFs produced in the AER facilitate changes in cell specification in a progress zone at the distal tip of the limb bud. We propose that FGF4 and FGF8 together function prior to the start of skeletal differentiation to establish the number of chondrocyte progenitors that will be available to form limb skeletal elements.

The progress zone model for patterning along the proximo-distal axis

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The progress zone model for patterning the limb along the proximo-distal axis assumes that the cells measure the time spent in the progress zone (about 300 microns) and this gives tham a positional identity . The progress zone is thought to be defined by signals from the apical ridge such as FGFs.The cells the give rise to the humerus leave early and those to the digits, last. Removal of the ridge gives rise to truncations and these are more proximal the earlier they are done. Up to stage 24 there are no significant differences in mitotic index between the progress zone but there is an higher index distally at later stages. Labelling of cells in the progress zone shows that subapical cells contribute to both digits and radius and ulna.The best evidence for the model comes from killing cells in the early bud by X-irradiation or chemical treatment - this results in the loss of proximal cartilaginous structures and the greater the damage the more the losses extend distally , until only digits form; these can be quite normal. A prediction of the model is that if the size of the progress zone is reduced all cartilaginous elements will be normal but smaller as the number of cell leaving per unit time will be reduced.

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Session 4: Patterning the appendages
Chair: Stephen Cohen

Molecular mechanisms that control apical ectodermal ridge morphogenesis and function

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Classical experimental embryology studies identified the apical ectodermal ridge (AER) as the important signaling center that controls limb growth and patterning along the proximal-distal (shoulder to digit) axis (Saunders, 1948; Summerbell, 1974). Our studies over the years have investigated the molecular mechanisms that regulate the formation and function of the AER. I will highlight our more recent and on-going research that addresses questions of AER morphogenesis and function.

Fibroblast Growth Factors (FGFs) are key signals from the AER and they can substitute for the AER to allow limb outgrowth and patterning. The Bone Morphogenetic Proteins (BMPs) regulate AER morphogenesis and function in a complex way. During the earliest stages of limb development, BMPs act as positive factors in the induction of the AER. Moreover, BMPs appear to mediate this function through the transcription factors of the Msx family. BMPs at this time also act upstream of another transcription factor, Engrailed-1, to regulate dorso-ventral patterning of the limb. Thus, AER formation and D-V patterning are linked by BMP but are separately controlled by two different sets of transcription factors (Pizette et al. 2001). Following AER formation, BMPs act as negative signals to limit AER function by controlling AER regression and Fgf4/Fgf8 expression (Pizette et al. 1999).

I will also discuss our recent molecular genetic studies related to an ENU-derived mouse mutant that displays defects in limb growth and patterning. This phenotype appears to arise, at least in part, due to defects in AER morphogenesis. These mice display defects along all three axes of the limb, exhibiting P-D shortening, A-P digit loss and fusions, and duplications of the remaining digits along the D-V axis. Our current mapping data and the unique phenotype suggest that the mutation reveals a novel gene necessary for limb development. (Scott Weatherbee, unpublished).

Finally, I will discuss a new evo-devo approach in which we are using the bat limb as a model to understand the molecular control of morphological diversity. Our current morphological and molecular analysis will be presented, including our experiments to test the underlying molecular basis of interdigital webbing. (Scott Weatherbee, Richard Behringer, Chris Cretekos, and John J. Rasweiler III; unpublished)

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Limb disorders and Shh regulation: a long-distance relationship

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Preaxial polydactyly (PPD) is a common limb malformation linked to chromosome 7q36 (Heus et al. 1999). PPD maps to a complex locus that includes the other limb disorders acheiropodia (Ianakiev, 2001), complex polysyndactyly (Tsukurov, 1994), and acropectoral syndrome (Dunbar et al, 2001). Identification of the basis for one of these abnormalities may lead to a broader understanding of this locus and its role in limb development; however, isolation of the gene responsible for any of these abnormalities has remained elusive. Here we combine genetic approaches in human and mouse to identify mutations responsible for formation of preaxial supernumerary digits.

In mouse, a number of different polydactylous mutants indicate that the ultimate misexpression of *Shh* is a common requirement for generating extra digits. We show that one of these mutants, called sasquatch (*Ssq*), is the counterpart to human PPD. We identify a translocation breakpoint in a PPD patient and the transgenic insertion site in the *Ssq* mutant. The genetic lesion in both mouse and human are located within the same intron of a gene termed *LMBR1/Lmbr1* (Clark et al. 2000) situated approximately 1Mb from *Shh*. We previously showed (Sharpe et al. 1999) that the reporter gene contained within the *Ssq* transgene insertion has acquired a limb-specific *Shh*-like expression pattern; not only in the appropriate posterior ZPA, but also at an ectopic site at the anterior margin of the limb bud. It was unclear whether the mechanism for *Shh* misexpression involves disruption of the *Lmbr1* gene and is therefore indirect or direct due to disruption of a long range *Shh* regulatory element. In order to distinguish the two possibilities we established a genetic *cis trans* test which revealed that Ssq mutation interrupts a long-range *cis*-acting regulator of *Shh* ~1Mb away. Thus PPD in human most likely results from similar acting mutations and supports the prospect that *Shh* regulatory elements underlie this complex locus. Subsequently we have shown that Shh regulatory information resides within this locus and we present a model to explain other limb-related defects that map to this locus.

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Characterisation of two new genes involved in antenna and eye development in *Drosophila melanogaster*

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The morphological diversification of appendages is a crucial aspect in development and evolution (Panganiban et al., 1995). A good model to gain insight into appendage variation is to study the development of antenna and leg in *Drosophila*. Antenna and leg are thought to come from an ancestral structure, but little is known about how their morphogenetic differences take place (Casares and Mann, 1998).

From a P-Gal4 screen done in the laboratory (Calleja et al., 1996), we isolated two lines which have a specific expression pattern in the third antennal segment, the arista and the eye. Those two lines are not expressed in the legs and correspond to insertions upstream of two similar new genes, in which the expression patterns reproduce the P-Gal4 ones.Their pattern of expression overlap with Dll transcription and is restricted to the region of the antennal imaginal disc giving rise to the more distal part of the antenna.

We reported here the study of those two genes in relation with genes like *distal-less*, *dachshund*, *homothorax* and *spalt*, known to play a crucial role in antennal development.

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Serial deletions and duplications of *Hoxd* genes lead to regulatory re-allocations which modify limb morphology

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Vertebrate *Hox* genes members of the *HoxA* and *HoxD* clusters are required for proper limb development. In particular, four contiguous *Hoxd* genes, from *Hoxd10* to *Hoxd13*, are similarly expressed in the most distal part of the developing appendicular skeleton ; the future digits. In order to decipher both the respective functions of these four genes in this context, as well as the underlying regulatory mechanisms, we embarked on an exhaustive series of deletions and concurrent duplications by using the TAMERE recombination strategy. We find that functional differences, as well as a hierarchy exist amongst these genes, and that their transcription likely result from the equilibrium between a remote enhancer sequence and a set of target promoters.

Modifications in either the number, or the genomic order, of these promoters re-allocated the regulatory interactions towards a novel equilibrium, with concurrent modifications in the number of digits and their morphology. These re-allocations are directional, with the gene positioned at the extremity of the complex being always the more strongly expressed, in response to the digit enhancer sequence. This suggests that the 5' located member can compete out other neighboring genes. This regulatory strategy, which provides an explanation for the 'quantitative colinearity' phenomenon (Dollé et al., 1991), is made possible through the presence of evolutionary conserved sequences whose deletions abrogate this kind of colinearity.

The impact of such a regulatory system upon the development and evolution of our digits will discussed, as well as the problem to assign an objective meaning to the concept of gene function in such a tight cluster of transcription units.

Tbx5 is required for limb bud initiation and later for limb outgrowth and patterning

Charalambos Rallis, Jo Brough and Malcolm Logan

Transgenic and conditional knock-out analyses in the mouse define the roles of Tbx5, Tbx4 and Pitx1 in limb outgrowth and patterning

We are studying the molecular differences that distinguish the fore limbs and hind limbs in the developing vertebrate embryo. We are focussing on three genes that have restricted expression patterns in either the developing fore limbs or hind limbs. Two closely related T-box family members, Tbx5 and Tbx4 are expressed in the developing fore and hind limbs respectively. The paired-related gene, Pitx1 is expressed in the hind limb. Since all three genes code for transcription factors we are interested in identifying the target genes that they regulate and how such regulation controls the development of limb-type specific elements.

We are currently using both chick retroviral and mouse transgenic methods to study the genetic pathways upstream and downstream of these three candidate genes. Using an enhancer element capable of driving gene expression in the developing limb buds we have generated independent transgenic lines in which either the hind limb–specific genes Pitx1 or Tbx4 are ectopically expressed in the fore limbs. We observe dramatic changes in fore limb morphology in such lines consistent with a role for these genes in specifying hind limb identity. We have also generated a transgenic line expressing cre recombinase in the developing limb buds. We are currently using the cre-expressing line in combination with a mouse carrying a conditional allele of Tbx5 to generate mice with a limb-specific knock-out of this gene.

Prolonging Hoxd gene expression during mesenchymal condensation causes limb reduction phenotypes and striking oligodactyly as an apparent indirect proximal-to-distal effect

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5' Hoxd genes regulate both formation and growth of chondrogenic condensations, particularly digits. Some of these growth effects appear to be a "late" function, related to persistent 5'Hoxd expression at the peripheries of condensing and differentiating mesenchyme. To evaluate Hoxd function selectively at later stages, we are using a conditional Cre-lox transgenic approach to misexpress Hoxd12 and Hoxd13 in early chondrogenic cells.

This approach will misexpress Hoxd12 and Hoxd13 both by prolonging expression in differentiating chondrogenic condensations relative to the cellular differentiation state (normally it shuts off within the condensation) and by spatially expressing these genes in anterior/proximal condensations where they are normally never expressed. We have begun to evaluate phenotypes using several different Cre lines to manipulate both the timing of transgene expression relative to differentiation state of condensing cells, and the spatial pattern of transgene expression in different condensations. Preliminary results indicate:

1. Hoxd12 and Hoxd13 produce a virtually identical range of phenotypes in this system.

2. Timing of transgene expression relative to the differentiation state of cells in condensing mesenchyme is critical. If transgene expression is started after the cellular chondrogenic differentiation pathway has begun, it has no effect.

3. The phenotypes show predominantly reductions and loss of specific limb elements, especially anteriorly. Mildly affected embryos show selective reductions or loss of the stylopod and of the anterior zeugopod (radius/tibia), and loss of up to several anterior digits (I-III in a strict A-P order with increasing severity). More severely affected embryos also show an overall growth inhibition that is much greater in proximal than in distal elements (ie. digit size is spared, although not number).

4. Surprisingly, even though more posterodistal elements are also affected by the transgene misexpression (including loss of up to 3 digits and reductions of fibula/ulna), phenotypes only occur when transgenic misexpression includes anterior/proximal condensations. Expression restricted to the posterior, or to distal limb elements (ie. digits) has no effect.

These results suggest a non-cell autonomous effect of proximal-anterior condensations on distal undifferentiated mesenchyme that normally serves to promote formation of distal elements. Such a 'positive' indirect effect has not been previously suspected, but may help regulate and coordinate growth.

Session 5: Morphogenesis
Chair: Lewis Wolpert

Analysis of the earliest steps of limb skeletogenesis

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The formation of the cartilaginous template of the limb skeleton is a key event of limb morphogenesis. Basically two main steps can be distinguished in the onset of chondrogenesis: condensation and differentiation. During the condensation stage the precartilaginous mesenchyme is segregated into chondrogenic and nonchondrogenic domains. Next in development, the prechondrogenic aggregate enters into the differentiation stage in which specific extracellular matrix components such as type II Collagen and Aggregan are produced. Sox genes and BMPs have been identified as the major regulatory molecules in both chondrogenic steps. In our communication we will present the results of our studies designed to clarify their role during the formation of the cartilaginous skeleton of the chick leg bud.

We have examined the expression of different members of the Sox family of transcription factors and members of the BMP signaling pathway during the formation of the limb chondrogeneic aggregates. In addition, using the experimental model of TGFß1-induced interdigital digits we have dissected out the sequence of events during in vivo chondrogenesis. We found that *Sox9* and *Sox8* are the most precocious markers of limb cartilage and its expression is independent and precedes the activation of BMP signaling. *Sox10*, which is a marker of the developing nerves, appears also to cooperate with *Sox9* and *Sox8* in the establishment of the digit cartilages. The expression of these *Sox* genes is regulated by the ZPA, retinoic acid- and TGFß-signaling indicating that they are implicated in the specification of the identity of the limb cartilages. In addition, we show that their experimental induction in the interdigital mesoderm is accompanied by loss of the apoptotic response to exogenous BMPs. *L-Sox5* and *Sox6*, are also expressed in the developing cartilages and are induced, respectively, coincident and after the expression of *Bmpr1b* in the prechondrogenic aggregate. The activation of those genes correlates with the induction of *Type II Collagen* and *Aggrecan* genes in the differentiating cartilages.

The expression of *Bmpr1b* precedes the appearance of morphological changes in the prechondrogenic aggregate and establishes a landmark from which the maintenance of the expression of all *Sox* genes and the progress of cartilage differentiation becomes dependent on BMPs. Moreover, we show that *Ventroptin*, precedes *Noggin* in the modulation of BMP activity in the developing cartilages.

In summary our findings fit with a cooperative role of *Sox8*, *Sox9* and *Sox10* in conferring chondrogenic competence to limb mesoderm in response to BMP signals. In turn, BMPs in concert with *Sox9*, *Sox6* and *L-Sox5* would be responsible for the execution and maintenance of the cartilage differentiation program.

Morphogens and cell proliferatlion in the *Drosophila* wing disc

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Centro de Biología Molecular "Severo Ochoa"

It has been postulated that morphogens play a dual role in morphogenesis: they delimit territorial gene expression patterns of target genes and act as mitogens in a graded fashion away from their sources.

We have carried out a detailed clonal analysis using twin clones to ascertain the trends in cell division and allocation of daughter cells in all the wing disc regions. The results do not reveal any differences in mitotic regimes close to compartment boundaries, the sources of the postulated morphogens. Moreover, ectopic expression of genes like *Notch* **(1)** or *vestigial* in clones are capable of generating extragrowths without including compartment boundaries. Experiments giving rise to territorial duplications (e.g. after removal of *engrailed* or of *apterous* or their overexpression in clones) can be interpreted as cell non-autonomous "accommodation" of positional values rather than resulting from simple growth. This is actually the situation in classical intercalar regeneration. More related to growth are local signals between proliferating cells to generate positional values and cell allocation. These signals are affected by a series of genes of the *Notch* **(2)** and *Egfr* **(3)** pathways among others.

The results will be discussed in the light of a morphogenetic model, called the "Entelechia" model **(4)** that emphasizes local cell interactions within compartments as the driving force in cell proliferation, and the control of size and shape.

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New approaches for studying limb development: Optical Projection Tomography, and computer modelling of limb bud tissue

James Sharpe

Vertebrate limb bud development involves phenomena operating at many different levels: from molecular regulatory events, through cellular behaviours, up to the large-scale mechanical properties of tissues. A full understanding is therefore likely to require the development of computer models which can integrate both molecular and mechanical processes. These models should be based on empirical quantitative data, so another goal is the generation of improved techniques for mapping 3D information such as gene expression patterns.

We have invented a new form of 3D microscopy, called Optical Projection Tomography (Science 2002, in press, 19th April). This was originally developed as a method for rapidly and accurately measuring the 3D shapes of limb buds as data for the computer model. However, we have now proven it to be an invaluable method for mapping the 3D distributions of RNA's, proteins and transgenic reporter expression within embryonic tissue. We are thus building up 3D datasets of gene expression patterns, and exploring its use in mapping proliferation rates and cell death distributions.

As a first step towards powerful computer models of limb bud development, we have used OPT data to build a 4D mechanics-based computer model of early vertebrate limb development (chick and mouse). The model allows us to explore different explanations for the movement of tissue through time and space, for example different distributions of proliferation rates or cell migration. Preliminary results are encouraging, suggesting that such models will be important for arriving at complete dynamic descriptions of the mechanics of limb bud development.

Session 6: Evolution
Chairs: Maria A. Ros and Denis Duboule

The roles of evolutionarily conserved genetic cassettes in development of the mouse external genitalia

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During animal evolution, the origin of novel appendages was facilitated by the co-option of ancient genetic cassettes into new developmental functions. Although the functions of these genetic cassettes in different organs are seemingly diverse, at a cellular level they are likely to control highly conserved, ancient behaviors. The novelty of function may result from the differential genetic and environmental contexts in which these cassettes operate, which will, in turn, influence how these signals are interpreted by cells at a given position in the embryo. In tetrapod vertebrates, development of the limbs bears a striking resemblance to development of the external genitalia. The limb emerges as a limb bud from the lateral edge of the body wall and the external genitalia emerge as a genital tubercle from the ventral midline. In each system, a field is specified and budding is initiated at specific position along the main body axis. Maintenance of outgrowth is coordinated with three-dimensional axial patterning of structures within the bud, and differentiation proceeds from proximal to distal, under the control of a distal epithelial signaling region (Saunders, 1948; Murakami and Mizuno, 1986). Previous work has shown that Hox gene expression in the limb buds and the genital tubercle is under the control of a common regulatory element, raising the possibilities that development of these two organ systems may be co-regulated, and that they may share an evolutionary history (Zákány and Duboule, 1999). Although a detailed picture of the genetic control of limb development has emerged in recent years, comparatively little is known about the molecular control of external genital development. We are interested in understanding the details of external genital morphogenesis, how genital development is regulated at a molecular level, and how evolutionary changes to this organ system have arisen within amniote vertebrates. To this end, we have characterized the embryology and investigated the molecular control of external genital development in the mouse.

External genital development begins with formation of paired genital swellings, which develop into the genital tubercle. Proximodistal outgrowth and axial patterning of the genital tubercle are coordinated to give rise to the penis or clitoris. The genital tubercle consists of lateral plate mesoderm, surface ectoderm and an endodermal urethral epithelium, which is derived from the urogenital sinus. Previous work showed that the genital tubercle has polarizing activity (Dollé *et al.*, 1991; Izpisúa-Belmonte *et al.*, 1992), but the precise location of this activity within the tubercle is unknown. We reasoned that if the tubercle itself is patterned by a specialized signaling region, then polarizing activity may be restricted to a subset of cells. Transplantation of urethral epithelium, but not genital mesenchyme, to chick limbs results in complete, mirror-image duplication of the digits. Moreover, when grafted to chick limbs, the urethral epithelium orchestrates morphogenetic movements normally associated with external genital development. Signaling activity of the genital tubercle is therefore restricted to urethral epithelial cells. Before and during normal genital tubercle outgrowth, urethral epithelium expresses *Sonic hedgehog* (*Shh*). In mice with a targeted deletion of Shh, external genitalia are absent. Genital swellings are initiated, but outgrowth is not maintained. In the absence of Shh signaling, *Fgf8*, *Bmp2*, *Bmp4*, *Fgf10* and *Wnt5a* are downregulated, and apoptosis is enhanced in the genitalia.

These results identify the urethral epithelium as the signaling center of the genital tubercle, and demonstrate that SHH from the urethral epithelium is required for outgrowth, patterning and cell survival in the developing external genitalia. Our findings are consistent with a parallel study of SHH function in genital development by Gen Yamada and colleagues (Haraguchi *et al.*, 2001).

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Development and regeneration of the cricket leg

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In order to understand the genetic basis of the development and regeneration processes, we have studied leg development and regeneration of a hemimetabolous short-germ insect, *Gryllus bimaculatus* (cricket). We focused on expression patterns of the three key signaling molecules, hedgehog (hh), wingless (wg), and decapentaplegic (dpp), which are essential during leg development of *Drosophila*, belonging to holometabolous insects. In *Gryllus* embryos, expression of hh is observed in the posterior half of each leg bud, while dpp and wg were expressed in the dorsal and ventral side of its anteroposterior (A/P) boundary, respectively. Their expression patterns are comparable to those of the three genes in *Drosophila* leg imaginal discs, suggesting the existence of a common mechanism for leg pattern formation. On the other hand, significant changes in their expression domains between the two were observed only for dpp. Since the leg size and shape differ from each other, the changes may reflect differences in the leg morphology, implying that dpp may play a key role to create diverse leg morphologies during evolution. In addition, we observed expression patterns of aristaless, Distal-less, and homothorax during limb formation. We found that although their expression patterns resemble in principle those observed in *Drosophila*, their expression timing and regions differ from those in *Drosophila*.

Similar expression patterns of the genes were observed in a regenerating leg blastema, supporting the modified boundary model proposed by Campbell and Tomlinson (1995). We also observed their expression patterns during formation of two supernumerary legs after grafting a distal half of a donor amputated left tibia onto the stump of the proximal half of a host amputated right tibia. The observed expression patterns are consistent with those predicted from the modified boundary model, i.e., formation of the proximal/distal axis of a regenerating leg is triggered at the site where wg-expressing cells abut dpp-expressing cells.

POSTERS

Role of buttonhead and D-Sp1 in the specification of ventral structures of *Drosophila*

Carlos Estella

The formation of the *Drosophila* limb primordia requires input of signals (Wg, EGF and Dpp) from the antero-posterior and dorso-ventral axes, which activate the homeobox gene Distal-less (Dll) in specific positions. We have found evidence that the early activation of Dll also requires the function of the zinc finger encoding genes buttonhead (btd) and D-Sp1. These two genes are adjacent and have partially redundant roles during head development. They are also expressed similarly during embryogenesis and imaginal development. We find that btd and D-Sp1 are expressed in the primordia of all ventral discs (antennal, labial, leg and genital), and their function is necessary for activation of Dll in these primordia: in the absence of both btd and D-Sp1, Dll is not expressed, and the Keilin's organs (the rudiments of larval legs) are not formed. The activation of btd and D-Sp1 is dependent on Wg signal, and possibly EGF and Dpp, suggesting that Dll activation by these signals is mediated by btd and D-Sp1. These genes are also able to induce Dll and probably other appendage forming genes, during imaginal development, giving rise to the formation of ectopic antennae and legs in eyes, wings and halteres.

Regulation of Wingless gradient formation by Torero

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Wingless, Dpp, and Hh act at a distance from their sources to activate target genes, organizing patterning and growth in the imaginal discs during development reviewed in (Strigini and Cohen, 1999). We have carried out an EP overexpression screen, to identify new genes involved in wing development (Rorth, 1996; Mata et al., 2000). We have identified a new gene called Torero (Andalusian for Dragonfly). Torero's expression is activated in response to high Wingless signaling levels during embryonic and imaginal disc development. The overexpression of Torero with the Gal4-system (Brand and Perrimon, 1993), shows a reduction of Wingless signaling. Ectopic Torero in early stages of wing development causes the repression of wing pouch specification resulting in notum duplication, as in the wingless[1] mutants (Sharma and Chopra, 1976; Morata and Lawrence, 1977). During embryonic development, it induces secretion of denticles in naked regions of the epidermis. Homozygous mutants for Torero develop an excess of naked cuticle during embryogenesis. Mutant larvae have wing pouch duplications, resulting in a loss of notum specification.

These results suggest that Torero antagonizes the Wingless signaling pathway, restricting the region of high and low response to Wingless and helping to shape the activity gradient in different contexts throughout development.

Molecular details of the mechanism of action of Torero will be provided.

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The tumor suppressor gene l(2)giant discs restricts the activity of Notch to the dorsoventral boundary during *Drosophila* wing development

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During the development of the *Drosophila* wing, the activity of the Notch signalling pathway is required to establish and maintain the organizing activity at the dorsoventral boundary (D/V boundary). At early stages, the activity of the pathway is restricted to a small stripe straddling the D/V boundary and the establishment of this activity domain requires the secreted molecule fringe (fng). The activity domain will be established symmetrically at each side of the boundary of Fng expressing and non-expressing cells. The requirement of a fringe expression boundary is the only known additional criteria so far known to define the activity domain. It is not known whether other factors or mechanism are required in addition.

Here I present evidence that the *Drosophila* tumor suppressor gene lethal (2) giant discs (lgd) is part of a mechanism that is required to restrict the activity of Notch to the D/V boundary. In the absence of lgd function, the activity of Notch expands from its initial domain at the D/V boundary. This expansion requires the presence of at least one of the Notch ligands, Delta, which probably can activate Notch more efficiently in the mutants. Surprisingly, the activity of fringe seems to be dispensable for wing development in lgd mutant wing discs. The results further show that Lgd appears to act as a general repressor of Notch activity, because it also affects vein, eye and bristle development. Furthermore, they provide *in vivo* evidence for the existence of a diffusible Notch ligand.

The anterior signaling cascade in limb formation; function of aristaless-related genes

Sanne Kuijper, Annemiek Beverdam, Carla Kroon-Veenboer and Frits Meijlink

Alx3, Alx4 and Cart1 belong to a subgroup of highly related genes that have overlapping expression patterns and are structurally similar to the paired type homeobox gene aristaless of *D. melanogaster*. Alx4 is expressed anteriorly in the early limb bud and Alx4 mutant mice exhibit preaxial polydactyly, which appears to be caused by an ectopic ZPA. This implies that Alx4 has an essential role in confining the ZPA to posterior limb bud mesenchyme, and is part of an "anterior restrictive cascade" along with for example Gli3. We and others have found that Alx3, Alx4 and Cart1 have redundant functions in the patterning of craniofacial structures. In contrast, it appears that in the autopod Alx4 has much more impact than Alx3 and Cart1. To further investigate redundant roles of these genes in the limb, we identified several defects in double mutants, which included defects in the shoulder and pelvic girdle of Alx3/Cart1 mutants. The highest priority of our research is to identify the molecular pathways that are responsible for the restricted expression of Alx4 in anterior limb mesenchyme and those that are responsible for the downstream events that depend on Alx4. To characterize these upstream mechanisms we try to identify regulatory DNA elements that specify the typical expression of Alx4, by analysis of transgenic embryos carrying reporter constructs. In addition, we analyze at the molecular level the limbs of Alx/Cart double and triple mutants and of other selected mutants. Finally, we apply a gain-of-function approach using transgenic mice to clarify aspects of the anterior cascade.

Genetic analysis of rat hypodactyly

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The Norway rat is not widely used as a model organism for limb development, however, there are several interesting spontaneous mutations leading to disturbances of limb development. We study rat hypodactyly (Hd), an autosomal recessive mutation causing preaxial autopodium reduction with accompanying abnormal spermatogenesis resulting in male sterility. Aiming to identify the gene, we mapped the mutation to rat chromosome 10, to 1.7 cM interval. According to rat-mouse-human homology, there are several positional candidate genes in this segment (Shbg, Syb2, Glut4, Sox15, Ybx2, Fxr2, Fgf11), but, surprisingly, no one has proven role in limb morphogenesis. This could indicate a novel, yet unresolved, step in limb morphogenesis regulation. We analysed expression of Shbg, Syb2 and Glut4 in testicular tissue by RT-PCR with no significant difference between hypodactylous animals and normal controls. To confirm position of the mutated gene, we are producing congenic strains SHR-Hd and BN-Hd. The Hd-containing segment of SHR-Hd is to date about 7 cM long. SHR-Hd has decreased penetrance and expressivity of limb affliction, but not spermatogenesis, which indicates an important role of modifying genes of genetic background in limb development of Hd animals.

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COE proteins function in vertebrate limb development

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Rodent Olf-1/ebf and *Drosophila* Collier (col) are the founding members of a new family of transcription factors, the COE proteins, which contain both a novel type of DNA-binding domain and an atypical HLH motif (reviewed by Dubois and Vincent, 2001). Col is the single COE factor in *Drosophila* while there are 3 to 4 known paralogs in vertebrates, all highly structurally related. appear to play a variety of important functions throughout development, in both. A major project of our lab is to understand the genetic and molecular circuitry underlying the functional diversity COE proteins in vertebrate and invertebrate development, focusing on limb/wing patterning. Our lab has previously shown that Col (also designated Kn (Knot)) mediates Hedgehog short-range patterning in the *Drosophila* wing (Vervoort et al, 1999). More recent results show that col coordinates a compex interplay between Hedgehog, Dpp and EGF signalling in the *Drosophila* wing A/P organiser (Crozatier et al., in preparation). I will present recent data on the developmental expression of each of three coe/ebf genes during limb formation in mouse and chicken, in relation to signalling centers. Based on these and *Drosophila* data, I will discuss a strategy of conditional expression of a dominant-negative COE protein to investigate the function of COE proteins in limb patterning and morphogenesis.

Expression of Meis genes during axolotl development and regeneration

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and Miguel Torres

Vertebrate limbs develop in a temporal proximodistal sequence: regions close to the trunk are specified and generated earlier than distal ones. Limb outgrowth is triggered by interactions between a distal organizing center, the Apical Ectodermal Ridge (AER), and the underlying mesenchyme, called Progress Zone (PZ). Fibroblast Growth Factor (FGF) family members released from the AER induce proliferation in the PZ. In this way, cells leaving the PZ contribute to progressively distalized cells that incorporate to the limb axis. The TALE homeobox genes Meis1 and Meis2 function as determinants of proximal limb compartments. During limb development, Meis1 and Meis2 function is restricted to the proximal limb region up to the stylopodal-zeugopodal boundary. Ectopic expression of Meis1 during chicken limb development inhibits PZ cell distalization and distal limb growth.

Retinoic acid (RA) is an upstream activator of the proximal determinant genes Meis1 and Meis2. Thereby, RA promotes the proximal character of limb cells. Meis expression initially spans the whole lateral plate mesoderm and becomes restricted to proximal limb domains by the AER activity following limb induction. We identify FGF's as the molecules responsible for such AER activity and propose a model that integrates FGF role in stimulating limb proliferation with a specific role in promoting limb distalization by inhibition of RA signalling.

Currently, we are analysing transgenic mice ectopically expressing Meis1 in the distal limb region and in addition, studying the role of Meis genes during urodele appendage regeneration.

Medio-lateral differences in cell affinities in the *Drosophila* wing disc: roles of Spalt and the LRR-proteins Capricious and Tartan

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Segregation of cell populations is required for proper tissue patterning during animal development. Cell segregation can be driven by heritable states of gene expression, which produce compartments. Compartments are separated by boundaries of lineage restriction. Cell populations can also be segregated by non-compartmental subdivisions. Proximal-distal subdivision of the leg and medial-lateral subdivision of the wing produce adjacent domains that are globally stable in terms of gene expression, but that do not preclude net movement of cells from one domain into the other. In the *Drosophila* wing primordium, Capricious and Tartan, transmembrane proteins with leucine-rich repeats, contribute to segregation of cells at the dorsal-ventral compartment boundary (Milán et al, 2001). Here we report that they also contribute to producing an affinity difference between medial and lateral regions of the developing wing. Thus, Capricious and Tartan contribute to both compartmental and non-compartmental cell segregation processes in wing development (Milán and Cohen, submitted).

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Dpp signaling is required to confine iro-C expression during notum specification

Isabel Rodriguez & Juan Modolell

During development, the imaginal wing disc of *Drosophila* is subdivided into territories that will give rise to body wall (notum and mesothoracic pleura) and appendage (wing). Expression of the Iroquois complex (Iro-C) in the most proximal part of the disc defines the notum. Here we show how the expression of Iro-C is confined to the notum territory. Neither Wingless signaling, which is necessary to define the wing territory, nor Vein/EGFR signaling, which is needed to activate Iro-C, delimit Iro-C expression. This is accomplished by repression by the TGF-homolog Decapentaplegic (Dpp). Later, Dpp again downregulates Iro-C in the proximal-most part of the notum to accomplish its subdivision into medial and lateral regions.

Expression and regulation of Colloid and Ventroptin during limb development

Rodríguez-León, J and Izpisúa Belmonte, J.C.

The BMP signaling pathway is one of the best conserved through evolution. BMPs exert its biological action by binding serin-threonin kinase receptors type I and type II which form dimmers and activate Mad/Smad cascade intracellularly. Several BMP inhibitors bind BMPs outside the cell and thus prevent the interaction with their receptors. Another level of BMP control occurs by cleavage of one of their antagonists (Chordin) by one specific metalloprotease (Tolloid), allowing the BMPs to trigger with their receptors Mad/Smad cascade. Several members of the Tolloid family have been cloned in *Drosophila*, mammals, Zebrafish, *Xenopus* (Xolloid) and chick (Colloid). The action of this metalloprotease has been shown by in vivo studies. Injection of Chordin mRNA in the ventral side of the *Xenopus* embryo induces secondary axis by inhibiting ventral BMP signalling, but this duplications do not occur when Xolloid and Chordin mRNAs are coinjected. We have used the chick limb bud as a model to study colloid expression and its relationship with other signaling molecules. We have found that colloid is expressed during early limb development in the mesenchyme and apical ectodermal ridge, then the expression is restricted to the chodrogenic areas and tendinous blastemas. As chordin transcripts are detected when digits begin to develop, we have cloned ventroptin, a BMP inhibitor belonging to the cysteine repeat family proteins. Since ventroptin expression pattern in the aerly stages is restricted to the chondrogenic areas we propose ventroptin as a target of Colloid protein during the first steps of chodrogenic differentiation.

Digit morphogenesis: Effects of Shh and the role of FGF signalling

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Digit identity is determined by its antero-posterior position in the limb and described in terms of morphology and number of phalanges. Early signalling from the polarising region dependent on Shh and mediated by BMPs is responsible for this patterning. However, the molecular and cellular mechanisms translating this early positional information into differential anatomy of the digits are not fully understood. We have shown that application of Shh to the interdigital spaces of the chick limb leads to alterations in digit morphogenesis, including either truncations or elongation of digits, sometimes with the generation of a new joint and thus an extra phalange. We will present the characterization of these effects in detail and will show evidence implicating FGF signalling in late steps of digit growth and morphogenesis. Also, the mechanisms involved in ending up a digit will be discussed, including some evidence that making the tip of a digit/toe might be a special process. All these data could be important in our understanding of morphogenesis and have practical implications in regeneration and evolution.

β-catenin regulates AER formation and dorso-ventral polarity in the limb

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The apical ectodermal ridge, a specialised structure at the dorsal-ventral boundary of the limb bud, regulates proximal-distal outgrowth of the vertebrate limbs. β-catenin, a downstream effector of the Wnt signaling pathway, is a key regulator of the limb initiation and AER induction in the vertebrate embryo. Conditional inactivation of the β-catenin gene in the limb ectoderm results in severe limb truncation. Analyses of Fgf8, Bmp4 and Msx2 expression suggest that formation and positioning of the AER along the dorsal-ventral axis are disrupted in the mutant limbs. Ectopic ventral expression of Wnt-7a and Lmx-1b leads to transformation of the ventral limb structures to dorsal phenotype in the β–catenin mutants. We propose that β-catenin plays an essential role in AER formation and in the establishment of the dorsal-ventral patterning during limb development.

Regulation of the Dpp expression during pupal development

Sol Sotillos and José F. de Celis

The differentiation of the veins in the *Drosophila* wing is directed by the dpp/TGFb signalling pathway (de Celis, 1997;Yu et al., 1996). Soon after puparium formation the gen *dpp* is expressed in the developing veins, in a process that depends on the activity of the short vein (dppshv) regulatory region (de Celis, 1997). The restricted expression of Dpp in pupal veins can be considered as the culmination of a patterning proccess initiated during larval development. At this early stage, the expression of several transcription factors such as Knirps, Iroquois and Abrupt is restricted to individual veins (de Celis, 1998). It appears that these regulatory proteins participate in localising the activity of two additional signalling systems required for vein formation: the Ras/MAP kinase signalling cascade and the Notch signalling pathway (de Celis et al., 1997; Díaz-Benjumea and Hafen, 1994). However it is not known whether these vein-specific transcription factors also contribute directly to the late expression of Dpp in individual veins. In support of this posibility, we have identified fragments of the Dpp regulatory region that direct the expression of a reporter gene in individual veins.

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Fin development in a cartilaginous fish and the origin of vertebrate limbs

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Recent fossil finds and experimental analysis of chick and mouse embryos prompted us to re-explore the lateral fin fold theory which suggests that two pairs of limbs in tetrapods evolved by subdivision of an elongated single fin. We examined fin development in embryos of the primitive cartilaginous fish, *Scyliorhinus canicula* (dogfish) by scanning electron microscopy and investigated expression of genes known to be involved in limb positioning, identity and patterning in higher vertebrates. Although we did not detect lateral fin folds in dogfish embryos, Engrailed-1 expression suggests the body is compartmentalised dorso-ventrally. Furthermore, specification of limb identity occurs via Tbx4 and Tbx5 genes as in higher vertebrates. In contrast, unlike higher vertebrates, we could not detect Shh transcripts in dogfish fin buds, although dHand (a gene known to be involved in establishing Shh) is expressed. In *S. canicula*, the main fin axis lies parallel to body axis. "Freeing" fins from body wall and establishing a separate "limb" axis is a crucial step in evolution of tetrapod limbs. We suggest that Shh plays a critical role in this process.

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