Abstracts

3rd Congress of the Spanish Society of Developmental Biology, Málaga, 2006

Hormone and light control of flowering

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Plants generate new organs during their whole life cycle, and developmental programs are triggered or modulated in part through interactions with the environment. In Arabidopsis, several growth phases can be distinguished, with different developmental traits associated to each of them. The most important phase change is the transition between the vegetative phase (during which leaves are produced) and the reproductive phase (in which flowers and secondary shoots are made). Leaves and flowers emerge from equivalent primordia, and the switch between the two developmental programs for the identity of these organs depends on the activity of floral meristem identity genes such as *LEAFY* (*LFY*), as indicated by two observations: first, *lfy* loss-of-function mutants undergo the transition to the reproductive phase but keep generating leaves instead of flowers; and second, overexpression of LFY causes premature transformation of leaves into flowers.

The timing of the transition to the reproductive phase of growth is controlled both by environmental and endogenous factors. Among the signals perceived by the plant, photoperiod (or daylength) has the strongest effect on flowering time. Among the endogenous factors, gibberellins (GAs) are the plant hormones that have been proposed to be involved in the promotion of flowering in the absence of inductive conditions, since the *ga1* mutant (defective in the synthesis of GAs) does not flower under short days and is late under long days, and exogenous application of GAs to wild-type plants accelerates flowering under short days.

An obvious hypothesis that can be derived from the previous observations is that *LFY* expression is under the control of both the inductive (photperiod-dependent) and the non-inductive (GA-dependent) pathways that promote flowering. To determine whether *LFY* expression was regulated by photoperiod, a fusion between the *LFY* promoter and the reporter gene *GUS* (*LFY::GUS*) was introduced into plants, and GUS activity was measured in single apices of plants grown under long or short days. Surprisingly, *LFY::GUS* was expressed during the vegetative phase in leaf primordia, and the activity increased with time. This upregulation was very fast in long days, while short days only caused a slow gradual increase. These results suggest that flowers would be initiated after a critical threshold of *LFY* activity has been reached. This hypothesis was validated by the observation that plants with increasing number of *LFY* copies flowered earlier, indicating that *LFY*, a floral meristem identity gene, has properties of flowering-time genes. The physiological relevance of the modulation of *LFY* expression in the control of flower initiation is also indicated by the reduction of *LFY* promoter activity observed in several late-flowering mutants. However, some of these mutants showed wild-type levels of *LFY* expression, which reveals another level of regulation of flower initiation, *i.e.* the competence to respond to *LFY* activity.

The LFY promoter was also subject to regulation by GAs, as supported by the fact that the *ga1* mutation considerably delayed upregulation of *LFY::GUS* under long days, consistent with the observed delay in flowering time in *ga1* mutants. Under short days, the *ga1* mutation abolished significant upregulation of *LFY::GUS*, which correlates well with the absence of flowering caused by *ga1* under these conditions. Moreover, constitutive expression of the *LFY* cDNA could rescue the flowering defect of the *ga1* mutant under short days, indicating that GAs accelerate flowering through the upregulation of the *LFY* promoter.

Since *LFY* expression was regulated by both a photoperiod-dependent and a GA-dependent signaling pathway, a relevant question was: how do plants integrate the information for the promotion of flowering time? Do these signaling pathways interact with a common signaling element that in turn activates *LFY*, or do they act on the *LFY* promoter through separate, parallel pathways? In the course of a deletion analysis of the *LFY* promoter, we identified at least two regions that are important to determine the actual level and the photoperiodic regulation of the expression: a 200-bp region located between positions –1800 and –1600, needed to confer maximal expression; and a 100-bp region between positions –300 and –400, necessary for expression during both the vegetative and the reproductive phase of growth. Interestingly, a consensus binding site for a Myb transcription factor that mediates regulation of gene expression by GAs in barley, is present in this 100-bp fragment. Mutagenesis of this site abolished expression of the reporter gene under short, but not under long days, indicating that the short-day GA-dependent pathway and the facultative long-day pathway that regulate flowering time merge at the level of the *LFY* promoter, and act through separable *cis* elements. Thus, *LFY* acts as the integrator of the information transduced by at least these two flowering-time signaling pathways.

An analysis of fruit development in *Arabidopsis thaliana*

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The fruit is a highly specialized plant organ that occurs in diverse forms among the angiosperms. Fruits of *Arabidopsis thaliana* develop from a gynoecium that consists of two fused carpels. The mature gynoecium of *Arabidopsis* is composed of an apical stigma, a short style, and a basal ovary that contains the developing ovules. Following fertilization of the ovules, the fruit elongates and differentiates a number of distinct cell types, allowing for the successful maturation and the eventual dispersal of the seeds.

The *FRUITFULL (FUL)* MADS-box gene is required for normal fruit development in *Arabidopsis.* All the tissues within the ovary walls are affected by the *ful* mutation: The valves fail to differentiate and expand and frequently break out before maturation of the seeds, the style displays abnormal morphologies, and the replum adopts a zigzag arrangement (Gu *et al.,* 1998). To better understand the role of FUL in carpel tissue development, we have generated gain-of-function alleles by constitutively expressing FUL under the control of the 35S CaMV promoter. 35S::FUL lines display a phenotype in which the ovary walls are largely converted into valve tissue. Moreover, the dehiscence of the pod does not occur upon desiccation (Ferrándiz *et al.,* 2000). Dehiscence, or pod shatter, is a carefully orchestrated event that occurs late in fruit development to assist in the dispersal of seeds. This process involves the formation of a dehiscence zone, a region that is only one to three cells wide, and extends along the entire length of the fruit at the valve-replum boundary. The indehiscent phenotype of the 35S::FUL fruits resembles the *shatterproof1 shaterproof2 (shp1 shp2)* double mutants (Liljegren *et al.,* 2000). We have investigated the possible interactions between FUL and SHP1/SHP2 at the genetic and molecular level (Ferrándiz *et al.,* 2000). To further explore the function of FUL, we have generated transgenic plants that have a constitutively activated version of FUL (FUL:VP16) under the control of the endogenous FUL regulatory sequences. The phenotypic and molecular analysis of FUL:VP16 gynoecia identify the SHATTERPROOF genes as direct targets of FUL repression in fruit valves, whereas other genes regulated by FUL seem to be controlled by more indirect ways. The FUL:VP16 allele also reveals a role of FUL in controlling both inflorescence and floral determinacy.

We have also undertaken a mutagenesis approach to identify additional loci involved in these processes. In an EMS mutagenized ful population, a suppressor of ful phenotypes in the valves has been identified and cloned. A new gain-of-function mutant obtained in an activation tagging experiment showed phenotypes resembling ful effects in fruit development (Weigel *et al.,* 2000). Corresponding loss-of-function alleles have been identified and are currently under functional characterization. Finally, in a T-DNA mutagenized population, a mutant that shows fruit phenotypes including small size and indeterminacy has been isolated. Its possible functional relationship with FUL/SHP genes will be investigated.

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Effects of exogenous application of cytokinin in wild type and ” roots of *arabidopsis thaliana*

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Cytokinins are a class of plant hormones originally identified by their ability to stimulate cell division in concert with auxin, and to act antagonistically to auxin in the control of shoot and root initiation in culture (1).Cytokinins also influence such processes as outgrowth of lateral branches(2). Plakidou-Dymock et al. (3) isolated an *Arabidopsis* gene (*GCR1*) that encodes a protein with seven predicted membrane-spanning domains and other characteristics of seven transmembrane domains (7TM). They suggest that *GCR1* encodes the first 7TM receptor identified in higher plants, related to the G-protein signalling pathway, and is involved in cytokinin signal transduction. To compare the wild type and the ”*gcr* plants, we first determined root length and lateral root numbers daily from the 4th day after the start of the experiment. Wild type (wt) and ”*gcr* seeds were grown for 11 days in MS medium. The root length at day 11 was similar in both groups of roots (52.6+/-5.2 mm in wt and 47.1+/- 9.6 in ”*gcr* roots). However the density (number of lateral root primordia per millimeter) was significantly smaller in ”*gcr* root (0.5 in wt and 0.15 in ~gcr). Another diference was the distance between the hypocotyl and the first emerged primordia. This distance was 3.5 +/-1.6 mm in wt plants and 24+/-6.8mm in ”*gcr.* This was also reflected in the daily observation of root primordia: in wt the first primordia appeared at day 4, while in ”*gcr* roots we did not see primordia until day 7. In light of these results we decided to grow seeds in the presence of BA (benzyadenine). Wt and ”*gcr* seeds were grown for 10 days in MS and MS with BA ($3.3x10^{-8}$M). In both groups, the root length reduction was similar. Ten-day-old wt seedlings had roots that were 61+/-2.4 mm long, and that were reduced by 50% to 30.5+/-2 mm when grown on $3.3x10^{-8}$M BA. The ”*gcr* seedlings had roots that were 51+/-3 mm long, and were reduced by 56% to 22.4+/-3 mm on BA. Comparing the number of lateral root primordia in wt and ”*gcr* treated roots, we detected a 76% reduction in wt roots treated with BA and a 95.5% reduction in ”*gcr* roots. This suggests that BA blocks lateral root

initiation in the ❞*gcr.* Preliminary experiments using an auxin (NAA) suggests that ❞*gcr* root respond normally to this plant hormone.

Acknowlegements

This work was supported by the Consejería de Educación y Juventud de la Junta de Extremadura (BRV98025); Consejería de Educación, Ciencia y Tecnología de la Junta de Extremadura (FIC99B028 and IPR00A093).

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Clonal analysis of stomatal development and patterning in *Arabidopsis* leaves

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Cell lineage has been used to explain the stomatal distribution in several plant species. We have used transgenic plants carrying a 35S*GUS::Ac* construct that produces clonal sectors to analyze the possible role of cell lineage during the establishment of stomatal patterning in *Arabidopsis* leaves. The analysis of sectors ranging from two to eighteen cells supports the conclusion that most stomatal complexes derive from a single and immediate precursor cell through a stereotyped pattern of three unequal cell divisions followed by a final equal one. In addition, they show that the successive cell divisions take place at a constant angle (approximately 60º) with respect to the previous ones. Interestingly, this angular dimension shifts from 60º to 0º in the last cell division that gives rise to the stoma. These sectors also reveal the development of both clockwise and counterclockwise patterns of cell divisions during stomatal development. Our clonal analysis indicates that cell divisions involved in the development of stomatal complexes are probably the last ones contributing to epidermal growth and development. Finally, the stereotyped pattern of cell divisions that culminates in the formation of stomatal complexes indicates that cell lineage plays a very important role during stomatal pattern establishment.

Role of Semaphorins and Slits in brain development

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In the developing central nervous system (CNS), axonal growth cones are known to be partly guided by diffusible molecules. Some of these secreted factors can attract the axons whereas others repel them. Chemorepulsive factors belong to the Netrin, Semaphorin and Slit families. Secreted semaphorins bind receptors of the neuropilin family, and Slits bind Robo receptors. We studied the role of semaphorins and slits in axon guidance. Using collagen gel co-cultures we have shown that hippocampal axons can be selectively repelled by Sema3A, Slit2 and Sema3F. An antibody directed against neuropilin-1 is able to block the repulsive action of Sema3A, but not Sema3F. In *neuropilin-2* knock-out Sema3F repulsion is abolished. Moreover, mossy fiber projection from the dentate gyrus to CA3 pyramidal cells is abnormal in *neuropilin-2 -/-*. These animals also exhibit strong defects of many axonal tracts, such as the anterior commissure. In the olfactory system, we showed that Sema3B, Sema3F and Slit1 and Slit2 are able to orient *in vitro* the growth of olfactory bulb axons: Sema3F, Slit1 and Slit2 are repulsive whereas Sema3A is attractive. The expression patterns of Sema3A, Sema3F and slits in the developing olfactory system, confirm that they may play a cooperative role in the formation of the lateral olfactory tract. We found that the expression pattern of semaphorins and slits is very dynamic and that most are highly expressed in the adult brain.We also explored the response of rat DRG axons to substrate-bound Slit2 fragments in the stripe assay. Slit2 fragments were avoided by DRG axons when expressed on membranes or coated as stripes on laminin. In addition, DRG axon response to Slit2 fragments could be modulated by cGMP and by a laminin-1 peptide. These results strongly support the idea that extracellular matrix proteins modulate the response of growth cones to chemotropic molecules by modulating cyclic nucleotide levels.

Secondary organizers and brain patterning

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In the central nervous system of vertebrates distinct neural identities are acquired through progressive restriction of developmental potential of neuroepithalial domains under the influence of local environmental signals. Evidence for the localization of such morphogenetic signals at specific locations of the developing neural primordium has suggested the concept of «secondary organizer regions», which regulate one step further the identity and regional polarity of neighboring neuroepithelial areas. In recent years, the most studied secondary organizer is the isthmic organizer, which is localized at the hind-midbrain transition and controls the anterior hindbrain and midbrain regionalisation. *Otx2* and *Gbx2* expressions are fundamental for positioning the organizer and the establishment of molecular interactions that induce *Fgf8* expression and then, stabilizes the autoregulative loop of *En1, Wnt1* and *Pax2* expressions (Wurst and Bally-Cuiff, 2001). Temporo-spatial patterns of such gene expressions are necessary for the correct development of the organizer that, by planar mechanism of induction, controls the normal development of the rostral hindbrain, from rh2, to midbrain-diencephalic boundary (Nakamura, 2001). *Fgf8* appears as the active diffusible molecule for the isthmic morphogenetic activity and has been suggested as the morphogenetic effector in other inductive activities revealed in other neuroepithelial regions, which are, therefore, considered candidates

for rostral secondary organizers. Double in situ hibridisation for *Fgf8* and *Shh* expression patterns showed interesting spatial relations between the expression domains of these genes, actively involved in mophogenesys and regionalization of vertebrate neural tube (Martínez, 2001). *Shh* shows abrupt spatial changes in its expression pattern, which is systematically sifted dorsally where *Fgf8* is expressed: the isthmus (I), the zona limitans (ZLI) and at the rostral pole of the brain, the presumptive commissural plate. It has been demonstrated an inductive activity in the isthmus (isthmic organizer) and in the rostral pole of the brain (Shimamura and Rubestein, 1997). The zona limitans is the region that appears now a suggestive area where a new organizer can display inductive and morphogenetic properties in diencephalic regionalization. This effect has been studied in our laboratory and seems to be related to a control of inductive opposite influences from the anterior pole of the brain and the isthmic organizer. In addition, recent experimental data demonstrate that an ectopic ZLI is induced at the ectopic border generated between prechordal and epichordal neuroepithelium, suggesting that, like the interaction between *Otx2* and *Gbx2* domains, prepatterning factors are underlying the specification and positioning the ZLI organizer.

The work has been supported by DIGESIC-MCT PM98-56; EC grants: BIO 98-309, QLG2 CT-99.793 and 99.3156

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Where is the thalamic eminence situated?

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Chick/quail chimeric embryos with various types of homotopic prosencephalic transplants were performed to localize in the neural tube of the 2-day-old avian embryos the area giving rise to the thalamic eminence (TEM). This structure situated between the telencephalic hemispheres and the ventral thalamus has been assigned to the prosomere 4 (P4) in the prosomeric model of forebrain subdivision (Bulfone *et al.,* 1993). But the diencephalic or telencephalic identity of this area remains unclear (Martinez & Puelles, 2000). We have observed that the histogenesis of this area undergoes a slight time-lag with respect to ventral thalamus and their cells seem to migrate as far as neighbouring structures. The performed transplants delimite a rough fate map. When the transplants were performed in embryos of 10 somites or younger the integration was better than those of 12 somites or older. This suggests a progressive neuroepithelial commitment. The precise grafted areas varies from case to case, but all survivor chick embryos showed quail cells in the telencephalon, in the diencephalon or in both. The location of quail cells in ependimal and mantle zones permit us to infer the migration routes. Most cells carry out a radial migration but tangencial migration is also important in the studied area. We have no evidence of cellular contributions from the TEM to the caudal amigdala however definitive conclusions on this point need additional experiments with smaller grafts.

Supported by: DGICYT PB 90-0296-CO2-02, DGICYT PB97-1472-CO3-02.

Prospective mapping of the *locus coeruleus* in the chick embryo at stage HH10, using quail- chick chimeras and genetic markers

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We studied the rostro-caudal and dorso-ventral neuroepithelial origin of the chick *locus coeruleus* (LoC) in rhombomere 1 (r1) at stage HH10, and its relationship with other adjacent nuclei. In order to determine the rostral and caudal limits of that nucleus, we performed quail-chick grafting experiments with different transverse portions of the neural tube included between the isthmic region and rhombomere 2. Our results consistently showed that the LoC primordium is restricted to r1. Its caudal limit coincided with the r1/r2 boundary and its rostral limit with the caudal limit of the isthmus, or overlapping a discrete caudal portion of the isthmus (depending how that limit is defined). In order to determine the dorso-ventral origin of r1, we produced a set of r1 chimeras with quail grafts of different dorsoventral extent. The results are consistent with a LoC origin in the middle third of the lateral wall of the neural tube, a region that probably belongs to the alar plate. If we compare the source of LoC neurons with that of adjacent nuclei, we observe that the LoC nucleus is originated caudal to the isthmic nuclei (isthmo-optic, parvocellular and ventral isthmic nuclei) and also to the dorsal nucleus of the lateral lemniscus. The rostral part of LoC partially overlapped in its origins with the semilunar nucleus. The LoC developed mostly at the same rostro-caudal level than the parabraquial nucleus, the principal sensory trigeminal nucleus (which overlaps with the caudal part of LoC), and the intermediate nucleus of the lateral lemniscus. While the parabraquial nucleus is mostly origined more dorsally than the LoC, the intermediate nucleus of the lateral lemniscus and the principal sensory trigeminal nucleus arise more ventrally.

In order to characterize molecularly the localization of the LoC, we performed double situ hibridizations, both in early embryos (from 3 to 6 days incubation; wholemounts) and in serial sections at later stages (from 6 to 10 days). In order to carry out these experiments, we labelled either Fgf8, which is expressed in the isthmus and negative in r1, or basal and alar plate markers (Nkx-6.1, Otp and Pax6), comparing with the markers that specifically identify the LoC nucleus in the alar plate (DBH and Phox2b, present in noradrenergic neurons). Our in situ results confirmed that the LoC nucleus origin is localized in the alar plate of r1, and its caudal limit is coincident with the r1-r2 boundary.

Supported by the contract BIO4-CT98-0112 and the project DGICYT PB98-0397.

The *Snail* gene family in neural crest development

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The *Snail* gene family of transcription factors plays crucial roles in different morphogenetic processes during the development of vertebrate and invertebrate embryos.

Loss of function experiments carried out for one of the family members, Slug, showed its involvement in early mesoderm and neural crest formation in the chick embryo[1]. Its role is mediated by the triggering of the epithelial-mesenchymal transition, a dramatic phenotypic change that allows cells to delaminate from an epithelium and migrate towards their destinations. We have carried out a series of overexpression experiments by electroporation in the neural tube of the chick embryo and show that an excess of *Slug* function induces an increase in neural crest production. The phenotypic analysis of electroporated embryos shows that *Slug* can directly or indirectly induce the expression of other neural crest markers such as *RhoB*, *Pax3* and *Hnk-1*, indicating that *Slug* lies upstream of them in the genetic cascade of neural crest development. The increase in neural crest formation after *Slug* overexpression is only observed in the head region, indicating that the mechanisms of crest induction somehow differ between head and trunk.

The expression of the two vertebrate family members, *Slug* and *Snail*, is peculiar with respect to the neural crest: the patterns are inverted between chick and mouse. *Snail* is not expressed in the premigratory neural crest in the chick, whereas it is expressed in this cell population in the mouse[2]. Recently, we have shown that, in the mouse, it is *Snail* rather than *Slug* the gene that induces the epithelial-mesenchymal transition[3]. Thus, this suggests that they could be functionally equivalent. In order to test this hypothesis both intra- and inter-species, we have performed a series of ectopic expression experiments by electroporating chick *Snail* and mouse *Snail* and *Slug* in the chick embryo neural tube. Preliminary data indicate that they can be functionally equivalent, although the embryos show a higher response to the endogenous gene.

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IGF-I regulation of inner ear neurogenesis

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We have investigated the role of IGF-I in the development of the neuronal population of the chick inner ear. IGF-I and its high affinity receptor (IGFR) are expressed in the otic vesicle. Blockade of IGF-I activity by anti-IGF-I antibodies or the inhibitory peptide JB1 unveils an endogenous IGF-I activity, which is essential for autonomous ganglion formation in otic vesicles cultured in serum and growth factor-free media. Conversely, over-expression of IGF-I by means of RCAS virus results in increased generation of neurones, reduction of apoptosis and accelerated maturation of neuroblasts into differentiated neurones. A more delayed effect of IGF-I on neuronal differentiation is revealed by the ability of exogenous IGF-I to induce neurite outgrowth and expression of neuronal markers in ganglion explants. Taken together, our results show that IGF-I activity in the otic vesicle supports proliferation of neuronal precursors, delamination of young neuroblasts and proliferation and differentiation into post-mitotic neurones. The local expression of IGF-I in the otic vesicle and its possible auto or paracrine activity may be one of the long-term searched factors that underlie the autonomy of the otic vesicle and the autonomous control of size and number of neurones of the inner ear.

Ectopic expression of *Ebf1* in the embryonic neural tube promotes migration of ventricular zone progenitors into the mantle layer

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Ebf genes belong to a new family of Helix-Loop-Helix transcription factors that includes *Ebf1*, *Ebf2* and *Ebf3*. Ebf1 was originally identified as a transcription factor necessary for B lymphocytes differentiation, but recently it has been shown that *Ebf* genes are widely expressed in the mouse developing central nervous system. In particular, they show a general activation in early post-mitotic cells suggesting a role in neuronal differentiation.

We have performed ectopic expression of *Ebf1* by *in ovo* electroporation of 20-somites chick embryos. Forty hours after electroporation transfected cells localise to the mantle layer of the spinal cord while in control electroporations most of the transfected cells remains in the ventricular zone (VZ). *Ebf1* ectopic expression also correlates with a reduction in expression of the VZ marker *neurogenin1* 20 hours after electroporation. Finally *Ebf1* induces expression of the neuronal marker *neurofilament* while forced expression of a dominant negative *Ebf1* leads to a reduction of the level of expression of this marker. Together these data support a role of Ebf1 in timing of neurogenesis and exit from the cell cycle.

Snail superfamily genes in brain development: are they involved in mechanisms of cell migration and differentiation?

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The Snail and Scratch gene families form the metazoan Snail superfamily of zinc-finger transcription factors. In vertebrates, these two families are respectively represented by *snail* and *slug*, and by *scratch1*, *-2* genes (Manzanares *et al.,* 2001). These genes

play multiple crucial roles in development. Snail-family members trigger epithelial-mesenchymal transitions, which are involved in the formation of mesoderm and neural crest as well as in the acquisition of invasive phenotype in epithelial tumours (Cano *et al.,* 2000; Locascio and Nieto, 2001). Scratch genes are the representation of Snail-superfamily expression in early neuroepithelium. In zebrafish and mouse embryos *scratch* RNA appears in young postmitotic neurons, suggesting a role in the radial migration and/or differentiation of these cells (Nakamura *et al.,* 2001; Blanco and Nieto, unpublished results).

In this work we have studied these genes in mouse and chick embryos, focusing on the following questions:

- the temporal-spatial relationship between *scratch* expression and that of markers of neuronal differentiation,
- the eventual role of Snail and Scratch genes in later migration/differentiation phenomena like those involved in cerebellar histogenesis,
- their possible involvement in the migration of glial and endothelial cells within the developing brain.

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Interkinetic nuclear movement provides spatial clues for the regulation of neurogenesis

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The vertebrate neuroepithelium initially is a monolayer of pseudostratified cells that, during the transition from S-phase to mitosis, first displace their nuclei and subsequently, migrate from the basal membrane to the apical surface, a phenomenon termed interkinetic nuclear movement (INM)[1]. Here we show that neuroepithelial cells acquire a neurogenetic potential, as defined by the expression of the neurogenic genes *Notch1* and *Delta1* and the proneural determination gene *Neurogenin (Ngn)2*, at the same time that they move towards the apical surface. Based on these data, we have developed a mathematical model which simulates neurogenesis in the presence or absence of INM. When INM was excluded from the model, neurogenetic and pre-neurogenetic progenitors could interact and an exacerbated rate of neurogenesis was predicted due to the reduction of inhibitory signals. Consequently, many precursors are lost from the neuroepithelium resulting in its diminished growth and the reduction in the late production of neurones. The pharmacological blockade of the INM in the developing retina confirmed some of the above-mentioned predictions. Thus, such a blockade resulted in (a) the distribution of the cells expressing *Notch1* interspersed among pre-neurogenetic cells, (b) the increase of cells positive for *Ngn2,* and (c) the overproduction of retinal ganglion cells. These findings suggest that INM is important for the efficient and continued production of neurones in G0. Moreover, it appears that INM is involved in defining a proneural cluster in the ventricular part of the neuroepithelium that contain precursors in stages of the mitotic cycle with the potential to differentiate as neurones.

Mechanism of radial migration of microglial cells during embryonic development of quail retina

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Radial migration of microglial cells from the vitrealmost part toward the inner and outer plexiform layers of the retina was studied with confocal laser scanning microscopy on wholemounts and sections of retina of quail embryos from 9th to 15th days of incubation (E9 to E15). They were treated immunocytochemically with the antibodies QH1, H5 and M1B4 that recognize quail microglial cells, Müller cells and tenascin, respectively. The actin-rich radial processes of Müller cells were also identified with fluorescent phalloidin. Microglial cells migrating in the vitrealmost part of the retina (level 0) migrated radially from E9 onwards to reach one of three levels where they ramified. These levels coincided with the vitreal border of the inner plexiform layer (level 1), the scleral border of this layer (level 2) and the outer plexiform layer (level 3). Generally, microglial cells arrived at each level from the immediately more vitreal level, but some cells jumped from level 0 to level 2. The mechanism of radial migration of microglial cells in the vitreal half of the retina (where they migrated from the inner limiting membrane to the scleral border of the inner plexiform layer) appeared to differ from that in the scleral half (where cells migrated through the inner nuclear layer to reach the outer plexiform layer). In the vitreal half, the radial migration and ramification of microglial cells were simultaneous, because the cells sent forth long thin radial processes that squeezed through the nervous parenchyma until they reached the next level, where they ramified well before the arrival at this level of the cell body containing the nucleus. Microglial cells ramifying in a specific level were able to emit further radial processes to climb to the next level, where they again ramified. Some microglial cells ramifying at level 2 traversed the inner nuclear layer to reach level 3. These cells retracted their processes to become ameboid and migrate between neurons of the inner nuclear layer with no emission of long thin processes. Microglial cell radial migration was apparently related to the microenvironment of the developing retina, which has a dense network of Müller cell processes and different extracellular matrix molecules such as tenascin. In fact, many radial processes of migrating microglial cells were closely adhered to radial processes of Müller cells, suggesting that microglial cells used the Müller cell processes as a substrate to migrate radially. Tenascin was strongly expressed in the inner and outer plexiform layers from the earliest appearance of both layers. Ramification levels 1, 2 and 3

coincided with zones of non-expression of tenascin located immediately adjacent to zones where it was strongly expressed. Thus, cell branches of ramified microglial cells appeared to avoid regions of strong tenascin expression, indicating that this molecule may inhibit microglial ramification.

Supported by grant PB97-0178 from the DGSIC of the Spanish MEC.

Influence of different substrates and media on quail embryo retina ameboid microglia cultured *in vitro*

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Ameboid microglia migrate tangentially in the vitreous part of the embryonic quail retina using Müller cell endfeet (MCEF) as substrate for migration. We isolated sheets of the vitrealmost part of the retina containing the inner limiting membrane (ILM) covered by a carpet of MCEF (ILM/MCEF sheets) on which migrating ameboid microglial cells remained adhered. These sheets were obtained on poly-L-lysine (PLL) coated coverslips and cultured for 4 days in DMEM containing serum (DMEM-CS). ILM/MCEF sheets were examined by immunocytochemistry with QH1 (which recognizes quail microglial cells) immediately after explantation and after different time intervals of *in vitro* culture (15 and 30 min, and 1, 3, 6, 12, 24, 48, 72 and 96 h). Immediately after explantation, microglial cells showed similar morphological features to those in the retina *in situ* (elongated cell bodies with broad processes) but with no lamellipodia. At 15 min of culture, many microglial cells had retracted their broad processes and were small and round with thin filopodia; a small proportion of cells remained elongated. At 30 min of culture, all microglial cells were rounded. A few small round microglial cells were in mitosis, revealing their proliferative capability. The cell proliferation rate increased with increasing culture time. At 96 h of culture, microglial cells were confluent in extensive zones of the culture and most were large and flattened and showed ruffled surface. Interestingly, microglial cells did not go beyond the borders of the ILM/MCEF sheet to reach the PLL-coated glass, suggesting a preference for their natural substrate. After 96 of culture, we then tested the influence of different substrates and culture media on morphological features of microglial cells. The cell cultures were treated with lidocaine and centrifuged. Isolated microglial cells were then plated on PLL-coated (PLL-C) or uncoated (U-C) coverslips and grown for a further 24 hours in DMEM-CS. For each substrate, the medium was then renewed with fresh DMEM-CS or changed to serum-free DMEM (SF-DMEM) or serum-free MEM with low concentration of glycine and serine (MEM-gly-ser), previously reported to regulate a ramified phenotype in rat microglia. Cells were grown in these media for 24 hours. The substrate had no effect on morphological features of cultured microglial cells because cells growing in specific media showed similar morphologies irrespective of the substrate. In contrast, the culture medium clearly influenced the phenotype of microglial cells. In cell cultures in DMEM-CS, microglial cells were large, flattened and ruffled, with a relatively high proportion of small cells in division. This could be due to an inhibitor effect of the serum on cell ramification. In SF-DMEM or MEM-gly-ser, microglial cells showed few short processes and many filopodia radiating from the cell body. Many cells left QH1-positive tracks and some dying cells showed thin portions of cytoplasm detached from their cell body. The inductive effect on cell ramification reported for SF-DMEM and MEM-gly-ser in cultured rat microglial cells was absent. Our observations suggest a high cell motility with intensive emission of cytoplasmic portions, which could exhaust the cells until their death.

Supported by grant PB97-0178 from the DGSIC of the Spanish MEC.

Pax6 is required cell autonomously in neuronal precursors of the developing cerebral cortex

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Mutations in the *Pax6* gene disrupt telencephalon development resulting in a thin cortical plate, expansion of proliferative layers and the absence of the olfactory bulb. We have used mouse chimeras containing a mixture of wild-type and *Pax6*$^{-/-}$ cells to determine the primary action of *Pax6* during the development of the cerebral cortex. This analysis revealed a dramatic reduction of *Pax6*$^{-/-}$ cells in the medio-caudal forebrain suggesting that *Pax6* specifies regional identity. In addition *Pax6* has cell autonomous affects on the cell surface properties of neuronal precursors and is required for efficient migration at the subventricular zone/intermediate zone boundary. We postulate that *Pax6* operates from the earliest stages of telencephalic development specifying regional identity with later affects on the migration of neuroblasts.

The Wnt-activated *Xiro-1* gene encodes a repressor that is essential for neural development and downregulates *Bmp-4*

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The *iroquois* (*iro*) genes encode a new family of homeoproteins evolutionary conserved. Both in *Drosophila* and vertebrates they have an early function required for the specification of large territories, and a late function required for the subdivision of these large territories in more restricted domains (reviewed in Cavodeassi

et al., 2001). Three *iro* genes have been identified in *Xenopus* (*Xiro1-3*). They are required for activation of proneural genes like *Xash3* and *Xnrgr1* and for neural plate formation (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Recently we have found that Xiro1, and probably Xiro2 and 3, are repressors. *Xiro1* overexpression promotes *Bmp-4* downregulation and neural plate expansion. Moreover, Xiro1 overexpression transforms competent ectoderm (animal caps), that is fated to become epidermis, towards neural tissue. We have also found that, even in the absence of BMP-4 signaling, *Xiro1* is esential for neural differentiation. This indicates that, in addition to *Bmp-4*, Xiro1 repress other genes that supress neural development. Finally, our data indicate that *Xiro1* is activated by Wnt signaling. Thus, Xiro1 is one of the factors that participate in Wnt-mediated *Bmp-4* repression (Gómez-Skarmeta et al., 2001). In summary, our results reveal that in *Xenopus* Xiro1 is not only required at later stages for proneural genes activation, but is also necessary at earlier stages for *Bmp-4* repression and neural plate specification. These and new data related to new genes repressed by Xiro will be commented.

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Homeobox-containing genes in head development

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Homeobox-containing genes are transcriptional regulators that control a variety of patterning and morphogenetic processes in many different organinsms, including vertebrates (Krumlauf, 1994). Members of this group of genes control segmental identity in different areas of the vertebrate head. In particular, *Hoxa2*, a gene expressed in the neural crest-derived mesenchyme of the second and more caudal branchial arches and in the hindbrain up to the second rhombomere, is essential for proper development of both, the second branchial arch skeleton and the preotic hindbrain (Gendron-Maguire *et al.,* 1993; Rijli *et al.,* 1993; Kanzler *et al.,* 1998; Davenne *et al.,* 1999). A series of genetic experiments will be discussed aimed at addressing the requirements of this gene for development of the mouse head. These experiments include the modification of the *Hoxa2* genomic locus to produce an allele suitable of controlled regulation. This allele, which behaves as a "knock down" allele, also allowed the analysis of the *Hoxa2* dosage requirements for development of the different embryonic areas where its activity is essential. The results showed that different areas and developmental processes require different levels of *Hoxa2* activity, being the hindbrain more resistant to *Hoxa2* deficiencies than the skeletogenic processes in the branchial area.

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Anterior patterning in mouse

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An increasing amount of evidence suggests that in mouse there are two signalling centres required for the formation of a complete neural axis: the anterior visceral endoderm (AVE), and the node and its derivatives. Embryological and genetic studies suggest that the AVE has a head-inducing activity. In contrast, the node appears to act first as a head inducer in synergy with the AVE initiating anterior neural patterning at early stages of mouse development, and later, node derivatives are necessary for maintenance and embellishment of anterior neural character. *Hex* and *Hesx1* are homeobox genes that are expressed in relevant tissues involved in anterior patterning. The analysis of the *Hex* and *Hesx1* mutant mice has revealed that the lack of these genes has little or no effect on the early steps of anterior neural induction. However, both genes are required subsequently for the proper expansion of the forebrain region. We suggest that disturbance in the specification of an FGF8 signalling centre in the anterior neural ridge may account for the anterior defects observed in these mutants.

Subtractive screen for the isolation of novel ave genes

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Xenopus *cerberus*, an extracelular antagonist of *Xnr1*, *Wnt8*, *BMP4* is expressed in the leading edge of the non involuting endoderm. Injection of *Xcer* mRNA in the ventral side of the embryo results in the formation of an ectopic head, without duplication of the body axes. Recent reports have shown that the topological equivalent to the leading edge endoderm in the mouse, the anterior visceral endoderm (AVE), also plays an essential role in the development of the rostral nervous system. Homozigotic mutant mice for *Lim1*, *Otx2* and *Hnf-3beta* fail to develop brain structures anterior to rhombomere 3. These transcription factors are known to be expressed in the AVE before prechordal plate formation and mouse chimeras experiments have shown that their expression in this extraembrionic tissue is necessary for the initial induction of a forebrain fate in the overlying ectoderm.

The mouse *Cerberus-like* gene was also found to be expressed in the AVE. This extracelular antagonist of Nodal and BMP4 is able to induce neural markers in animal caps experiments but the homozigotic mutant mice don´t show any phenotype. With the purpose of isolating new genes involved in forebrain induction and patterning, two transgenic mouse lines were generated in which EGFP is expressed in the AVE, under the control of the promoter region of the *Cer-l* gene. The AVE GFP positive cells of E5.5-6.0 embryos were isolated by cell sorting, and a library highly enriched for cDNA transcripts differentially expressed on the anterior side was generated by PCR cDNA subtraction between libraries of the anterior and posterior side. Clones that were shown to be differentially expressed were isolated and identified by sequencing.

The temporal and spatial expression patterns of the newly identified genes were determined by *in situ* hybridization and preliminary biochemical studies of their products will be reported.

Genomic approaches to the study of the mechanisms of vertebrate head induction

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Mouse *cerberus-like*, a member of the *Cerberus*/*Dan* gene family is a secreted factor expressed in the Anterior Visceral Endoderm (AVE) of the pre-streak mouse embryo. This region has been implicated in anterior neural specification. The neural inducing and mesoderm inhibition activities of Cer-*l* result from specific inhibition of BMP4 and Nodal molecules respectively. These activities are shared with the closely related Xenopus *cerberus* (a gene implicated in head induction in *Xenopus* experiments) and chick *Caronte.*

We have generated the plasmid vector pPMcerl.EGFP, which contains the fluorescent marker EGFP under the control of the upstream promoter sequences of *cer-l*. In the generated transgenic mouse lines, expression of EGFP can be observed in the AVE. When this plasmid is injected in dorsal blastomeres of *Xenopus* embryos, the *cer-l* promoter was able to drive the expression of EGFP in the deep endodermal cells of gastrulating embryos, overlaping with the domain of expression of *Xcer*. Electroporation of pPMcerl.EGFP in chick embryos also resulted into a *Caronte* like type of EGFP expression. By this we are now studying the regulation of *cerberus-like* promoter and using it to drive the expression of Xwnt8, Xnr-1 or XBMP4 factors in the *Xenopus* embryo anterior endoderm. We hope to gain further insight into the mechanism by which *cerberus* is required for the induction of the head in the *Xenopus* embryo and the role of the controlled inactivation of specific signaling molecules in the anterior endoderm of the vertebrate embryo.

To investigate the extent of evolutionary conservation between the regulatory sequences of mouse, *Xenopus* and chick *cer-like* genes, intra and cross species promoter studies are being performed

BMPs in growth control and cell death during limb development

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The developing limb is one of the best model systems to analyze the molecular control of morphogenesis in vertebrate embryos. The early limb bud consists of a core of mesenchymal cells covered by an ectodermal jacket. In the course of development, the limb bud undergoes outgrowth and at the same time the cells within the bud follow a patterned process of differentiation into cartilage which gives rise to the limb skeletal primordium. Several members of the BMP family of growth factors in coordination with a variety of BMP antagonists are responsible for the control of outgrowth and differentiation of the limb mesoderm, including the regulation of programmed cell death. In this communication, we review the molecular basis for those different functions of BMPs in limb morphogenesis.

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

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The formation of the veins, specialized structures of the wing of *Drosophila* and pterigotes, depends on the activity of different

intracellular signalling pathways. In one of these pathways, the *decapentaplegic* gene (*dpp*), a member of the TGFβ superfamily, acts as a ligand. *dpp* participate in both vein positioning and differentiation during pupal development. *dpp*, during larval development, stage at which vein position is determined, transcriptionally regulates the *spalt* gene complex which codes for two Zn-finger proteins. These proteins regulate other genes which individually define each vein. Later, during pupal development, *dpp* is expressed in the veins controlling their differentiation. *dpp* expression in veins, depends on a regulatory region, 5´ to the coding sequence, named *short vein*.

In this presentation, different aspects of *dpp* function in vein pattern formation will be described. Special attention will be paid to the regulation and function of *spalt* gene during imaginal development and the transcriptional regulation of *dpp* gene during pupal development.

Study of genetic interactions with mouse *cerberus-like*

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Mouse *cerberus-like (cer-l),* a gene related to *Xenopus cerberus* encodes for a secreted factor of the Cerberus/Dan family, a member of the TGF-β superfamily. This molecule can prevent BMP-4 and Nodal signaling by direct binding to these ligands in the extracellular space. In addition, this gene has neural inducing capabilities and is expressed in the Anterior Visceral Endoderm (AVE), a tissue that has been implicated in head organizer activity. The analysis of generated *cer-l* null animals revealed no abnormalities, since the mutants were born normal and were fertile. This result led us to propose the existence of other factor(s) capable of compensating the loss of function of *cer-l*.

Since the BMP-4 inhibitors noggin (nog) and chordin were shown to compensate for the loss of each other during mouse development, we proposed that *noggin* could be compensating for the loss of function of *cer-l* in *cer-l*$^{-/-}$ mutants. Despite of having functional similarity, these genes share domains of co-expression in the prechordal and notochordal plates. To test our hypothesis *cer-l*$^{-/-}$;*nog*$^{-/-}$ mutants were generated.

The second interaction studied was with the homeobox gene *goosecoid* (*gsc*). *Gsc* and *cer-l* are co-expressed in the AVE and in the prechordal plate. *Gsc* was shown to be essential for craniofacial morphogenesis and to interact with HNF-3β (a gene also expressed in the AVE) in neural tube patterning and head development. To assess for the existence of functional redundancy between *gsc* and *cer-l* we generated *cer-l*$^{-/-}$;*gsc*$^{-/-}$ null mutants and the resultant data will be shown.

Cell fate determination and patterning of the *Drosophila* tracheal system

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The *Drosophila* tracheal system is a complex network of tubular structures that develops in a stereotyped manner. Tracheal development begins when antero-posterior and dorso-ventral signals position ten segmental clusters of cells on ectoderm. These so-called tracheal placodes invaginate and migrate to give rise to the tracheal system, without cell division. We have studied how different tracheal branches migrate through other embryo tissues and found that mesoderm tissues are implicated in tracheal migration. We show that mesoderm myoblasts are a substrate of tracheal branches migrating along the dorso-ventral axis of the embryo, whereas the fat body, another mesoderm structure, acts as a substrate of the major antero-posterior branch (the dorsal trunk). We suggest that interactions between substrate mesoderm tissues and different tracheal branches are mediated by specific cell-surface molecules.

We are also studying the molecular mechanisms that specify the different tracheal branches. Previous work has shown this process depends on several regulatory signals, including those with similarities to vertebrate FGF and TGFb signaling mechanisms. We have implicated other regulatory factors in tracheal specification and morphogenesis, and we will discuss their relationship with TGFβ signaling.

Supressor of Hairless is the core of a molecular switch for Notch target gene regulation

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Cell-cell signalling mediated by Notch is critical during many different developmental processes for the specification or restriction of cell fates. Currently the only known transduction pathway involves a DNA binding protein, Suppressor of Hairless [Su(H)] in *Drosophila* and CBF1 in mammals, and results in the direct activation of target genes. It has been proposed that, in the absence of Notch, Su(H)/CBF1 acts as a repressor and is converted into an activator through interactions with the Notch intracellular domain. Recently we have also suggested that the activation of specific target genes requires synergy between Su(H) and other transcriptional activators. Here we have designed an assay that allows us to directly test these hypotheses in vivo. Our results clearly demonstrate that Su(H) is able to function as the core of a molecular switch, repressing transcription in the absence of Notch

and activating in the presence of Notch. In its capacity as an activator, Su(H) can cooperate synergistically with a DNA-bound transcription factor, Grainyhead. These interactions indicate a simple model for Notch target-gene regulation that could explain the precision of gene activation elicited by Notch signalling in different developmental fate decisions.

DWnt genes in developing tracheae

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The tracheal network consists of interconnected epithelial tubes that arise from 20 clusters of ectodermal cells. The tracheal cells invaginate inside the embryo and formation of the tracheal tree occurs without cell proliferation by cell migration and branch fusion. It has been already shown that the *wingless* (*wg*) gene is required for the normal tracheal development. However, *wg* is not responsible for all *Wnt* functions there, as the tracheal phenotype of other members of the Wnt pathway is stronger than the *wg* phenotype. Sequencing of the *Drosophila* genome has revealed that there are seven *DWnt* genes, including *wg*. We have tested each one of the seven *DWnts* in different ways to find out whether they are also required in the developing tracheae. We have evidence that only a subset of the 7 *DWnt* genes acts or can act in the tracheae. In particular, we have found that DWnt2 may assist Wg to specify the main tracheal branch, the so-called dorsal trunk. The pattern of expression of *DWnt2* is consistent with a role in the trachea, as it is expressed near the tracheal cells at the appropriate time. In addition, overexpression of *DWnt2* in the tracheal cells produces a similar phenotype to that of overexpressing *wg.* Finally, we also show that DWnt2 and Wg have different effectivity in specific tissues, as the epidermis responds differently to these two genes.

Role of Bmps in the specification of the left-right axis in the chick embryo

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During gastrulation the three main embryonic axes, anterior-posterior, dorso-ventral and left-right (L/R), are specified. In the chick, several important signaling molecules have been shown to exhibit small asymmetric domains of expression in the node (Levin *et. al.,* 1995; Boettger *et. al.,* 1999; Shamim and Masson, 1999; García Castro *et al.,* 2000; Monsoro-Burq and LeDouarin, 2000). Multiple regulatory relationships between these molecules control L/R asymmetries.

Bmp4 is asymmetrically expressed at low levels in the right side of Hensen's node in a pattern complementary to that of *Shh* (Monsor-Burq and LeDouarin, 2000). We have performed an analysis of the role Bmp4 plays during initial steps of left right development in chick. Exogenous application of Bmp4 to the left side of Hensen's node (stage 4-5HH) caused a downregulation of *Shh* expression both in the node and in the nascent notochord. Conversely, blocking Bmp signaling by noggin resulted in symmetric *Shh* expression in the node. Bmp4 treated embryos showed perturbed patterns of *Nodal* expression thus confirming alteration of laterality. This effect is dose-dependent and also elicited by Bmp2 and Bmp7. Our results support endogenous Bmp4 participation in the left-side restriction of *Shh* expression.

We will also show molecular and morphological alterations of the midline due to Bmps overexpression. These results will be discussed in the context of current models for L/R development.

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Expression and regulation of the chick Tolloid metalloprotease, Colloid, during limb bud development

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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. One level of control of BMP signaling occurs by the action of several BMP inhibitors that bind BMPs outside the cell and then preventing the interaction with their receptors. Another level of BMP control demonstrated in the last years occurs by cleavage of one of their antagonist (Chordin) by one specific metalloprotease (Tolloid), allowing the BMPs to interact with their receptors. 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. The injection of Chordin mRNA in the ventral side of the Xenopus embryo induces secondary axis by inhibiting ventral BMP signalling, this duplications do not take place when Xolloid and Chordin mRNAs are coinjected. Since BMPs have a critical role during limb morphogenesis inducing cell death, chondrogenesis, muscle

diferentiation and controlling A/P patterning, we have used the chick limb bud as a model to study Colloid expression and its relationship with other signalling molecules. We have performed whole mount in situ hybridizations in order to determine the expression pattern of Colloid in control limbs. Colloid shows a dynamic expression pattern during limb development from stage 20 to 35. Transcripts are detected first in distal limb mesenchyme and from stages 24 to 27 this domain is subdivided in anterior, posterior and central domains. From stage 28 to 29 Colloid expression is detected in the interdigital space and tip of digits. Later on, during stage 30 to 35 the areas of expression are restricted to the joints, tip of digits and tendinous blatemas. This pattern of expression is closely related with that of BMPs and Chordin. We studied the interactions of Colloid with SHH, FGFs and BMPs. Grafts of SHH expressing cells in the anterior part of limb bud increase Colloid expression after 6 hours. The removal of apical ectodermal ridge is followed by downregulation of Colloid expression in the anterior part of limb bud and this phenotype can be rescued by aplication of FGF after AER removal suggesting that Colloid expression is maintained by the FGFs released from the AER. Treatments with BMP protein leads to Colloid downregulation surrounding the bead. These preliminary results suggest that all the studied molecules influence Colloid expression during limb development.

Digit morphogenesis: characterization of the effects of late ectopic Shh signalling and the formation of the digit tip

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The formation of digits/toes is a final step in the development of limbs. 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. Here we characterize these effects in detail and will present evidence implicating modulation of both BMP (as it has been proposed recently) and FGF signalling as well in these processes. Also, joint formation and spacing and 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.

Signal transduction and morphogenesis in *Drosophila* development

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The interest of our group is focused in the role of cell communication mechanisms in development. In particular, we are analysing such mechanisms in two model systems in *Drosophila*. The first model is the torso (tor) receptor system that allows us to study two basic features in the signal transduction mechanisms: the spatial control of their activation and their potential to generate more than one response. The tor receptor is distributed along all the blastoderm membrane but it is only activated at the termini by a diffusible ligand generated at each pole; the tor receptor not only transduces the spatial signal but also ensures its correct localisation by sequestering the ligand. *trunk* (*trk*), one of the genes required for tor activation, codes for a protein that may be secreted and cleaved to yield a c-terminal fragment similar to several types of growth factors. This fragment is sufficient to activate tor signalling and bypasses the requirements for the other genes involved in the activation of the tor receptor. These results suggest that a cleaved form of the trk protein acts as a signal for the tor receptor. Thus, the restricted activation of the tor receptor appears to be defined by the spatial control of the proteolytic processing of trk. We are at present investigating the role of two extracellular proteins, the products of the *fs(1)N* and *fs(1)ph* genes, that have a dual role in mediating eggshell assembly and enabling tor receptor activation.

On what concerns the potential of the tor pathway to generate more than one response, it has been shown that changes in the number of activated tor receptors at the cell surface can be one of the mechanisms to generate differential gene expression. More precisely, tor signaling induces gene expression by inactivating at the embryonic poles an uniformly distributed repressor activity that involves the groucho (gro) corepressor. A newly identify gene, *capicua* (*cic*), encodes an HMG-box transcription factor that interacts with gro in vitro. Cic acts as a repressor of the terminal genes and is inactivated by tor signaling at the embryonic poles. *cic* has been evolutionarily conserved, suggesting that cic-like proteins may act as repressors regulated by RTK signaling in other organisms.

The other system is the formation of the tracheal system and in particular the mechanisms involved in tracheal cell migration. This system arises from the tracheal placodes, clusters of ectodermal cells that appear at each side of ten embryonic segments. The cells of each cluster invaginate and migrate in different and stereotyped directions to form each of the primary tracheal branches.

The general conclusion from many studies is that the direction of migration of the tracheal cells relies on a set of positional cues provided by nearby cells. Cells in different branches exploit different strategies for their migration. Indeed, the alternative migratory pathways of the tracheal cells are associated with distinct subsets of mesodermal cells suggesting a model for allocation of different groups of tracheal cells to form different branches.

At present, we are investigating the role of cell surface proteins in the definition of these pathways.

Immortal cells in the germ line of *Drosophila*

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The production of functional gametes during oogenesis in *Drosophila* requires the existence of germline stem cells which divide asymmetrically to give rise to another stem cell and to a differentiating cystoblast. In *Drosophila*, the maintenance of the germline stem cells requires the activity of adjacent somatic cells. These somatic cells provide a developmental 'niche' with all the growth factors needed for the stem cell to remain undifferentiated and to produce cystoblasts throughout the lifetime of the female. In our laboratory, we study the genetic and molecular requirements for the development of stem cells, using the oogenesis of *Drosophila melanogaster* as a model system.

Control of cytoskeleton and cell adhesion during morphogenesis in *Drosophila*

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Epithelial cells tend to associate in polarized, monolayer sheets and develop into various forms, such as layers, tubes or vesicles. Cell movement and contact are implemented 1) by intercellular interactions which involve surface properties of cells such as attraction, repulsion and differential adhesivity; and 2) by intracellular forces generated by the cytoskeleton, which are responsible for changes in cell shape. The movement and fusion of epithelial cells during development is an essential and ubiquous morphogenetic event. Many of these processes, such as epiboly in teleosts, amphibians and birds, neural tube and palate closure in vertebrates, ventral enclosure in *C. elegans*, embryonic dorsal closure and imaginal disc fusion in *Drosophila* and the related tissue movements of adult and embryonic vertebrate wound healing, involve extensive and directed sweepings of two epithelial faces. Although extensive morphological descriptions of these processes represent classical paradigms of embryology and pathology, the genetic basis and cellular behavior underlying these events are still poorly understood.

During embryonic dorsal closure in *Drosophila*, the mechanical forces leading the spreading of the epidermis are purse-string and amnioserosa contraction. Actin and non-muscle myosin (NMM) accumulate at the leading edge and in the absence of this contractile machinery, e.g. in mutants for *zipper* (the gene coding for NMM), embryos show dorsal-open phenotypes. We and others have shown that actin and NMM accumulation depends on the activity of Rho GTPases and JNK signaling. JNK activity triggers the expression of Puc and Dpp. Puc is a JNK phosphatase that functions to negatively feedback on JNK, regulating the level of its activity. The role of Dpp is less clear. In mutants for members of the Dpp cascade, cells elongate correctly, but the actin cytoskeleton suffers an extreme compaction, generating epidermal bunching.

During metamorphosis, forward locomotion of imaginal cells involves extensive filopodia. These, which protrude out of leading cells, expand over the larval surface and form actin bridges. We have found that during imaginal closure, JNK signaling affects the adhesion between imaginal and larval epidermal cells and in mutant conditions larval cells detach, disrupting the continuity of the epithelium. This defect is also observed during embryonic dorsal closure. Interference with JNK appears to affect the accumulation of integrins at the epithelial leading edge leading to dorsal open phenotypes and lack of imaginal fusion.

To identify new genes affecting imaginal fusion, we have already initiated a gain of function screen. We are using P elements with a UAS module targeted by the transcription factor GAL4 (EP screening). This allows the inducible expression of genes adjacent to P element insertions. In pilot experiments, 1000 new EP lines were tested with Pnr-GAL4, a line expressed in a dorsal domain including imaginal and larval cells. This analysis identified more than 30 genes whose overexpression affects the spreading/fusion of wing discs.

Functional analysis of *sonic hedgehog* during fin regeneration in zebrafish

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Teleost fish has the ability to regenerate their fins after injury. *Sonic hedgehog* (*shh*), its receptor *patched1* (*ptc1*), and the bone morphogenetic factor *bmp2b*, which are not expressed in the normal adult fin, become re-expressed during regeneration in patterns that implicate shh and bmp2b in the patterning of the dermal bones within the regenerating fin. To better understand the role of shh signaling pathway and of bmp2b during fin regeneration, we have performed a functional analysis. We have shown that ectopic expression of *shh* or *bmp2b* using an in vivo transfection approach, results in excessive bone formation that disrupts the normal patterning of the regenerating fin. Over-expression of shh, but not bmp2b, results in a down-regulation of endogenous *shh*. In addition, we demonstrate that exposure of regenerating fins to the alkaloid cyclopamine, which specifically interferes with hedgehog signaling, results in a dose dependent reduction of cell proliferation in the regenerative mesenchyme, resulting in an inhibition of fin regeneration. Therefore, the ectopic bone deposition following over-expression of shh and bmp2b and the effects on fin outgrowth following treatment with cyclopamine implicate shh signaling in the proliferation and/or differentiation of the specialized bone-secreting cells in the blastema during regeneration. In addition, gene expression analysis suggests that the domains of *shh* expression within the regenerating fin may be controlled by regulatory feedback mechanisms that function to define the region of bone secretion in the outgrowing fin.

Supported by a grant from the CIHR.

Myogenesis, regeneration and cell renewal in freshwater planarians

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We have characterized the body musculature and monitored the process of muscle differentiation during anterior regeneration in freshwaters planarians (Platyhelminthes, Turbellaria, Tricladida). These was performed in order to answer questions rised when monitoring the processes of cell renewal and restoration of body wall musculature. The monoclonal antibody TMUS-13, which binds specifically to the planarians myosin heavy chain, was used as a molecular marker. From the first day of regeneration we find determined myocytes (detected by «in situ» hybridization using the myosin heavy chain gene as probe) at the postblastema region, from where they seem to actively migrate to the inside of the blastema carrying on their differentiation.

We've stated determination and differentiation are separated processes both in time and space, since the early myocytes are already detected in the regeneration blastema from the second day of regeneration. The new muscle fibers differentiation seems to be proximo-distal or maybe in a intercalatory way being the pattern reestructuration different whether the muscle wall is dorsal or ventral.

The regeneration of the pharyngeal musculature proceeds intercalating myocytes throughout the organ. These myocytes are determined in the body parenchyma of the pharynx implantation zone, from where they migrate inside as they differentiate.

GtDap-1: a molecular marker to follow apoptosis in planarian regeneration

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Planarians have a great morphological plasticity supported by a continuous grow and shrink in volume and lenght and a high regenerative capacity. In cellular terms, growth and degrowth are tightly coupled to the rates of cell proliferation and apoptosis, and its balance is influenced by food availability, temperature, body size and the genetic characteristics of the planarian. During starvation there is an increased rate of cell death and a decreased rate of cell proliferation. On the other hand, planarian regeneration requires a wound healing and an increase in the proliferation rates of totipotent cells (neoblasts), already presents in the adult. Such mitotic activity is higher in regions close to the wound to produce the new tissue on blastema (epimorphosis), while the rest of the planarian fragment adjust to the new proportions by a programmed cell death and new cell production (morphallaxis). Such cell death has not been demonstrated yet.

We have studied the apoptosis as an evolutionary conserved form of cell death during starvation and regeneration. In that way, we have characterized a Death-Associated Protein called *GtDap-1* that belongs to a novel family of pro-apoptotic genes. From whole mount *in situ* hybridization we have observed its activation during regeneration and from that we can follow the apoptotic process in the whole regenerating planarian fragment or in starved adults.

Epithelial-mesenchymal interactions are perturbed in developing fins *of m565* and *m180* zebrafish mutants

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m565 and *m180* mutations were obtained by chemical mutagenesis in zebrafish. These mutations do not complement and show conspicuous blister formations in the fin fold accompanied by a fin growth arrest during early fin development stages (between 30 and 144 hpf). These phenotypes are transient, adult homozygote mutant fish are viable and do not show any phenotype in their fins, except for the loss of dorsal fins of some individuals probably due to a defect in the dorsal fin fold at the time when the fin fold is re-growing. Epithelial-mesenchymal interactions were studied by in situ hybridization using anti-sense RNA probes for genes involved in epithelial-mesenchymal interactions such as *fgf8*, *msxC* and *dlx3*. Expression of these genes is modified in *m565* and *m180* mutants. During blister formation, a local loss of *fgf8* and *msxC* expression is observed. Later, at 10 days post fertilization when fin growth is resuming, a re-expression of *msxC* and *dlx3* is detected although at this stage expression in wild type embryos is absent. Acridin orange staining is enhanced at the distal ectoderm surrounding the blisters and in the surrounding regions suggesting that cell death is occurring in those regions showing a loss of *fgf8* and *msxC* expression. At 10 dpf, cell death is similar to that observed in wild type embryos. Preliminary results from electron and light microscopy studies suggest that the basal lamina is present in the detached ectoderm. Based on these results, we propose that fin blisters and growth arrest phenotype observed in *m565* and *m180* mutants is due to a transient perturbation of epithelial-mesenchyme interactions.

Ancestral and derived functions of the *Snail* gene superfamily

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The *Snail* family of zinc-finger transcription factors plays an important role in early embryonic development of vertebrates and

invertebrates, with an evolutionarily conserved function in gastrulation and mesoderm patterning, and further roles in neural differentiation and vertebrate neural crest formation.

Several observations make the study of this gene family extremely interesting from an evolutionary point of view. Firstly, the neural crest is believed to have been crucial in the formation of the "new head" of vertebrates. In addition, we have reported a unique interchange in the expression patterns and the roles of the two vertebrate family members (*Snail* and *Slug*) between avian and mammals. Thus, we have carried out the study of the distribution and the evolutionary history of the Snail family.

Until recently, four members of the *Snail* family had been described in *Drosophila* (*snail*, *scratch*, *escargot* and *worniu*) and up to three in vertebrates (*Snail*, *Slug* and *Smuc*), whereas only one had been found in nematodes, echinoderms and non-vertebrate chordates (ascidians and amphioxus). This led to the suggestion that a unique gene was present before the protostome/deuterostome divergence and subsequent independent duplication events within the arthropod and vertebrate lineages gave rise to the present situation.

We have identified new family members from different model organisms that has led us to define a new group of scratch-related genes present in all metazoans. Thus, we have proposed the existence of the Snail superfamily that groups the Snail and Scratch families and have made new hypotheses about common ancestor states and functional changes during evolution.

These distinct duplication events and the subsequent divergence of the duplicated genes raise interesting questions as when and how the inversion in expression sites, observed for Slug and Snail between chicken and mouse embryos, occurred during evolution. We have isolated Snail family members from lizards and turtles and analysed their expression pattern during embryonic development. We will discuss whether the regulation of these genes is a derived feature of the avian lineage or a general characteristic of all the non-synapsid lineage, that includes turtles, avians and reptiles. This approach will offer a glimpse on how duplication and divergence within gene families during evolution correlate with early development and patterning of vertebrates.

Pitx genes in tunicates provide new molecular insight into the evolutionary origin of the pituitary

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We have initiated a project aimed at documenting molecular and cellular changes underlying the emergence of the hypothalamo-hypophyseal axis in Chordates. Considering the phylogenetic position of Tunicates and the "pan-hypophyseal" expression pattern of *Pitx* genes in Vertebrate pituitary, we searched for a *Pitx*-related homeobox gene in the ascidian *Ciona intestinalis*, and identified *Ci-Pitx* (*Ciona intestinalis Pituitary homeobox* gene). We also isolated *Cs-Pitx* and *Bs-Pitx*, the *Ci-Pitx* respective counterparts of *Ciona savignyi* and *Botryllus schlosseri,* two other Tunicate species. *Ci-Pitx* mRNA encodes a putative protein exhibiting the diagnostic K50-Paired-class homeodomain and a conserved C-terminal Aristaless domain. Embryonic expression pattern of *Ci-Pitx* revealed a conserved expression domain in the anterior neural ridge and subsequently in the pharyngeal primordium, defined in Vertebrates as the stomodeal ectomere, which encompasses the presumptive pituitary territory. This shows that expression at early steps of pituitary development is a feature of *Pitx*-related genes that was already present in the last common ancestor of Chordates.

A comparative study of the patterns of division of neuroblasts at the proliferative centers of the larval optic lobe of *Drosophila* and neuroepithelial progenitors of the vertebrate CNS

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The genetic and molecular analysis of neurogenesis in the *Drosophila* embryo has provided a great deal of information on the processes of segregation of neural progenitor cells and their specification. Although homologous genes have been found in vertebrates, the pattern of division of embryonic neuroblasts (Nbs) is very different from that of neuroepithelial progenitors in the vertebrate CNS. In order to explore an alternative experimental model where to study the genes and molecular mechanisms that govern neural proliferation and the specification of CNS progenitors, we have studied the proliferative centers of the larval optic lobes of *Drosophila* from which most cells of the adult brain originate. We have particularly focused on the outer proliferation center that presents a neuroepithelial-like arrangement of Nbs on the surface of the optic lobe. We have studied three aspects of these proliferative processes: the pattern of cellular division, the switch from symmetric to asymmetric divisions, and the expression/localization of asymmetric cell fate determinants that may be involved in the specification of neural cells. Although larval Nbs are of embryonic origin, our results indicate that their properties appear to be modified during development. Thus, our experimental data suggests interesting similarities with proliferative properties of neuroepithelial progenitors. For instance, in early larvae, Nbs divide in a proliferative manner (symmetric divisions) increasing the number of Nbs while in the late larval brain most Nbs divide asymmetrically in a stem cell manner to generate postmitotic cells (Ceron *et al.,* 2001).

This work has been supported by grants PM97-0083 fron the DGESIC (MEC) and the Fundacion Mafre-Medicina.

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Multiple roles of *Minibrain* on the development of *Drosophila* and vertebrate brain

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One of the most relevant problems in Neurobiology is to understand the mechanisms for the generation of neuronal diversity. The molecular analysis of mutants carried out in model organisms can be a useful approach to get insight into this problem. The *Minibrain (Mnb*) gene encodes a new protein kinase family involved in postembryonic neurogenesis (1).

Adult *Mnb* flies show a very characteristic phenotype which consists on reduction of the size of defined brain areas (especially optic lobes) with no gross alterations in neuronal architecture and connectivity.

This is due to a decrease in the number of neurons that are produced during the postembryonic proliferative pediod. Pulse-chase BrdU-labelling experiments carried out with first and second instar larvae demonstrate that Mnb mutations do not cause modifications in the timing of proliferation of embryonic quiescent neuroblast.

Nevertheless, the number of BrdU-labelled neurons that were chased in the adult brain after a pulse at late third instar larvae was clearly reduced in the optic lobes of Mnb flies. Consequently, we focused our analysis on the proliferative centers of the third instar larve (2).

We found modifications at the larval outer proliferation center (opc) of the optic lobes in *Mnb* mutants. Nbs and postmitotic cells are arranged in a neuroepithelial-like structure in this proliferative centre.. Interestingly, Mnb is specifically expressed at the opc by both in situ hybridization and immunocytology. Several homologous genes to *Mnb* have been cloned in vertebrates. We have demonstrated that they share both structural and functional similarities with Drosophila *Mnb* and belong to a new protein kinase subfamily (3).

However, despite the fact that the human *Mnb* gene is located in the Down Syndrome critical region, there have not been clear experimental data indicating that *Mnb* may play a similar role in vertebrate neurogenesis.

In order to find functional correlations, we have studied the spatio-temporal expression of *Mnb* during the embryonic development of chicken and mouse brain. Altogether our data support a role of *Mnb* in neuronal proliferation and differentiation at the vertebrate CNS, thus providing evidence for a conserved role during brain evolution.

This work has been supported by grants PM97-0083 from the DGESIC (MEC) and the Fundacion Mafre-Medicina

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What's at the base of the bilaterians?

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The origin of bilaterian is an open question in evolutionary biology. Which would be the closest living representative of the first bilaterally symetrical organism? In order to put some light upon that question, it is necessary to find an animal which may be in the base of the phylogenetic tree in the bilaterian clade. Molecular phylogenetic analysis based in 18S rDNA sequences point to the acoela as those suposed living ancient bilaterian, although this results are controversed.

A new approach to find out where in the tree should the acoela be placed is the study of certain genes which may have developmental universal functions. For this reason, we have undergone a new Hox and ParaHox-based approach to identify and characterize the Hox complement of acoela. The analisys of sequences and chromosomal location of these genes should place correctly the acoela in the metazoan tree.

Using a degenerate PCR approach, we have cloned by now several Hox and ParaHox genes from different species of acoela and we are currently analyzing their possible relationships with other sequences of this class of homeobox-containing genes from other bilaterians. The presence of molecular signatures in such genes will place the acoela in or out of the lophotrocozoan group and will clarify whether these animals are really at the base of the protostomates or, as they were catalogued before the sequences analysis, are real platyhelmintes.

Casanova B, an essential factor for zebrafish endoderm formation

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Endoderm originates from a large endomesodermal field requiring Nodal signalling. The mechanisms that ensure segregation of endoderm from mesoderm are not fully understood. We first show that the timing/dose of Nodal activation is critical for endoderm formation and the endoderm versus mesoderm fate choice, since sustained Nodal signalling is required to ensure endoderm formation but transient signalling is sufficient for mesoderm formation. In zebrafish, downstream of Nodal signals, three genes, *faust, bonnie and clyde* and the recently identified gene *casanova*, encode transcription factors, activated by Nodal signalling, which are required for endoderm formation/differentiation, but their respective positions within the pathway is not completely estab-

lished. In the present work, we show that *casanova* represents the earliest specification marker for endodermal cells and that its expression requires *bonnie and clyde*.

Furthermore, we have analysed the molecular activities of *casanova* on endoderm formation. *cas* can induce endodermal markers and repress mesodermal markers during gastrulation as well as transfate marginal blastomeres to endoderm. *casanova* overexpression also restores endoderm markers in the absence of Nodal signalling. In addition, *cas* efficiently restores later endodermal differentiation in these mutants, but this process requires, in addition, a partial activation of Nodal signalling.

Role of the transcription factor *Pitx-2* during cardiac development: a search for downstream genes

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The homeobox transcription factor *Pitx-2* plays a key role during left/right signalling, conferring leftness to asymmetric organs such as the heart and the stomach (Piedra et al., 1998). Recent studies have shown that *Pitx-2* remains asymmetrically expressed during at the heart tube stage (Campione et al., 1999). We have characterised thoroughly the expression pattern of *Pitx-2* during subsequent stages of cardiac development in rat and mice. *Pitx-2* is expressed in the left atrium, left region of the atrioventricular canal, ventral region of the ventricles and the ventro-lateral (left) of the outflow tract during early stages of development (E8.5-E11.5). The expression of *Pitx-2* decreases during fetal stages, being only detectable within the atrial myocardium. These findings lead to the suggestion that *Pitx2* represents a molecular marker of the left cardiac crescent-derived cells. DiD labeling experiments in chicken embryos have permitted to corroborate this hypothesis (Campione et al., 2001). With the goal of finding and characterising *Pitx-2* downstram genes in myocardial cells, we have designed a strategy to overexpress *Pitx2* in primary cultures of cardiomyocytes and embryonic stem cells. At present, we have cloned *Pitx-2c* under control of a cardiac muscle-specific promoter (alpha myosin heavy chain; a-MHC) and we have overexpressed it in primary cultures of rat fetal cardiomyocytes (E17.5). Expression analyses of *Pitx-2c* (endogenous, rat and y exogenous, mouse) by *in situ* hybrididization and RT-PCR show that exogenous *Pitx-2c* is able to induce expression of endogenous *Pitx-2c* during 12 to 24 hours while the transient expression of the exogenous construct remains being expressed even after 96 hours. These data allow us to use this approach to generate, and later on compare, cDNA libraries obtained from *Pitx-2* ovexpressing primary cultures and/or ES cells (adding a IRES-neomycin cassette) to controls, and therefore identify *Pitx-2* target genes.

Embryonic vascular development of quail mesonephros: paracrine and autocrine VEGF production

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It is generally accepted that embryonic blood vessels are formed by two distinct morphogenetic mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the neoformation of a vessel by angioblasts which differentiate *in situ* or by angioblasts which originate at a distant localization and migrate to the site of vessel formation. In contrast, angiogenesis is the formation of new vessels from pre-existing vessels. Two angiogenic processes have been described, sprouting and intussuceptive capillary growth.

We demonstrated that vasculogenesis and angiogenesis are implicated in the development of the mesonephric subcardinal plexus of quail embryo. The former occurs between days 3 and 4 of incubation (22-25 HH), while the latter takes place from day 5 to day 7 (28-35 HH). Examination of vascular corrosion casts and whole mounts, and tissue sections labelled with specific markers for hemangioblast lineage (QH1, LEP100 and AcPase activity) allowed us to distinguish six phases in the formation of the subcardinal plexus.

(1) Appearance of isolated angioblasts where subcardinal plexus will be formed.
(2) Alignment of angioblast*s*, which constitute cellular strands.
(3) Formation of compact endothelial cords by association with angioblast strands.
(4) Polygonal interconnection of endothelial cords to constitute the primary subcardinal plexus. In this stage, isolated angioblasts were present inside inter-vascular spaces.
(5) The splitting of primary inter-vascular spaces by angiogenic sprouts to form the secondary subcardinal plexus (outward angiogenesis). Isolated angioblasts were not present in this stage.
(6) Expansion of the secondary subcardinal plexus by insertion of slender transcapillary tissue pillars (inward angiogenesis) and angiogenic sprouts. We also describe three morphogenetic gradients during the development of the subcardinal plexus: ventro-to-dorsal, cranial-to-caudal and lateral-to-medial. Furthermore, using a polyclonal antibody, VEGF protein was detected in 3 and 4-day old quail embryo mesonephros. At day 3 of incubation, VEGF was restricted to epithelial lineage (tubular cells, podocytes and coelomic epithelium), while at day 4 it was also expressed in endothelial cells. In conclusion,

VEGF is involved in the development of mesonephric blood vessels in a paracrine and autocrine manner.

EphrinB2 mRNA expression during chick mesonephros development and degeneration

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The cellular and molecular mechanisms underlying vasculogenesis and angiogenesis are still poorly understood. However, an increasing number of intercellular signaling molecules have been identified that play an essential role in this process. Prominent among these are transmembrane receptor tyrosine kinases and their ligands (angiopoietins, platelet-derived growth factors, vascular endothelial growth factors and ephrins). Moreover, it is known that repulsive signaling between arteries and veins is decisive for angiogenic remodeling during the functional maturation of the vascular system. Recently, ephrinB2 has been shown to be required for the remodeling of the embryonic blood vessels. Because of its exclusive expression on arteries and the complementary expression of one of its cognate receptors, EphB4, on veins, it was suggested that ephrinB2 acts both as a ligand and as a receptor for EphB4. However, it is not known the role of ephrinB2 during the tremendous vascular remodeling that occurs when an embryonic organ is functionally substituted by the definitive one.

Avian mesonephros is an embryonic transitory organ with a complex, but well known, vascular system. Degeneration of the mesonephros leads to vessel disintegration. However, mesonephric vessels that communicate with metanephros, the definitive kidney, and gonads do not degenerate. The aim of this study is to analyze ephrinB2 mRNA expression throughout the development and degeneration of chick mesonephros, in order to understand the function of ephrinB2 during vascular mesonephric remodelation.

In 24 HH embryos, ephrinB2 transcripts were observed in endothelial cells of dorsal aorta, vitelline, femoral, umbilical and intersomitic arteries. Mesonephric arteries with the ephrinB2 signal were only seen in the cranial part of the mesonephros. At stage 30, ephrinB2 mRNA was only observed in the dorsal aorta and the caudal mesonephric arteries.

In 38 HH embryos, ephrinB2 expression was only present in the aorta and mesonephric arteries that will not degenerate. We found no ephrinB2 transcripts in glomerular capillaries or veins at any stage. In conclusion, the ephrinB2 temporal expression pattern matches the cranial-to-caudal morphogenetic gradient of mesonephros development. Totally differentiated mesonephric arteries do not express ephrinB2 and, when mesonephric degeneration occurs, only mesonephric arteries that remain expressed ephrinB2. Our results confirm the morphogenetic role of ephrinB2. Only arteries under remodelation expressed ephrinB2.

Wnt signalling is regulated by an extracellular sulfatase

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Heparan sulfate proteoglycans (HSPGs) have well established functions in the extracellular transmission of developmental signalling molecules to control the patterning and specification of cell lineages in vertebrate and invertebrate embryos and the growth of tumours in adults. The sulfation states of N-acetyl glucosamine residues in HSPGs participate in regulating FGF and Wnt signalling. Developmental mechanisms that regulate HSPG sulfation, however, have not been described. We isolated a novel N-acetyl glucosamine 6-sulfatase (*QSulf-1*), that was cloned as a somite-activated gene using differential display technology. As Sonic hedgehog and Wnt signalling are activated in newly formed somites, this screen was predicted to identify immediate early Sonic hedgehog (Shh) and Wnt response genes that function in the specification of somite cells to muscle, sclerotomal and dermal stem cell lineages. *QSulf-1* is expressed at sites of myogenic and neuronal stem cell specification and responds to Sonic hedgehog signalling. For example, its expression is enhanced by the Shh signal secreted by the axial structures that could be mimicked by the ectopic expression of N-Shh protein. The antisense oligos to Shh inhibit the expression of *QSulf-1*. In contrast to the lysosomal sulfatases, *QSulf-1* has a N-terminal signal peptide and cleavage site predicted to target its secretion to the cell surface and an internal hydrophilic domain that is unique to *QSulf-1* and its homologues. *QSulf-1* is thus secreted and docked to the cell surface of expressing cells through its distinctive hydrophilic domain. *QSulf-1*, a secreted enzyme, is specific for the release of 6-O sulfates from N-acetyl glucosamine on cell surface HSPGs. This could therefore regulate Wnt and FGF signalling in Drosophila and vertebrate embryos. Antisense inhibition of *QSulf-1* expression in avian embryos selectively blocks the localised activation of the muscle master regulatory gene, *MyoD*, which is mediated by Wnt signalling, and expression of *QSulf-1* in C2C12 skeletal myoblasts modulates their response to heparan sulfate-dependent Wnt signalling. In contrast to wild type *QSulf-1*, mutant *QSulf-1* has no activity and therefore does not enhance Wnt signalling in C2C12 Wnt signalling assay. *QSulf-1* functions as an active sulfatase enzyme in its Wnt signalling activity and thus is a new extracellular regulator of HSPG-mediated Wnt signalling for embryo patterning.

Chicken Vascular Endohelial Growth Factor D revealed in limb development using microarray technology

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Growth and patterning along the Antero-Posterior (AP) axis appear to be tightly coordinated with the development of the Proximal-Distal (PD) axis, in a process mediated by specific interactions between the AP organizer (the Zone of Polarizing Activity) and the controller of limb outgrowth (the Apical Ectodermal Ridge). Some genes involved in AP patterning have been described but mechanisms regarding the molecular and cellular mechanisms that control limb patterning remain to be understood. In order to study AP patterning during limb development we have made a cDNA microarray containing 4.608 genes from a chicken limb library (stages HH20 to HH24). We have hybridized microarray using fluorescence-labeled cDNAs reverse transcribed from mRNAs isolated from anterior and posterior part of limb buds. We have found clones that present statistically different fluorescence intensities. By whole mount in situ hybridization we have tested if those genes that are differentially detected in the microarray are differentially expressed along the AP axis of the limb bud. The sequence of one of the clones analyzed corresponds to the VEGF-D, that had not been described in chicken before, and is expressed in the posterior part of the limb bud from HH18 to stage 25 and later on is localized in different domains: proximal, distal and posterior. *Sonic hedgehog* (Shh) gene, located in the posterior limb bud, and retinoic acid (RA) are able to induce limb duplication if misplaced in the anterior part limb bud. RA and Shh beads misplaced in the anterior part of the limb bud induced ectopic expression of VEGF-D in the anterior part of the limb bud suggesting that VEGF-D is downstream of RA and Shh. In order to elucidate VEGF-D function during limb development in situ hybridizations of VEGF receptors types 2 and 3 are being performed. As it has been shown recently in mouse embryos, VEGF-D seems to be very important in lymphangiogenesis.

A molecular study of the *dHAND* mutant limb

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dHAND is a bHLH transcription factor expressed in the posterior of the limb bud in a pattern similar to that of the *5'Hoxd* genes (Fernandez-Teran *et al.,* 2000; Charité *et al.,* 2000). We have shown that *dHAND* plays an important role in limb development and can activate the Shh pathway when misexpressed in the anterior of the bud (Fernandez-Teran *et al.,* 2000). In addition, *dHAND* null mice have rudimentary limb buds that do not express detectable *Shh* (Charite *et al.,* 2000). The Zebrafish *dHAND* mutant limbs also lack expression of the most 5' *Hoxd* genes (Yelon *et al.,* 2000). Taken together these results support the hypothesis that *dHAND* acts upstream of Shh in the process of anterior-posterior polarization of the limb bud. Furthermore, *dHAND* initiation and polarization of expression does not require Shh, since *Shh* null mice express restricted and polarized low levels of *dHAND* in the posterior limb bud.

To further dissect the relationship between *dHAND* and the Shh pathway, we have performed a molecular characterization of the *dHAND* mutant limb. Our results show that formation of the apical ectodermal ridge is impaired in the mutant limb. A weak *Fgf8* expression is only transiently detected in the distal limb ectoderm, while *Fgf4* is never expressed. The domain of *Gli3* expression, considered a negative regulator of *Shh*, expands along the whole anterior-posterior axis of the *dHAND* mutant bud indicating possible implication of *dHAND* in the anterior restriction of *Gli3*. We will discuss our findings in relation to the Shh pathway.

Another feature of the mutant limb is the apoptosis that occurs in the core of the limb bud at stage 10 dpc. In an attempt to overcome the hearth failure that causes death we have started the *in vitro* culture of mutant embryos, before overt apoptosis, trying to extend limb development. Our results with the cultured embryos will also be discussed.

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Medial and lateral PSM cells are differently commited to somite segmentation

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In the vertebrate embryo, segmentation is built on repetitive structures, along the A/P axis, the somites. These epithelial spheres are formed progressively from the rostral part of presomitic mesoderm (PSM) and they appear on both sides of the axial structures in a synchronous way, every 90 minutes in the avian embryo (Gossler and De Angelis, 1998). Several experiments have demonstrated that PSM has an intrinsic segmenting property, since its cells always incorporate a somite at a given time, independently of

environmental tissues or anterior/posterior orientation (Packard, 1978; Palmeirim et al., 1997, 1998). So far, this sequential pattern of segmentation had never been perturbed. A recent quail/chick fate map of a 6-somite-stage embryo determined that medial and lateral PSM cells have distinct prospective territories (data submitted) in agreement with a previous DiI fate map of stage 4 (HH) Hensen's node (Selleck and Stern, 1991). By using an *in vitro* culture system, we show, for the first time, that medial and lateral PSM cells are not equally committed to form somites. Medial PSM cells are unique in possessing the information for somite formation and molecular segmentation. In order to form somites, we suggest that medial PSM cells have to recruit lateral PSM cells.

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Synthetic loss of trunk and tail in *knypek* and *no tail* zebrafish double mutants

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The development of the vertebrate posterior body requires coordination of morphogenetic movements and differentiation of cell subpopulations within the tail bud, that take place soon after gastrulation. Convergent extension movements that narrow embryonic tissues mediolaterally and elongate them anteroposteriorly are thought to drive tail formation. It has been shown that the genes of T-box family play an important role during trunk and tail development. In zebrafish *no tail* (T-*brachyury* homologue) mutant embryos are deficient in notochord and tail is shorter than in wild-type embryos. It has been demonstrated that *Brachyury* in mouse and *Xenopus* is involved in the control of convergent extension movements. In zebrafish mutations in the *knypek* gene encoding heparan sulphate proteoglycan impair convergent extension movements of all tissues during gastrulation and segementation *Knypek* mutant embryos exhibit shortened anteroposterior axis although anteroposterior and dorsoventral patterning of the three germ layers are correct. In order to adress functional interactions between *no tail* and *knypek* genes in convergent extension and tail formation we constructed *knypek*;*no tail* (*kny:ntl*) double mutant lines. Development of the posterior body region is severely affected in *kny;ntl* double mutants. Double mutants fail to form the most posterior trunk and tail regions whereas the development of the head and the anterior trunk appear normal.Gene expression analyses of different markers during early somitogenesis reveal that mesoderm formation and patterning (except notocord defect due to *ntl* function) occur relatively normally in *kny;ntl* double mutant embryos until the onset of segmentation. However, at later developmental stages the expression of specific genes related to tail development is reduced or absent and tail rudiment extension does not take place in *kny:ntl* mutant embryos. These results show that *knypek* and *no tail* might act in parallel pathways regulating normal development of the posterior body.

Axial skeleton defects and *Hox* gene expression alterations reveal genetic interactions between the murine *Ring1A* and *M33* Polycomb genes

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The products of the Polycomb group (PcG) of genes act as transcriptional repressors involved in the maintenance of homeotic gene expression patterns during development (Gould, 1997). Initially identified in D. melanogaster, both molecular and genetic evidence show that the PcG system is conserved throghout evolution (Gould, 1997). In our study of the mammalian PcG system we cloned two genes which encode the Ring1A and Ring1B proteins, by means of their interaction with M33 (Schoorlemmer *et al.,* 1997), an ortholog of the D. melanogaster, yet. Mice lacking or overexpressing Ring1A showed homeotic transformations and other alterations of the axial skeleton (Del Mar Lorente *et al.,* 2000). Transformation of vertebral identities in Ring1A-deficient mice, however, are anterior instead of posterior which are the usual transformations seen in other PcG mutant mice (Van Der Lugt *et al.,* 1994; Akasaka *et al.,* 1996; Coré *et al.,* 1997; Takihara *et al.,* 1997). In addition, the penetrance and expressivity of phenotypes in Ring1A+/- mice indicate a singular dependency on Ring1A gene dosage. Previous evidence in both D. melanogaster and mice shows genetic interactions between PcG products (Bel *et al.,* 1998), which is consistent with their action as macromolecular complexes. Because of the unex-

pected phenotypes of Ring1A-deficient mice, we wished to study mice doubly deficient for the Ring1A and M33 genes. Here we report the analysis of axial skeleton of these mice, which shows stronger M33-related phenotypes, whereas Ring1A-specific alterations are almost lost. Analysis of Ring1A -/- ;M33 +/- and Ring1A +/-; M33 +/- mice showed the dosage dependence of such interaction. However, these skeletal alterations were not accompanied by shifts in the mesodermal expression domain of Hox genes, at least in 11.5 dpc embryos. Instead, we observed anteriorization of the rostral domains of expression of some Hox genes in the neuroectoderm. Taken together, these results provide evidence that genetic interactions between PcG genes, such as Ring1A and M33, is more complex than anticipated.

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Short term effects of heptanol on *Xenopus* embryos at blastula stage

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Different gap-junctional uncouplers have been reported to behave as teratogens and/or non-mutagenic carcinogens and the involvement of cell communication by gap junctions in embryogenesis is supported by increasing experimental evidence. Gap junctions can be efficiently and reversibly uncoupled by heptanol and octanol. In an attempt to characterize the effect of such chemicals on *Xenopus* development at different stages, *Xenopus* embryos of stages 4 to 20 were exposed to different mM concentrations of heptanol and octanol during 5 hours. Heptanol at 2.0 and 2.5 mM and octanol at 1.0 and 1.5 mM gave a high proportion of malformed embryos. These effects were temperature dependent. A complete account of these results will be published eelsewhere. Here we report short term effects of heptanol on cell communication and cell adhesion at blastula stages. Short time effects of heptanol on embryo structure were studied in blastulae kept in 2.5 mM heptanol from 30 min after fertilization onwards. At stage 8 (5 hours after fertilization), blastulae were fixed in 3,4% paraformaldehyde. In order to assess gap-junctional intercellular communication in control and in 2.5 mM heptanol-treated blastulae (from 30 min after fertilization onwards), 4.6 nl of a solution containing 5% LY and 1% RLD was injected to a dorsal animal blastomere at stages 4 (8-cell stage) and 5 (16-cell stage). As LY goes through gap junctions and RLD does not, this two-tracer method rules out false positives due to cytoplasmic bridges between blastomeres. In agreement with the results of Landesman *et al.* (2000). *J. Cell Biol.* 150: 929-936, we found only pairs of labelled blastomeres with colocalization of both dyes, indicating communication through cytoplasmic bridges. In contrast, in heptanol treated blastulae, only the injected blastomere was labelled, showing that no gap-junctional nor cytoplasmic-bridge communication was present. Furthermore, as it has been shown previously, using other uncoupling procedures, by Paul *et al.* (1995). *Development* 121: 371-381 and by Lee *et al.* (1987) *Cell* 51: 851-860, in this study, we report cell separation and cell delamination at blastula stages after few hours of heptanol treatment. This effect on cell adhesion - additionally to the gap-junctional uncoupling action - could account for some of the patterning perturbations reported.

Nuclei and microtubule asters stimulate MPF activation in *Xenopus* eggs

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In many amphibians, during the period of mitosis of early cell cycles, Surface Contraction Waves (SCWs) cross the egg from the animal to vegetal poles, contributing to the progressive concentration of germ plasm to the vegetal pole region. We showed previously that waves of MPF ("maturation/ M-phase promoting factor") activation and inactivation accompanies SCWs at first mitosis in *Xenopus* eggs (Pérez-Mongiovi *et al.,* 1998, *J.*

Cell Sci. 111: 385-393.). This spatio-temporal correlation suggest that the SCWs are cortical reorganisations provoked locally by MPF. In this work, we demonstrate a causal relationship between localised MPF activation and the SCWs by microinjection of human recombinant cyclin B-Cdc2 (MPF subunits) into different regions of the egg, to provoke ectopic reorganisation waves. We present evidence to show that the localised animal-half MPF activation can at least partially be explained by enhancement of MPF activation by the sperm-aster and the zygote nucleus, localised in this region of the egg. It was analysed the timing of MPF activation and/or crossing SCWs (as a reporter of MPF activation) in egg fragments with separated nuclei and centrosomes, in anucleate eggs microinjected with centrioles and in cycling egg cytoplasmic extracts. We conclude that nucleus and the sperm aster act independently but appear to cooperate to trigger MPF activation locally.

Function of *Pax1* and *Pax9* in sclerotome development

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Pax1 and *Pax9* transcription factors are expressed in the sclerotome of the somites and play a fundamental role in axial skeletal development. The analysis of mice deficient for *Pax1* and *Pax9* have shown that they act synergistically in the formation of ventral structures of the vertebral column. Furthermore, no direct target genes of *Pax1* or *Pax9* are known.

In search for their target genes we have analyzed the expression of several somite markers in *Pax1/Pax9* double mutant mice. We have found that the somitic expression of the homeobox gene *Nkx3.2* is lost in double mutant embryos. Other *Nkx3.2* positive domains are not affected by the lack of the *Pax1/Pax9* genes. On the other hand, the somitic expression of *Nkx3.1*, the most related paralog of *Nkx3.2*, is not affected by the absence of *Pax1/Pax9*. In order to investigate the regulatory relationship between *Nkx3.2* and *Pax1/Pax9*, we have performed transient transfections assays with *Pax1/Pax9* expression vectors and reporter vectors containing *Nkx3.2* putative regulatory regions. The results show that *Pax1* and *Pax9* are able to activate *Nkx3.2* promoter. Furthermore, by overexpressing *Pax1* or *Pax9* by retroviral infection in explants of chick presomitic mesoderm, we have observed that *Pax* genes are able to induce *Nkx3.2* expression, as well as chondrogenic differentiation.

The results higlight the importance of *Pax1/Pax9* in sclerotome differentiation and suggest that they are main mediators in *Shh* signalling for sclerotome development.

The molecular clock at the chicken organizer

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Somitogenesis is a very well time and space controlled process in which metameric structures are formed, every 90 minutes, in the Chick embryo. Somites appear progressively, in a rostral to caudal manner, as pairs of epithelial spheres that bud off on both sides of the axial structures, at the cranial end of the presomitic mesoderm (PSM). Concomitantly with epithelial somite formation at the anterior end of PSM, gastrulation is taking place in the most posterior part of the embryo, at the level of Hensen's node and primitive streak, causing progressive PSM and embryo elongation Subsequently, development allows somites to give rise to all the muscles of the body, except those of the head, as well as the dermis of the back and the axial skeleton (vertebrae, intervertebrae discs and ribs) (Reviewed by Christ and Ordahl, 1995; Gossler and Hräbe de Angelis, 1998). A previous study (Palmeirim *et al.,* 1997) presented evidence for the existence of a molecular clock underlying the process of chick somitogenesis by showing that PSM cells undergo several 90 minute cycles of *c-hairy1* gene expression until they incorporate a somite. Later, this same type of behaviour was also described for a gene coding for a closely related transcription factor, *c-hairy2* (Jouve *et al.,* 2000), and for another gene encoding a secreted protein, *lunatic-fringe* (Mc Grew *et al*, 1998; Aulehla and Johnson 1999). In the latter region the hybridised embryos showed

Since all these studies were performed at the level of PSM, we wondered whether this oscillatory behaviour would start before cells enter the PSM tissue, i.e. in their prospective territories. We therefore performed a detailed whole-mount and cross-section analysis of the expression patterns of cycling genes (*c-hairy1*, *c-hairy2* and *lunatic fringe*), at the level of *sinus rhomboidalis*. Our results report very different patterns of expression which demonstrate that cycles of expression of *c-hairy1, c-hairy2* and *lunatic fringe* genes are already occurring in cells of the somitic prospective territory. At this level, a "wave" of expression of the cycling genes spreads along the longitudinal axis of the whole somitic prospective territory, which corresponds to the future medial/lateral somitic axis. Furthermore, by using the quail-chick chimera technique we demonstrate that medial PSM cells arise from a region located just behind Hensen's node and that medial and lateral PSM cells have distinct prospective territories. This expression pattern analysis correlated with the quail/chick fate-map presents evidence for a molecular clock underlying somitogenesis already operating in somitic precursor cells.

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Analysis of promoter and cis-regulatory elements of Pax9

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Pax9 is expressed during mouse embryogenesis in the sclerotomes, in the limb buds, in the facial mesenchyme and in the pharyngeal pouch endoderm and it is involved in the normal development of the deriving organs. In order to clarify the molecular mechanisms regulating Pax9 expression we are analyzing the promoter and cis-regulatory elements of Pax9 by two parallel approaches: BAC transgenesis and comparative genome sequencing. We isolated 10 Pax9 genomic clones from a mouse BAC library, each of which has an insert of about 200 kb, and successfully modified two of them with ET cloning by introducing a LacZ reporter. We are now in process of generating transgenic mouse lines with these BAC clones. The analysis of the LacZ staining pattern in the transgenic mice and rescue experiments conducted by crossing the transgenic lines to the existing Pax9 knock-out mice will allow us to identify the genomic region that contains all the elements required for the Pax9 embryonic expression. In parallel we carried out a sequence comparison among the human, mouse and pufferfish sequences. This allowed us at a first instance to describe the structure of the gene and to observe the conserved genomic organization in relation to the neighboring genes raising interesting theories about the maintenance of the sinteny during evolution. We were also able to identify several conserved non-coding sequences. These are individually tested for regulatory functions in a conventional transgenic approach using again the LacZ gene as a reporter. Moreover a luciferase assay is carried out on Pax9 expressing cell lines using genomic fragments upstream of the transcription start points in order to identify the promoter region(s).

Finally we are conducting the analysis of a newly identified gene lying in the vicinity of Pax9 as a potential antisense gene that could contribute to regulate Pax9 expression.

Study of *Colloid-like1* gene expression and function during somite differentiation

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The chicken orthologue of the tolloid-related metalloproteases, Tld-l1, in humans (Scott *et al.,* 1999); mTld-l1, in mouse (Takahara *et al.,* 1996) and Tld/Mini fin, in zebrafish (Blader *et al.,* 1997) was identified and named Colloid-like1 (Liaubet *et al.,* 2000). This metalloprotease, of the astacin family, acts by specifically cleaving Chordin (Piccolo *et al.,* 1996) therefore establishing the graded activity of BMPs, genes involved in the determination of the vertebrate embryonic dorsoventral axis.

In the vertebrate embryo, somites constitute the basis of the segmental pattern of the body and give rise to all striated muscles of the body, except those of the head. During differentiation, somites become polarised according to a mediolateral axis. BMP4 is produced by the lateral plate mesoderm and has been proposed to play an important role in lateral somite specification (Pourquié *et al.,* 1996). Determination of the dorsomedial mesodermal lineages requires the inhibition of BMP4 activity by proteins such as Noggin, Chordin or Follistatin. We analysed *colloid-like1* gene pattern of expression in detail, during somitogenesis. In embryos, until st.10HH this gene is broadly expressed in all formed somites and in the lateral plate flanking the most rostral part of the PSM and the epithelial somites. As development proceeds and somites differentiate, *colloid-like1* gene expression becomes restricted to the dorsomedial part of the 8-10 most anterior somites. Furthermore, we compared *colloid-like1* gene expression with that of *c-chordin*, *c-noggin* and *BMP4*. Surprisingly, *c-chordin* and *colloid-like1* are never co-expressed in somites as they are in the notochord. However, *c-noggin* and *BMP4* transcripts co-localise with those of *colloid-like1* gene in the medial part of somites. By using a tridimensional embryo tissue culture and *in ovo* operations we determined the role of surface ectoderm and neural tube, notochord and lateral plate in the induction and maintenance of colloid expression. Preliminary results implicate the neural tube in the maintenance of colloid-like1 gene expression.

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