Instituto Juan March
de Estudios e Investigaciones

97 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

NICHD NATIONAL INSTITUTE OF CHILD HEALTH
AND HUMAN DEVELOPMENT

Workshop on

Molecular Nature of the Gastrula Organizing Center:

75 years after Spemann and Mangold

Organized by

E. M. De Robertis and J. Aréchaga

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Introduction

Eddy M. De Robertis

Seventy-five years ago, in 1924, Hans Spemann and Hilde Mangold performed one of the best known experiments in embryology. They transplanted a small region of the amphibian gastrula embryo, the dorsal lip of the blastopore, into the opposite (ventral) side of a host embryo. They found that the graft, or "organizer", was able to induce a Siamese twin. The twinned axis was largely recruited from neighboring cells, indicating that vertebrate development takes place through a series of cell-cell inductions. Spemann's organizer had three activities:

1) It was able to induce central nervous system (CNS) on the overlying ectoderm.

2) It dorsalized the mesoderm, forming somite and muscle at the expense of neighboring cells that would normally form mesenchyme and blood.

3) It induced a secondary gut with all its endodermal derivatives such as liver and pancreas.

This experiment had an enormous impact on the biological thinking of the twentieth century. The organizer graft had to release substances responsible for the induction and patterning of the ecto-, meso- and endoderm, the three germ layers of the embryo. Isolating these inductive substances became the Holy Grail for experimental embryologists.

With the advent of molecular cloning, it became possible for researchers to isolate genes specifically expressed in Spemann's organizer and to study their properties. A number of genes were shown to function as transcription factors in the organizer, such as goosecoid, Lim-1, Xnot, Otx2, Siamois, Xanf-1, and HNF3-b. The function of these homeobox genes is to control the expression of secreted factors that in turn pattern the embryo. These secreted proteins include Noggin, Follistatin, Chordin, Frzb-1, ADMP, Dkk, Nodal-related, Cerberus and others.

Whereas some of this work started in Xenopus, mutagenesis screens in zebrafish identified mutations in organizer-specific genes such as Xnot (floating head) and chordin (chordino). In the mouse the homologues of most of the aforementioned genes have been inactivated by targeted gene mutation. In chick grafting experiments and implantation of beads expressing secreted factors have led to a renaissance of the study of gastrulation. Thus in 1999 the time was ripe to bring together a group of leaders in the field of vertebrate development to discuss recent progress, to integrate the information from diverse experimental animal models, and to discuss the questions that remained for future pursuit.

The workshop was not a celebration of the landmark Spemann experiment from a historical point of view but rather a discussion of the latest findings on the field. One of the unexpected results has been that many of the secreted molecules that pattern the embryo are themselves inhibitors of well-known growth factors, such as BMPs, Nodal-related genes and Wnts. These secreted antagonists act in the extracellular space and their activity can be controlled by proteolysis.

With so many components recently discovered, it was no surprise that the workshop did not provide final answers concerning the molecular nature of the gastrula organizer.

Some aspects, however, seem to be well settled. For example, the recent genetic screens in zebrafish show that BMP signals act at late gastrula to promote ventral development in the three germ layers. Studies in Xenopus have shown that the earliest signals are provided by beta-catenin, which promotes dorsal development already at early cleavage stages. During the intervening period - spanning from early cleavage to late gastrula - a plethora of molecules come into play and their exact functional relationships are being unraveled. Mouse knockouts revealed the unique and redundant function of the various genes, suggesting that we are only at the beginning of the dissection of the complexities of embryonic patterning. Studies in chick embryos pointed to the importance of cell movements and morphogenesis, an aspect that deserves much more attention than has been given to date. Studies in the diverse model systems provided a great synergy in understanding the common mechanisms of vertebrate gastrulation.

The workshop covered the latest aspects of the molecular biology of early patterning in vertebrates. The advances in the past few years have been astounding. New molecules and regulatory principles have been discovered. Some of the novel secreted factors will no doubt be useful to induce cell differentiation in vitro and may find applications in tissue replacement therapy. The gastrula organizer controls pattern formation of the vertebrate body plan and therefore, we can expect that Spemann's organizer will keep yielding new secrets well into its hundredth anniversary.

E. M. De Robertis

Session 1: Forming Spemann's organizer

Chair: Juan Aréchaga

Historical and evolutionary perspective on the organizer

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I. Steps of formation of the organizer, as revealed by "classical" intervention experiments by others and us.

A. Oogenesis: organizing the vegetal hemisphere
   UV of vegetal pole of oocyte, ventralized embryo (Wylie, Elinson).

B. First cell cycle: cortical rotation
   UV, cold, pressure, nocodazole to destroy microtubules. No cortical rotation, ventralized embryo. Role of rotation to align microtubules along which materials move. (Ancel and Vintemberger, Elinson, Scharf, Vincent, Rowning)
   Rescue UV'd eggs by tipping or centrifugation. Double forced rotations: twins. (Penners, Schleip, Morgan, Pasteels, Scharf, Black)
   Heavy water: random microtubules, no cortical rotation, but hyperdorsal embryos. (Scharf)
   The Curtis experiment-H. Kageura's results.
   Lithium treatment. (Kao, Elinson)

C. Blastula period: meso-endoderm induction.
   The Nieuwkoop-Boterenbrood experiment.
   Blastomere transplants into UV'd hosts for rescue of axis formation. What does this say? (Gimlich, Kageura)
   What is the minimal Nieuwkoop center?
   When does induction occur? How much is maternal, how much is zygotic? (Wylie, Heasman)

D. Late blastula: what is the late blastula organizer?
   Late grafts of ectoderm from UV'd embryo, to get notochord. How late can chordamesoderm be induced? (Stewart, Domingo, Keller).
   XFD embryos (Amaya)

E. Early gastrula: Spemann's organizer
   The Spemann-Mangold experiment, the head organizer, the trunk-tail organizer.
   Diminished organizers: why is the head not formed? (Stewart)

II. Evolution of the organizer:

A. The organizer as a chordate characteristic. Less familiar examples from sturgeon, lamprey, amphioxus, ascidian.

B. The organizer's role in the development of the 4 defining chordate anatomical traits: notochord, gill slits (branchial arches), post-anal tail, dorsal hollow nerve cord.

C. What does the organizer add to endo-mesoderm induction? The connection to extensive morphogenesis during gastrulation.

D. Is it the trunk-tail organizer that is unique to chordates? A new kind of signaling center. Convergent extension and Shh

E. Does the hemichordate embryo have a head organizer but not a trunk-tail organizer? What does the echinoderm embryo have?

A post-fertilization asymmetry in beta catenin accumulation regulates dorsal axis formation in Xenopus

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Based on our initial observation that ectopic stimulation of a Wnt signaling pathway elicits formation of a complete embryonic axis in Xenopus embryos (1), we have sought to test the hypothesis that a Wnt signaling pathway is normally involved in the specification of the endogenous dorsal-ventral axis. In this presentation we will review evidence that this hypothesis has now been confirmed, by focusing on data establishing the following points:

1. Endogenous ß-catenin accumulates preferentially on the prospective dorsal side prior to the first cleavage, in a manner dependent upon cortical rotation (2). Ectopic Wnt signals, but not Vg1 or noggin, elevate ß-catenin levels on the prospective ventral side, consistent with the possibility that endogenous Wnt signaling pathway(s) are involved in regulating the accumulation of endogenous ß-catenin. In both zebrafish and Xenopus, ectopic ß-catenin is sufficient to stimulate ectopic axis formation, pointing to its regulatory capacity. Importantly, work from Heasman, Wiley and colleagues establish that ß-catenin is required for axis specification. Thus ß-catenin is necessary, sufficient, and expressed in the right place and time to mediate axis specification.

2. We have conducted experiments to test the involvement of upstream components of the Wnt signaling pathway(s) in regulating the dorsal accumulation of ß-catenin, and will provide evidence that Dishevelled is indeed an important regulator of ß-catenin accumulation (3). We find that endogenous Dishevelled is enriched in dorsal regions relative to ventral regions of cleavage stage embryos. This asymmetry is dependent upon cortical rotation, as is the accumulation of ß-catenin. Both endogenous and ectopic GFP-tagged Dsh exist as small particles. Importantly, Dsh-GFP accumulates in the vegetal hemisphere, and is transported upon microtubule arrays after fertilization to the prospective dorsal side of the embryo. Thus, there is likely a central role for microtubules in promoting dorsal accumulation of a dorsal-determining factor, Dsh. On the dorsal side, Dsh interacts with a complex containing Axin, likely resulting in the downregulation of GSK-3 activity. Since GSK-3 had been promoting the degradation of ß-catenin, its inhibition results in the stabilization and accumulation of ß-catenin (4).

3. Dorsal ß-catenin then regulates the expression of the homeobox gene *siamois*, which in turn is required for specification of the Spemann Organizer (5). In the absence of ß-catenin, *siamois* is negatively regulated by complexes of TCF and C-terminal binding protein (CTBP) (6).

4. Specification of the dorsal-ventral axis also involves ventral signals that antagonize dorsalizing signals (7). We have found that vertebrate Wnts signal through two distinct and antagonistic Frizzled signaling pathways. The Wnt/ß-catenin pathway is well-known, while the details of the Wnt/Ca++ pathway are only now becoming elucidated. One of the targets of the Wnt/Ca++ pathway is calcium/calmodulin-dependent protein kinase II (CamKII). Our data suggest that this pathway is activated by Wnt-11 and Wnt-5a in the early embryo, and CamKII promotes ventral cell fate, and antagonizes dorsal cell fates (7).

In summary, the data will summarize how multiple Wnt signaling pathways actively promote both dorsal and ventral cell fates during the cleavage stages of Xenopus, thereby regulating target genes that specify the Spemann Organizer.

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Molecular interactions that specify and maintain Spemann's organizer in the chick

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ABSTRACT

Spemann's organizer is a unique region in the gastrulating embryo that can induce a complete embryonic axis when transplanted to another site, and is also defined by the axial fate of its cells and by its characteristic expression of a number of organizer-specific genes. Numerous studies in amphibian embryos have provided strong evidence that the organizer is specified by signals emanating from a neighbouring regon, the Nieuwkoop centre. Specifically, two signalling pathways have been implicated in this process: Vg1/activin and Wnt. It is generally believed that the activity of the Nieuwkoop centre is complete before the start of gastrulation, once the organizer has been set up.

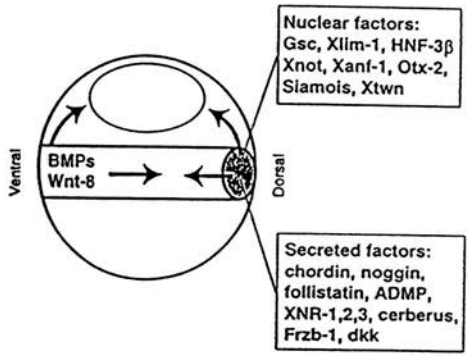
First, we will present evidence that in pre-primitive streak stage chick embryos, the posterior marginal zone is functionally homologous to the Nieuwkoop centre. As in amphibians, its activity appears to rely on Vg1 and Wnt signals but the source of these signals is determined zygotically, not maternally.

Remarkably, chick embryos at the mid- to late gastrula stage deprived of organizer cells can regenerate a fully functional organizer, and subsequently develop normally. We show that feedback mechanisms exist to sense the presence of Hensen's node, the chick organizer, involving an interplay of inducers and inhibitors secreted by cells in and around the node. The primitive streak secretes Vg1 and Wnt signals, which cooperatively induce organizer properties when the node is absent. The periphery secretes BMPs, which restrict the response to the center of the embryo. The node itself secretes ADMP, which represses induction. This mechanism resembles the interactions that position the primitive streak and organizer before gastrulation, even though the cells of the posterior marginal zone do not contribute to the primitive streak or to any other part of the embryo.

What could be the normal function of such a phenomenon? During gastrulation, some cells continuously enter and leave the organizer territory and appear to possess organizer properties only while located in the node region. We propose that "regeneration" of the node is an extension of the normal processes that convey positional information to these moving cells.

Patterning by secreted inhibitors in mouse and frog embryos
E. M. De Robertis, HHMI, UCLA

A large number of genes that function in specific parts of the Xenopus gastrula have been isolated by several laboratories. Many of these genes are regulatory transcription factors that in turn control the expression of secreted factors. Starting as a search for downstream targets of a homeobox gene, *Goosecoid*, the work to be described has contributed to the realization that cell-cell signalling in the vertebrate gastrula embryo is regulated by novel secreted inhibitors that bind to and inactivate growth factors in the extracellular space. The salient discovery was the identification of a secreted protein, Chordin, that binds to and antagonizes BMPs. Chordin is considered a major player in dorso-ventral (D-V) patterning of the ectodermal and of the mesodermal germ layers. In double knockout mice, removal of the Chd and Noggin genes leads to drastic reductions of the prosencephalon and of mesodermal midline structures. Another gene identified, *Frzb-1*, was the founder of a large family of secreted inhibitors of Wnt signalling. Finally, Cerberus was found to be a multivalent inhibitor of growth factors (Nodal, BMP-4 and Xwnt-8) which are involved in trunk development. *Cerberus* mRNA is expressed in anterior endoderm and has the property of inducing ectopic head structures in microinjected *Xenopus* embryos. A construct consisting of only the COOH-terminal cystine knot of Cerberus is a specific inhibitor of Nodal-related signaling and supports a central role for Nodal in mesoderm induction.



In addition to functioning as secreted inhibitors, these novel molecules may be subjected to a second layer of regulation. In the case of Chordin, inactive Chordin/BMP complexes are specifically cleaved by the Xolloid extracellular protease, releasing active BMP. Together with the work of others in *Drosophila*, these findings have led to the concept that dorsoventral patterning is controlled by proteolysis. The conservation of molecular mechanisms in arthropods and vertebrates suggests that the machinery for D-V specification was present in the urbilaterian common ancestor of these two lineages early in animal evolution. Chordin encodes a large protein containing four cysteine-rich (CR) domains of about 70 amino acids. We have recently found that the BMP binding activity resides in these individual modules. Xolloid cleaves Chordin close to or within the CR1 and CR3, which have the maximal BMP binding activity. Thus, the Xolloid

metalloprotease may release BMP by cleaving the active sites of Chordin. Other extracellular proteins also contain CR domains, and we and others have recently found that the CR repeat in the NH_2 propeptide of procollagen II, the main cartilage collagen, is able to bind BMPs as well. This is of interest because it indicates that extracellular growth factor binding CR modules, and perhaps specific proteases as well, may participate in tissue homeostasis.

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Session 2: Molecular composition of the organizer

Chair: Benjamin Lewin

Regulatory interactions in the organizer: The *Xlim-1* gene

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The elucidation of molecular mechanisms in organizer function have been approached by the analysis of regulatory genes that are expressed in this region during gastrulation. The *Xlim-1* gene is one of the transcription factor-encoding genes known to be expressed in the organizer just before and during gastrulation in Xenopus, and orthologous genes have been found in all vertebrates studied. Here we summarize several aspects of the function and regulation of this gene in the early embryo.

Xlim-1 expression is initiated shortly after the midblastula transition (MBT) in the dorsal region of the marginal zone, and then becomes localized to the organizer (1). In other vertebrates, a somewhat broader early pattern is seen, such as expression in the entire margin of the zebrafish (2) and initial expression in the anterior visceral endoderm in the mouse (see 3, 4), before concentration in the organizer is achieved. During gastrulation, *Xlim-1* is expressed in the entire involuting axial mesoderm.

Functional studies in animal caps showed that Xlim-1 requires either an activating mutation (5) or the cooperation of a cofactor named Ldb or NLI (6) to exert a biological effect. The biological effect detected is the activation of other organizer genes such as *goosecoid* (*gsc*) and *chordin*, and the ability to convert animal tissue into organizer-like tissue that can carry out neural induction (5). Direct evidence for the requirement of the *Lim-1* (or *Lhx-1*) gene in head formation was obtained through the study of a targeted mutation in the mouse (7). Using the method involving chimeric proteins with the Engrailed repressor domain (8) we have obtained evidence for the requirement of *Xlim-1* for head formation in the frog.

The activation of *gsc* expression by Xlim-1 is likely to be direct. The *gsc* promoter can be activated in animal caps by expression of activated Xlim-1, and most effectively by the combination of wild type Xlim-1 in combination with its cofactor Ldb and Otx2. Gsc protein itself, and PV.1/Vent.1 repress *gsc* transcription in this system. These observations, and the expression patterns of *Xlim-1* and *otx2*, can explain the maintenance of *gsc* expression in the prechordal plate during gastrulation.

Xlim-1 expression can be induced by activin or similar growth factors such as activated Vg1 and nodal-related factors. We found that the activin response element (ARE) in *Xlim-1* is located in the first intron and acts as an activin-sensitive silencer (9). This behavior is quite different from regulation in the *gsc* or *Mix.2* genes where the ARE acts as a standard enhancer (10, 11). Nevertheless, recent experiments show that the *Xlim-1* ARE contains similar sites to the *Mix.2*

ARE (11), such as Smad4 and FAST.1 binding sites, and that FAST.1 is required for the transcriptional activation of *Xlim-1* by activin.

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Organizing the avian embryo by means of homeobox genes

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Ectodermal patterning of the chick begins in the intrauterine embryo and continues during gastrulation, when cells around the anterior primitive streak adopt a neural fate, and cells in the periphery become fated to the epidermis. We applied the homeobox genes DLX5 as a marker for epidermal ectoderm, and GANF (Hesx1) for anterior neuroectoderm.

We demonstrate that the development of a neural plate is not only dependent on signaling from the midline, i.e. Hensen's node, the young head process, the prechordal plate, and the neural plate itself. In addition, it involves signals from the lower, nascent meso- and endodermal layers. The establishment of an epidermal territory, on the other hand, is not only dependent on secreted factors in the periphery (BMP2 and BMP4), but paradoxically also on signaling from the developing neural plate.

Previous findings in mice suggested that anterior neural induction and/or patterning could occur from the anterior primitive endoderm (hypoblast). We demonstrated by transplantation of anterior primitive endoderm from pre-streak rabbit embryos to cultured chick embryos that signals for neural induction, concomitant with GANF induction, are present in this mammalian tissue. In contrast, such activities were not detected in primitive endoderm (hypoblast) from chick embryos. Here, the signals were found in the node and its early derivatives. Thus, the head inducing capacity appears to be shifted from the node to the extraembryonic, primitive endoderm during the evolution of mammalia. Such a heterochronic shift could only be possible, if a head and a trunk organizer existed as separate entities. Our data would predict that the nodes of birds and mammals are non-homologous structures.

Functional redundancy and specificity between OTX1 and OTX2 gene products

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Murine *Otx1* and *Otx2* gene products share extensive sequence similarities even though in *Otx1*, downstream the homeodomain, these regions of homology to OTX2 are separated by stretches of additional aminoacids including repetitions of alanine and histidine residues. In mouse, *Otx1* expression is first detected at the 1-3 somite stage throughout the fore- and midbrain neuroepithelium. *Otx2* is already transcribed before the onset of gastrulation in the epiblast and in the visceral endoderm (VE), and at the end of gastrulation in the axial mesendoderm and rostral rostral neural plate. During brain regionalization, *Otx1* and *Otx2* show largely overlapping expression domains with a posterior border coincident with the mesencephalic side of the isthmic constriction. Furthermore, *Otx1* is transcribed in neurons of deep layers of the adult cerebral cortex. *Otx1* null mice show spontaneous epileptic seizures and multiple abnormalities affecting proper brain and sense organs development as well as pituitary functions. *Otx2* null mice die early in embryogenesis, show heavy gastrulation abnormalities affecting VE, primitive streak and axial mesendoderm, and fail to specify rostral neuroectoderm fated to give forebrain, midbrain and rostral hindbrain.
In order to determine whether these contrasting phenotypes reflect differences in temporal expression or biochemical activity of *Otx1* and *Otx2* gene product we generated two mouse models replacing *Otx1* with *Otx2* and vice versa.
-Homozygous mutant mice replacing *Otx1* with *Otx2* ($Otx2^{1}/Otx2^{1}$) fully rescued epilepsy and corticogenesis abnormalities and showed a significant improvement of mesencephalon, cerebellum, eye and lachrymal gland abnormalities. In contrast, the lateral semicircular duct of the inner ear was never recovered, strongly supporting an *Otx1*-specific requirement for the specification of this structure.
- Homozygous mutant mice replacing *Otx2* with *Otx1* recovered anterior neural plate and proper gastrulation but failed to maintain fore-midbrain identities, displaying a headless phenotype from 9 days post coitum (d.p.c.) onwards. A more deep analysis revealed that, unexpectedly, in spite of the RNA distribution in both visceral endoderm (VE) and epiblast, the hOTX1 protein was synthesized only in the VE. This VE-restricted translation was sufficient to recover *Otx2* requirements for specification of the anterior neural plate and proper organization of the primitive streak, but failed in maintenance of anterior patterning.
Altogether these data indicate an extended functional homology between OTX1 and OTX2 gene products and provide evidence that, *Otx1* and *Otx2* null mice contrasting phenotypes stem from differences in expression patterns rather than in aminoacid sequences.
Moreover, our data lead us to hypothesize that the differential post-transcriptional control highlighted existing between VE and epiblast cells in $Otx1^{2}/Otx1^{2}$ embryos may potentially contribute to fundamental regulatory mechanisms required for head specification.

Signals from the Yolk Cell induce Notochord, Neuroectoderm and the Trunk-Organizer in the Zebrafish Embryo

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We have analyzed the role of the yolk cell in establishing the organizer in the early zebrafish embryo. Ablating the vegetal pole prior to first cleavage results in completely ventralized embryos which lack an embryonic shield, a notochord and all neuroectoderm. These embryos also fail to form the anterior-most 14-15 somites, demonstrating that components at the vegetal pole of the zebrafish zygote are essential for the formation of a trunk-, but not a tail-organizer.

We have previously shown that the dorsalized mutant *swirl* encodes the zebrafish homologue of the BMP-2 gene (Kishimoto et al., 1998), while the ventralized mutant *chordino* lacks the BMP-antagonist Chordin (Schulte-Merker et al., 1997). Mutant *swirl* embryos exhibit an enlarged notochord precursor and expanded somites at the expense of ventral tissues such as blood and nephros, which are completely missing. In contrast to phenotypes obtained upon ablating the vegetal pole of wildtype embryos, removal of the vegetal yolk in mutant *swirl* embryos results in embryos which do form neuroectoderm and anterior trunk somites. However, as in wildtype embryos, experimental *swirl* mutant embryos also lack a notochord.

These ablation experiments in wildtype and *swirl* mutant embryos demonstrate that in the zebrafish embryo dorsal determining factors originate from the vegetal part of the yolk cell. These factors set up two independent activities: one induces the dorsal-most mesoderm (the notochord), the other one is involved in the formation of the neuroectoderm and the trunk-region by counteracting the function of *swirl/BMP-2*.

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Title: BMP antagonists in vertebrate development

The Xenopus egg become a complex embryo due to a cascade of signaling mechanisms. Early signals from the wnt pathway sensitize the dorsal side of the embryo to organizer induction and neural induction. Subsequent signals from the organizer act on both mesoderm and ectoderm to induce progressively finer pattern. In the neurula, reciprocal signaling between mesoderm and neural plate continues to elaborate and stabilize the organization of the embryo. Many of the signals are inhibitory, suppressing the activity of signal transduction pathways. Signaling by Bone Morphogenetic Proteins (BMPs) is suppressed by a surprisingly diverse set of antagonists. Selected examples of these diverse inhibitory mechanisms will be discussed.

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Session 3: Genetic Approaches

Chair: Igor Dawid

Localisation of bicoid RNA, that organises anterior pattern in the Drosophila Embryo

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The formation of anterior pattern in the Drosophila embryo is dependent on a gradient of Bicoid protein (Bcd), that spreads from an anteriorly localized mRNA source. The products of three known maternal genes, exuperantia (exu), swallow (swa) and staufen (stau), are required for the localization and anchoring of bcd RNA at the anterior pole during oogenesis. In the absence of Exu or Swa, bcdRNA is not localised at the anterior of the growing oocyte. Electron microscopy revealed that Exu protein is present in the nurse cell cytoplasm in special particles, sponge bodies, that are transported into the growing oocyte. Swa protein colocalizes with bcd RNA at the anterior pole of the oocyte in stage 10 ovaries. We identified the cytoplasmic dynein light chain 1 (Ddlc1) as a strong and specific Swa binding protein with the two hybrid system and also in in vitro studies. The dynein protein complex is a molecular motor transporting cargo to the minus ends of microtubules. In the freshly laid egg, bcdRNA is tightly localized at the anterior pole and translation begins, resulting in the formation of a gradient of Bcd protein that controls transcription of target genes in a concentration dependent manner. Stau, a dsRNA binding protein, associates with the 3`UTR of bcdRNA to form particles that are transported along microtubules. In this process, the three-dimensional structure of the localisation domain of the bcdmRNA plays an important role, suggesting that dimerisation or oligomerisation is a prerequisite for the formation of RNA-protein localisation particles.

Genetic analysis of BMP signaling in dorsoventral patterning of the zebrafish embryo.

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We previously identified 6 genes with dorsalized mutant phenotypes in the zebrafish.[1] At least 4 of these genes function as key players within a bone morphogenetic protein (BMP) signaling pathway to pattern ventral regions of the embryo. Mutant embryos of *swirl*, *somitabun*, and *snailhouse*, which produce the strongest dorsalized phenotypes in our series, can be rescued by overexpression of several components in a BMP signaling pathway. We, and others, have shown that *swirl* is a mutation in the zebrafish *bmp2b* gene.[2,3] We investigated the cell autonomous nature of *snailhouse* to assess whether it acts in the generation of a signaling molecule or as a receptor or intracellular factor. Our results indicate that *snailhouse* functions cell non-autonomously, thus implicating it as a BMP ligand or factor that generates the ligand. Molecular-genetic mapping, cloning, and linkage analysis of *snailhouse* to several Bmp ligand genes revealed that it is a mutation in a different Bmp ligand subclass to that of *swirl/bmp2b*.

Based on alterations in gene expression in mutant gastrula of *swirl*, *somitabun*, and *snailhouse*, we have suggested that graded BMP activity induces differential gene expression along the dorsal-ventral axis, leading to the specification of different cell types along this axis.[2] In particular, we hypothesize that low BMP activity specifies the laterally-derived neural crest cell fate. We tested this hypothesis by modulating BMP signaling levels in wild type and mutant embryos. By suppressing BMP signaling in wild type embryos, we expanded the presumptive neural crest, while in mutants lacking neural crest progenitors, we induced neural crest by providing low BMP signaling activity. Thus, the results strongly support our hypothesis that low BMP activity levels specify the neural crest cell fate.

In dorsoventral axis formation, BMP ligand activity is modulated extracellularly by BMP antagonists. The activity of the BMP antagonist Chordin is negatively regulated through proteolytic cleavage by the Tolloid metalloprotease.[4,5] We overexpressed *tolloid* in our dorsalized mutant embryos and found that it could only rescue the phenotype of *mini fin* mutant embryos. Linkage analysis, molecular cloning and DNA sequence analysis demonstrate that *mini fin* is a mutation in the *tolloid* gene. We found that *mini fin* establishes the most ventral cell types of the tail: the ventral fin, somites, and vasculature. Based on gene expression studies, we propose a model whereby Mini fin/Tolloid is required at the end of gastrulation and within the tail bud to inhibit Chordin function, thus generating high BMP activity levels, which specify ventral tail cell fates.

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The mouse gastrula organizer: Cell fate and patterning activity

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Several genes that are expressed by cells in the gastrula organizer of the zebrafish, frog and avian embryos are also expressed in the epiblast of the mouse early gastrula. Although the transcripts of these organizer-associated genes are localised to different parts of the epiblast, their expression domains overlap in a group of cells in the posterior epiblast that is positioned between the progenitors of the extraembryonic and embryonic mesoderm. Fate-mapping and cell tracking studies have revealed that the descendants of these cells contribute to the mesendoderm (head process and notochord) and the ectoderm (floor plate) of the midline and to the node. This population can also induce a secondary body axis following ectopic transplantation. All the experimental evidence therefore points to the presence of an early gastrula organizer (EGO) in the mouse embryo well before the formation of the node in the late gastrula. Similar to the outcome of node transplantation, the new axis induced by the EGO does not display any molecular or structural features that are characteristic of the neural tube rostral to the hindbrain. However, the expression of anterior neural markers can be induced by combining the EGO with the anterior germ layer tissues (visceral endoderm and epiblast) in the ectopic transplantation, though not by any other combinations of these tissues. This may suggest that the full axis organizing activity is generated by an interaction of the EGO and its derivatives, and the tissues that are reputed to possess anterior patterning signals. The functional role of the EGO derivatives in the late gastrula is tested by selective tissue ablation. The midline tissues associated with the head folds are found to be required for the morphogenesis of the forebrain during neurulation. Post-gastrulation morphogenesis of the neural axis is, however, independent of the continuous activity of the node or the notochord. This may suggest that the essential information for anterior-posterior patterning of the body axis is in place when gastrulation is concluded.

Synergistic Regulation of Mesoderm Formation in Zebrafish

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Genetic studies of zebrafish and mouse demonstrate that the formation of different antero-posterior regions within the paraxial mesoderm depends upon the functions of different T-box transcription factors. In zebrafish, trunk mesoderm requires *spadetail (spt)* function, whereas tail mesoderm depends upon *no tail (ntl)*. Several lines of evidence demonstrate that these restricted mutant phenotypes result from complex regulatory interactions rather than simple differential gene expression. For example, *ntl* interacts genetically with the *one-eyed pinhead (oep)* mutant demonstrating that the *oep* gene product and *ntl* are functionally redundant in trunk paraxial mesoderm formation. We show that this genetic interaction is accounted for by the regulation of *spt* expression, since *spt* expression is not maintained in *oep;ntl* mutants.

To address whether *spt* might perform an analogous, conditional function in tail mesoderm, we constructed the *oep;spt* double mutant. *Oep;spt* mutant embryos are totally deficient in muscle in the trunk and the tail, and also lack posterior notochord. This interaction occurs downstream of *ntl*, since *ntl* expression is not affected; we are currently addressing whether it involves *tbx6*, a third mesodermally-expressed T-box transcription factor, likely to play an important role in tail paraxial mesoderm. Since *spt* and *ntl* are both regulated, in part, by FGF and circumstantial evidence implicated *oep*-related proteins in the regulation of FGFR activity, we asked whether *oep* might interact with the FGF pathway in zebrafish mesoderm. Consistent with this, *oep* dramatically enhances the mild mesodermal defects found in *ace/FGF8* mutant embryos; *ace;oep* embryos lack muscle entirely whereas both single mutants have well-formed trunk and tail mesoderm. However, experiments using a specific inhibitor of the FGFR signaling indicate that *oep* does not interact with the FGF pathway directly, but rather acts in a distinct parallel pathway that synergises with FGF at the level of downstream targets such as *spt*. This is in accord with the recent demonstration that oep is an essential co-factor for signaling by *nodal*-related *TGFßs*. Thus, formation of trunk and tail mesoderm in zebrafish depends upon synergistic signaling by nodals and multiple members of the FGF family. Furthermore, since *ntl* and *spt* are likely to be upstream regulators of FGF expression, this mechanism explains the dramatic genetic interactions found in *oep;ntl* and *oep;spt* mutants.

The role of the node in early patterning of the mouse embryo. J. Rossant, J. Klingensmith, D. Dufort, J.Pearce, J. Partanen, S-L Ang, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, CANADA

The establishment of the basic body plan of the vertebrate embryo begins at gastrulation. It involves generation of the three germ layers, ectoderm, endoderm and mesoderm, and the elaboration of the three major body axes- anterior-posterior (A-P), dorsal-ventral (D-V) and left-right (L-R). Much of our current understanding of the mechanisms of establishing the body axes is based on the concept of the 'organizer', first proposed by Spemann and Mangold in the 1920s. They showed that a small piece of tissue from the dorsal lip of the blastopore in the amphibian gastrula could induce a whole new body axis, when transplanted ventrally. Transplant studies have identified a region of the embryo with similar properties in Zebrafish, the chick and the mouse (the node), suggesting that the organizer is a conserved element in vertebrate embryonic patterning.

However, evidence that the organizer signals are not as dominant as originally thought comes from examining embryos lacking an organizer. We generated a mutation in *HNF3β*, which produces embryos lacking the structures of the organizer- the node and notochord. Detailed examination of this phenotype shows that mutants express no organizer-associated *noggin* and only very transient levels of *chordin* mRNA and yet neural tube forms and is correctly patterned in the A-P axis. The dorsal mesoderm derivatives, the somites, also form, although they are fused in the midline in the absence of the notochord.

These observations have suggested that the classic organizer is not the only source of signals for axis patterning. Beddington and colleagues have proposed that, in the mouse, there is a separate source of anterior patterning signals associated with the anterior region of the visceral endoderm(AVE). A number of genes, including a mouse gene related to the head-forming gene Cerberus, *Cer1*, is expressed in this AVE. Experiments in support of the importance of the AVE for anterior patterning include our previous demonstration that anterior endoderm plus overlying mesoderm can induce *Engrailed*, a midbrain marker, in naive ectoderm, and Beddington's experiments showing that some anterior abnormalities occur when the AVE is scraped away. Clearly, there is need for more direct genetic and embryological evidence for the role of the AVE and its signalling molecules, and we are currently undertaking experiments of this sort.

So far we have deconstructed the mouse organizer role into two separable functions; an anterior head organizer (the AVE)and the classic organizer (the node), which is presumed to provide signals to pattern the posterior of the body axis. However, even here, the story gets more complicated. *HNF3β* mutant embryos manage to pattern

not only anterior structures but also more posterior structures of the hindbrain and spinal cord quite correctly, as judged by regional gene expression, and yet have no node or notochord. There is evidence from multiple systems that the embryo does indeed need posteriorizing signals to develop the trunk, but that these signals need not only come from the organizer, but also from the paraxial mesoderm or lateral mesendoderm

These non-organizer sources of posterior signals presumably use non-organizer associated signalling pathways. Strong candidates for involvement in posterior signalling are the retinoids, the FGFs and later-acting Wnts, based on experiments in multiple systems. All these pathways are still active in HNF3β mutants. We have genetic evidence in mice for involvement of FGF signalling in posteriorizing the developing paraxial mesoderm. Hypomorphic or gain of function alleles of *Fgfr1* cause homeotic transformations of the vertebrae consistent with a posteriorizing role for Fgfr1 signalling. It is not yet clear, however, whether this posteriorization extends to the nervous system.

All these experiments leave open the true endogenous role of the node. We suggest that there is only a transient requirement for early node activity interacting with the AVE to establish the basic organization of the body axis in mice. The later role of the node is to generate the notochord, which acts as a source of sonic hedgehog signalling to pattern the D-V axis of the nervous system and of the somites. D-V patterning of the neural tube and somites is severely disrupted in *HNF3β* mutant embryos, which fail to express Shh in the midline. The node and notochord also play a critical role in L-R asymmetry and asymmetric expression of the *TGFβ*-related genes, *nodal* and *lefty*, fails to occur in *HNF3β* embryos.

Embryos doubly heterozygous for *HNF3β* and *Otx2* show a cyclopic phenotype in midgestation, very reminiscent of the *Shh* mutant phenotype, implicating all these genes in the genetic pathways of midline patterning. A genetic screen for more loci that interact with *HNF3β* in the heterozygous state should identify more genes involved in the pathways upstream and downstream of *Shh*, and also provide candidates for involvement in the human holoprosencephaly syndromes.

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Session 4: Patterning signals

Chair: Nicole M. Le Douarin

T-box targets

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Brachyury is a member of the T-box gene family and is required for formation of posterior mesoderm and for differentiation of the notochord during vertebrate development (Smith, 1999). The Brachyury gene product functions as a transcription activator and binds as a dimer to a 20 base-pair palindromic sequence (Kispert and Herrmann, 1993; Kispert et al., 1995; Muller and Herrmann, 1997). Although the ability of Brachyury to activate transcription is essential for its biological function (Conlon et al., 1996), little is known about its target genes. In this presentation I describe two approaches designed to identify targets of *Xenopus* Brachyury (*Xbra*), both of which have proved successful. The targets thus isolated prove to be activated not only by Xbra but also by the maternal T-box protein VegT (Zhang and King, 1996; Stennard et al., 1996; Lustig et al., 1996; Horb and Thomsen, 1997).

The first approach involves simple guess-work, and this has succeeded in identifying *eFGF* as a gene which is directly regulated by Xbra (Casey et al., 1998). Inhibition of *Brachyury* function interferes with expression of *eFGF*, and the *eFGF* regulatory region contains Brachyury binding-sites which are essential for Xbra-mediated transcriptional activation. Interestingly, these sites comprise half of the palindromic sequence previously identified by Herrmann and colleagues, and appear to bind a single Brachyury molecule, rather than a dimer. These results provide further evidence that *Xbra* and *eFGF* are components of an indirect autoregulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995).

The second approach has involved the use of a hormone-inducible Xbra construct (Tada et al., 1997) to make cDNA libraries enriched for Brachyury targets. Screening of these libraries has resulted in the identification of several putative T-targets, including *Xwnt11* and a group of homeobox-containing genes we call *Bix1–4* (Tada et al., 1998). *Bix4* proves to be a target of VegT as well as Xbra, and examination of the *Bix4* regulatory region has identified Xbra and VegT binding sites which in transgenic *Xenopus* embryos are essential for the normal expression of *Bix4*.

Ablation of maternal VegT transcripts inhibits endoderm formation in *Xenopus* and vegetal pole cells lose the ability to induce mesoderm (Zhang et al., 1998). Expression of *Bix4* in such embryos rescues, to some extent, both endoderm and mesoderm formation, but does not restore mesoderm-inducing ability to the vegetal pole blastomeres (E. Casey, M. Tada, L. Fairclough, C. Wylie, J.

Heasman and JCS, in preparation). We are now searching for VegT targets which do restore mesoderm inducing capacity to the vegetal cells.

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NEURAL FATE IN CHICK EMBRYOS: ORGANIZING CENTERS CONDUCTING CELL BEHAVIOR.

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Hensen's node (HN) is probably the most significant organizing center in vertebrate development. The chick embryo, and more precisely the chick embryo developing in culture, is undoubtedly the ideal system for analyzing this organizer center by experimental embryology. One of the functions assigned to this center is the formation of the cell territory in the blastoderm that will give rise to the nervous system (neural plate). Several genes have been recently involved in the molecular pathways that lead to neural induction in Xenopus embryos. Based on this data, a default model for neural induction has emerged in the literature (1). In that model, blastoderm cells are fated to become neural "unless told otherwise" and the previously discovered neural inducers (chordin, noggin and follistatin) are molecules that compete at the molecular level with epithelial signals (BMPs) that become from ventral (lateral) blastoderm cells. When the default model was tested in the chick embryo, this mechanism for neural induction was questioned: the expression patterns of neural and epithelial inducers did not match with the expected territories, and there was no neural induction when chicken homologues for neural inducers were ectopically expressed (2). This opened up the possibility of different mechanisms for neural induction in different classes of vertebrates (3).

To provide insights into the mechanisms underlying neural induction in chick embryos, a unique neural inducer had to be found. The surprising solution was a class of molecules that are widely known for their mesodermal inducing activity: the fibroblast growth factor (FGF) family. Besides their role in the formation of mesoderm, FGFs had been implicated in neural patterning, but only some controversial findings in Xenopus had provided clues that FGFs could be involved in neural induction (4). To check whether FGFs can mimic the neural inducing ability of HN we delivered different FGF members (by soaking heparin beads with recombinant protein) to the periphery of chick blastoderms. This territory is, under normal circumstances, fated to become epithelial tissue (5,6) and a change to neural phenotype can be considered as neural induction. Beautiful small neural tube-like structures appeared expressing early (N-CAM, *Gsx, Sox*) and more advanced (*Hox-B9, Krox-20, EphA7*) neural markers (7, 8). The results have been confirmed by other groups and in the less biased territory of the area opaca (9). FGFs are present in the node at the time when neural induction is taking place (10) and therefore fulfill the last requirement to be neural inducers (i.e., they are present in the neural organizer at the right moment). There remained the possibility that FGF acts by a secondary induction: mediating either the formation of new organizing center or the production of new mesodermal cells. Although few mesodermal markers can be induced by FGF under some circumstances, neural ectopic plates form without induction of either a secondary HN or mesoderm (7,8).

The analysis of the antero-posterior (A-P) and dorso-ventral (D-V) pattern of the ectopic neural plates developed has demonstrated that FGF does not induce a complete nervous system. Only dorsal phenotypes can be found in the ectopic neural plates after 24 h of development and anterior neural markers (*BF-1, Tlx*) have never been induced by FGFs in our hands. The formation of a new border between the epithelial endogenous system and the ectopic neural plate can account for the dorsalization of the FGF induced plate. On the contrary, the absence of prosencephalic structures is difficult to explain in our experimental system. One possibility that we propose is that FGF is a candidate for exclusively posterior neural induction. The consequence of this hypothesis

is that we now need either an anterior neural inducer (by direct induction of cells with anterior character) or an anterior modifier (anteriorizing neural cells that otherwise would become posterior). Molecules expressed in the prechordal plate and/or notochord would be responsible for this mechanism (11) and they need to be identified.

If FGFs are neural inducers, and they are not working by direct competition with BMPs (also epithelial inducers in the chick), we have a wide field open to study: How does FGF induce neural cells? FGFs are growth factors that increase the rate of proliferation in most of the cells in which they have been checked. On the contrary, BMPs are well known to be involved in cell death processes. Do FGFs and BMPs play a competition game in synchronizing blastoderm cells´ behavior (proliferation and death)? We have detected cells in the epiblast undergoing cell death (is their suicide induced by BMPs?). Would the default model be true also for the chick embryo but affecting the regulation of cell behavior in blastoderm cells? The cellular mechanisms that convert a thin epiblast into a thick pseudo-stratified neural plate are largely unknown (although we know several of the genes that must be involved) and the fact that FGF is able to produce such a transformation could make it a suitable tool to study then. Finally, the role of FGF in Xenopus neural induction seems to be less important (12), so that one still has to deal with the possibility of different neural induction mechanisms depending on the phylogenetic group chosen as model.

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Patterning the organizer: The role of one-eyed pinhead and nodal signaling

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The organizer gives rise to prechordal plate anteriorly and notochord posteriorly. To determine how these distinct cell types are specified and how the organizer is patterned, we have employed fate mapping and studied the role of one-eyed pinhead (oep) and TGF-beta signaling in zebrafish. Fate map analysis using laser-mediated uncaging of caged fluorescein dextran indicates that the zebrafish organizer is patterned along the anterior-posterior axes already before gastrulation. Prechordal plate progenitors are located in the vegetal region of the organizer, whereas notochord precursors reside more animally. Mutations in the EGF-CFC gene oep result in the transformation of prechordal plate precursors into notochord progenitors. This defect is apparent at blastoderm stages when prechordal plate progenitors express the notochord gene *floating head* instead of the prechordal plate marker *goosecoid*. These results suggest that *oep* is required for the anterior specification of organizer cells before gastrulation. Previous studies have implicated the TGF-beta signals activin, Vg1 and/or nodal in anterior-posterior specification of organizer derivatives. To determine the relationship of TGF-beta signaling and EGF-CFC proteins, we have carried out detailed genetic and misexpression studies. Our results suggest that oep acts as an essential extracellular co-factor for nodal signaling during organizer patterning. We will discuss how differential activity of oep-mediated nodal signaling patterns the organizer.

Intrinsic and extrinsic events in the early steps of endoderm determination.

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Recent data indicate that dorso-anterior endoderm is a key component of the organiser. We have investigated the early steps of endoderm formation between fertilisation and the onset of gastrulation, using the genes Sox17a, Mix.1, Mixer and GATA4 as endodermal markers.

We find that these genes can be grouped into two categories. Activation shortly after MBT of Sox17a and Mix.1 is insensitive to cell dispersion and therefore appears to be cell autonomous. Accumulation of the transcripts for these genes in late blastulae, however, requires cell communication. In the case of Mixer and GATA4, which are first activated in late blastulae, both activation and accumulation of transcripts are strictly dependent on cell-cell communication.

We first analysed the molecular nature of the signals involved in the expression of the genes studied. Overexpression in vegetal cells of a dominant negative form of a type 2 receptor for activin, which blocks signalling via many TGFß factors, has the same effect as cell dispersion. Conversely, overexpression of a constitutively active type 1 TGFß receptor is sufficient to rescue the effect of cell dispersion on gene expression. This indicates that the vegetal signals belong to the TGFß family of secreted factors. Two potential candidates are Xnr1 and Xnr2 which are expressed in the vegetal pole of blastulae and, like Sox17a and Mix.1, are activated in a cell autonomous manner. Overexpression of Xnr1 and Xnr2 in animal caps reveals that Xnr1 is a qualitatively better inducer of endoderm markers than Xnr2 as only the former can activate expression of GATA4 at the beginning of gastrulation.

We then analysed the nature of the cell intrinsic factors that activate Sox17a, Mix.1, Xnr1 and Xnr2. Inhibition of protein synthesis from MBT onwards reveal that these factors are of maternal origin. One candidate is the T-box transcription factor VegT, which was shown to be maternally required for endoderm formation. Overexpression of VegT in animal cap cells is sufficient to activate Mix.1, Sox17a and Xnr2 at MBT but has no effect on the expression of Xnr1. 2 hours later, this activation of early genes leads to the contact dependent activation of Mixer, but not of GATA4. This suggests that the sequential activation of Xnr1 and GATA4 requires a maternal determinant distinct from VegT.

Taken together, our results indicate that two steps are required for the full activation of the enedodermal programme by the early gastrula stage. First cytoplasmic determinants, probably including VegT but not limited to this factor, directly activate early endodermal genes such as Sox17a, Mix.1, Xnr1 and Xnr2. Second, TGFß factors, possibly Xnr1 and Xnr2, act during the late blastula stages to activate later genes such as Mixer and GATA4, and to maintain and reinforce their expression as well as that of Sox17a and Mix.1.

SOME PRINCIPLES OF LONG RANGE SIGNALLING IN XENOPUS DEVELOPMENT

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During the early stages of development of amphibia and probably of other vertebrates secreted signalling molecules have an important role. A number of these are believed to have long range properties, such that a molecule secreted by a cell or cells in one position can influence the fate of other cells located several cell diameters away. An important characteristic of some long range singalling molecules is that they behave like a morphogen in that responding cells follow different fates according to the concentration of the morphogen that they receive.

In some apparent cases of long range morphogen action, it has not been established that the same signalling molecule affects cells near and far from the source. In some cases proximal responses are elicited by one kind of molecule and distant responses by another. In other apparent examples of morphogen action, the responding tissue is undergoing cell division and cell rearrangement on a substantial scale, and this complicates the interpretation of the process, especially if there is a long time interval between the release of the morphogen and the appearance of the responses to it.

During the first few hours of Xenopus development, it is believed that a Nieuwkoop signal is released from the dorso-vegetal region of early blastulae, and that this signal helps to determine the fate of the mesoderm as a high (Spemann organizer), middle (muscle), or low (blood) response to Nieuwkoop signalling concentration. The TGFβ class molecule activin provides a very good model of this concentration dependent response by animal cap cells. We have used the effects of activin on animal cap cells as a model by which to analyze the concentration-dependent response to activin.

In this case, it has been established that the same activin molecule is responsible for proximal and distal effects, that the concentration is formed by diffusion and not by a relay process, and that cell movement does not contribute to the distant effects. We have used naturally labelled activin, synthesized in Xenopus oocytes, to carry out binding studies on dissociated responsive cells of a blastula. By this means it is possible to

determine the numbers of receptors and of bound ligands per cell, the on- and off-rates of receptor-ligand binding, and to show that internalization of the receptor is not involved in this case. As a result of these measurements, we have established the numbers and proportion of receptors that need to be occupied by ligand for cells to switch from a nil to low gene response (Xbrachyury, Xantipodean), and from a low to high gene response (Xgoosecoid, Xeomesodermin). As a result of these studies, we propose a novel mechanism of morphogen gradient interpretation in embryonic cells. We believe that cells sense the concentration of an external morphogen continuously as its concentration rises, and that the gene response selected by a cell depends upon a ratchet mechanism depending on the high affinity and long occupancy of activin receptors. We consider the choice of gene activation by a cell to be determined by the absolute number of occupied receptors over a particular period of time. We propose that a morphogen gradient is read by cells long before a steady state is reached and that cells continuously monitor morphogen concentration around them.

Our results lead to a new concept of how cells interpret their position in a concentration gradient, and we suspect that the general principles revealed by this work may be generally applicable in early stages of development.

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Session 5: Organogenesis and the Organizer

Chair: John B. Gurdon

Control of Organogenesis and Embryonic Body Axis Formation in Amphibian Development

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In the process of early vertebrate development, formation of an embryonic body pattern is established through cell division, gene expression, morphogenesis and cell differentiation. The mechanism of body patterning is complex and includes multiple induction events. In the induction events occurring between two cells or tissues, factors secreted by the inducing cells may cause the directive differentiation of the reacting cells. In mesoderm induction the expected factors from vegetal cells are referred to as mesodermal inducing factors (MIFs). The assays have led to the identification of several endogenous molecules that are responsible for mesodermal and neural inductions, such as activin/Vg1, follistatin, FGFs, BMPs, chordin and noggin.

Activin, a member of the TGF-B-super family, can induce several kinds of mesodermal and endodermal tissues in *Xenopus* and newt animal caps. The effect of activins on animal caps is distinctly dose-dependent, with induction of more dorsal tissues such as muscle and notochord and endodermal tissues as the concentration increases.

In a recent study of the role of activin in organ formation, we succeeded in raising a beating heart by treating animal caps with a high concentration of activin A. Renal tubules were induced in *Xenopus* animal caps treated with a combination of activin A and retinoic acid (RA) at a high frequency (100%). The renal tubule explants induced by activin and RA *in vitro* could also function *in vivo* when the explant was transplanted into the presumptive kidney.

When an activin-treated animal cap was sandwiched between two non-treated animal caps, the treated animal cap obviously behaved as Spemann's organizer. They induced embryo-like explants with multiple endodermal and mesodermal tissues and a central nervous system. Activin-treated animal caps become artificial organizers which act as "trunk-and-tail organizer" or "head organizer", depending on the preculture time after the treatment of activin on animal cap.

To examine the signal transduction pathways of activins, we injected truncated activin type I or type II receptors into the egg. The phenotypes of the embryos changed depending on the receptor. These results could also be obtained with specific smad-2 antibodies; Smad-2 is a the mediator of activin signals in the cell. Activins or related proteins seem to be the one of the first important induction signals responsible for establishing the fundamental embryo body plan.

Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction

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The Spemann organizer in amphibian embryos is a tissue with potent head inducing activity, the molecular nature of which is unresolved. We showed previously that simultaneuos repression of BMP and Wnt-signalling results in head induction and our observation that the head inducer *cerberus* inhibits Wnt-signalling is consistent with this proposition. We recently identified *dickkopf-1* (*dkk-1*), which encodes a secreted inducer of Spemann's organizer in *Xenopus* and which is member of a novel protein family. Expression of *dkk-1* occurs predominantly in prechordal plate mesoderm, both in mouse and Xenopus embryos, which is thought to harbor head organizer activity. Coexpression of *dkk-1*and inhibitors of BMP signalling induces formation of complete head structures in Xenopus. Antibodies were raised against dkk-1 protein and their injection into the gastrula blastocoel results in microcephalic and cyclopic embryos. The results indicate that *dkk-1* is necessary and sufficient for head induction. *Dkk-1* is a potent antagonist of Wnt signalling, suggesting that *dkk* genes may encode a novel family of secreted Wnt-inhibitors.

MOLECULAR AND CELLULAR ASPECTS OF THE ISTHMIC ORGANIZER

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The Isthmus Organizer (IsO) controls the rostral hindbrain and the midbrain regionalisation. Experimental mutations of developmental genes in mice and in ovo isthmic grafts have demonstrated the existence of an important inductive activity of this organizer in normal and ectopic midbrain and cerebellar development. One possible effector molecule for the morfogenetic activity of the IsO is the product of the gene Fgf-8. Insertions of heparin beads soaked in recombinant FGF-8 into rostral neuroepithelial areas of midbrain or diencephalon generated induction, by planar effects, of an ectopic isthmic region in the host (Crossley et al. 1996, Martinez et al., 1999). Therefore the induced organizer determines an isthmic, cerebellar and mesencephalic development in the presumptive tectal or thalamic neuroepitheium. Our results demonstrated that the organizer is defined by a precisse pattern of locally expressed melecules. In addition, morphogenetic properties of thie territory are derivated from the correct topological pattern of expressions, that likely determines the vectorial direction of inductuve influences and characteristic cellular events at the isthmic constriction. Experiments in progress are exploring the mechanisms controling the especification of the istmic organizer at the midbrain/hindbrain junction. Our experimental hypothesis is that the interaction between Gbx2 and Otx2 expressing domains in the early neural plate regulates the expression of Fgf8. Then the inductive properties of Fgf8 regulate the molecular pattern that defines the isthmic organizer.

DIFUSIBLE SIGNALS ACTING IN THE ORGANIZER ARE ALSO INVOLVED IN DIGIT MORPHOGENESIS

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The formation of the digits in the vertebrate limb provides an excellent model for analyzing the molecular basis of morphogenesis. Digits develop as radial chondrogenic condensation in the autpodial limb region (hand/foot plate). The mesoderm located between the digital rays (interdigital regions) has also digit-forming potential but in the course of normal development they are eliminated by programmed cell death. In our talk we provide evidence showing that the digital versus interdigital fate of the mesoderm is controlled by secreted factors. FGFs produced by the apical ectoderm riming the distal margin of the limb (AER) mantain the subjacent mesoderm in an undifferentiated and proliferating state. These undifferentiated cells are commited to cartilage differentiation by proximally acting signals including : TGFß2, Activin A , Activin B and/or Activin AB (1-2). BMPs expressed in the undifferentiate mesoderm induce apoptosis in the cells lacking Activin/TGFß chondrogenic stimula while promote growth of the digital cartilages (3-4). The chondrogenic and the apoptotic effects of BMPs are modulated respectively by the BMP-antagonists Noggin and Gremlin (5). Retinoic Acid active metabolites are locally produced in the autopodial mesoderm and control interdigital cell death promoting BMP gene expression and simultaneously repressing the chondrogenic potential of these factors (6).

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A novel view on neurulation in Amniotes

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The process of neurulation has been studied in the avian embryo through the use of the quail-chick chimera technique. By substituting Hensen's node material in a 5-6ss chick embryo by its counterpart from a stagematched quail, we demonstrate that it yields three superposed structures on the embryonic midline : the floor plate, the notochord and the dorsal endoderm on the whole length of the embryo. We establish that the cordo-neural-hindge as defined by Pasteels (1937) in the tail bud is the remnant of Hensen's node. We show that HNF3β is expressed in Hensen's node and its derivatives throughout development thus contradicting the claim of previous authors that this gene is induced in the floor plate by the Shh protein produced by the notochord. We therefore present a new model to account for both primary and secondary neurulation in Amniotes. Moreover, we have identified a particular zone corresponding to the junction between Hensen's node and the extreme anterior tip of the primitive streak that is critical for regression of Hensen's node and thus elongation of the embryo.

POSTERS

THE WINGED-HELIX TRANSCRIPTION FACTOR HNF-3ß IS REQUIRED FOR EXPRESSION OF THE SIGNALLING MOLECULES FGF8 AND SHH EXPRESSION IN MOUSE EMBRYOS.

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The winged-helix transcription factor, *HNF-3ß*, is expressed in the anterior primitive streak and the visceral endoderm of mouse embryos at E6.5. During gastrulation, *HNF-3ß* expression is maintained in descendants of the anterior primitive streak, the node and notochord cells and it then spreads to include cells in all three germ layers, including the floorplate, axial mesoderm and all definitive gut cells. *HNF3ß* is also expressed at later stages in ventral diencephalon and ventral mesencephalon. To study the function of *HNF-3ß* in vivo, we have previously generated a null mutation of this gene by homologous recombination in ES cells. Homozygous mutant embryos die at E9.5, and fail to generate an organized node and to produce any notochord cells. In addition, they show gastrulation defects due to an earlier function of *HNF-3ß* in the visceral endoderm. In order to bypass the gastrulation phenotypes and early embryonic lethality and to study later functions of the gene in the brain and gut, we have generated a conditional allele of *HNF-3ß* flanked by loxP recombination sites (*HNF-3ß^{floxed}*). The *HNF-3ß^{floxed}* allele has been introduced into the germline of mice, and mice homozygous for this allele are viable and fertile. We have crossed these animals with Ball *Nestin::Cre* transgenic mice, which express the bacteriophage Cre recombinase in all tissues starting at E7.5. *Nestin::Cre/+;HNF-3ß$^{-/floxed}$* embryos do not present the gastrulation and visceral endoderm defects observed in *HNF-3ß$^{-/-}$* embryos, but they exhibit defects in midline tissues as early as E7.5. Using molecular markers, we have observed that patterning molecules, like SHH and FGF8 are not expressed or severely reduced in *Nestin::Cre/+;HNF-3ß$^{-/floxed}$* mutant embryos, and as a consequence these embryos show both antero-posterior and dorso-ventral patterning defects in the neural tube.We have also found that *HNF-3ß* is required autonomously in floor plate cells for expression of SHH by co-culturing neural plate isolated from *Nestin::Cre/+;HNF-3ß$^{-/floxed}$* mutant embryos with SHH-expressing P19 cells. We are currently investigating the mechanisms leading to the loss of FGF8 in *Nestin::Cre/+;HNF-3ß$^{-/floxed}$* mutant embryos also using explant culture experiments.

THE ROLE OF CERBERUS IN ANTERIOR-POSTERIOR PATTERN FORMATION

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Anterior-posterior patterning of the neuroectoderm as well as the endoderm is controlled by the Organizer region, as originally shown by Spemann and Mangold in amphibia. The modulation of growth factor activity by secreted antagonists appears an important mechanism of pattern formation in vertebrates. Several proteins have been identified that inactivate particular classes of growth factors by extracellular sequestration.

Cerberus is a secreted glycoprotein containing a cystine-knot module that acts as a multivalent growth factor antagonist. It is expressed in anterior endodermal cells (presumptive extraembryonic) that during the course of gastrulation are in direct contact with rostral neuroectoderm, fated to give rise to fore- and midbrain, and involuting definitive endoderm, fated to give rise to foregut. Simultaneous inactivation of BMP and Wnt activities is required for the formation of ectopic heads by Cerberus. Here we have addressed the role of Nodal-related factors in mesoderm and endoderm formation by overepressing a mutant allelle of Cerberus that inactivates this class of growth factors. From these functional as well as expression data we extrapolate that Cerberus might control the positioning of the marginal ring of mesodermal cells by antagonizing Nodal-related factors. Furthermore we propose that during gastrulation progressive trapping of Nodal-related growth factors by Cerberus might be important for imposing anterior-posterior pattern on the definitive dorsal endo- and mesoderm.

Regionalization of the neuroectoderm is believed to be a multi-step process, by which anterior cell fates aquire progressively more posterior fates under the influence of caudalizing factors. We have previously identidied a novel forkhead-related gene that is epressed in a highly dynamic fashion in the neuroectodermal midline. Comparative expression data provide evidence that the neuroectodermal midline (floorplate) originates, at least in part, from cells close to the blastopore that undergo drastic convergence and extension movements. Furthermore we will present aspects of the function, by a dominant interference strategy, and regulation of this transcriptional regulator during neuroectodermal patterning.

Involvement of the small GTPases XRhoA and XRnd1 in morphogenesis and head formation in early *Xenopus* development

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The Rho family of small GTPases regulates a variety of cellular functions, including the actin cytoskeleton, cell adhesion, transcription, cell growth and membrane trafficking. We have isolated the first *Xenopus* homologs of the Rho-like GTPases RhoA and Rnd1 and examined their potential roles in early *Xenopus* development. We found that *Xenopus* Rnd1 (XRnd1) is expressed in tissues undergoing extensive morphogenetic changes, such as marginal zone cells involuting through the blastopore, somitogenic mesoderm during somite formation and neural crest cells. XRnd1 causes a severe loss of cell adhesion in overexpression experiments, making it a potential regulator of morphogenetic movements in early embryos. *Xenopus* RhoA (XRhoA) appears to increase cell adhesion in the embryo and reverse the disruption of cell adhesion caused by XRnd1. In addition to the potential roles of XRnd1 and XRhoA in the regulation of cell adhesion, we find a role for XRhoA in axis formation. When coinjected with dominant-negative BMP-receptor in the ventral side of the embryo, XRhoA causes the formation of head structures and induces anterior neural markers, resembling the phenotype seen after coinjection of wnt-inhibitors with dominant-negative BMP-receptor (tBR). Since dominant-negative RhoA is able to reduce the formation of head structures, we propose that RhoA activity is essential for head formation. Thus, RhoA may have a dual role in the embryo by regulating cell adhesion properties and pattern formation.

XENOPUS BRAIN FACTOR-2 CONTROLS MESODERM, FOREBRAIN AND NEURAL CREST DEVELOPMENT

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The forkhead type Brain Factor 2 from mouse and chicken help pattern the forebrain, optic vesicle and kidney. We have isolated a *Xenopus* homolog (*Xbf2*) and found that during gastrulation it is expressed in the dorsal and dorsolateral mesoderm, where it helps specify this territory by downregulating *BMP-4* and its downstream genes. Indeed, *Xbf2* overexpression caused partial axis duplication. Interference with BMP-4 signaling also occurs in isolated animal caps, since *Xbf2* induces neural tissue. Within the neurula forebrain, *Xbf2* and the related *Xbf1* gene are expressed in the contiguous diencephalic and telencephalic territories, respectively, and each gene represses the other. Finally, *Xbf2* seems to participate in the control of neural crest migration. Our data suggest that XBF2 interferes with BMP-4 signaling, both in mesoderm and ectoderm.

XFKH5, A FORK HEAD GENE, IS INVOLVED IN THE EARLY PATTERNING OF *XENOPUS* EPIDERMIS AND ITS EXPRESSION DEMARCATES THE LIMIT OF INVOLUTION DURING GASTRULATION

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The fork head domain is a monomeric DNA motif that defines a rapidly growing family of transcriptional regulators. Some of them are involved in embryonic pattern formation. We have isolated a novel forkhead gene, *XFKH5* that is involved in formation of ciliated cells in the *Xenopus* epidermis. Its expression begins in the animal third of the blastula stage embryos in a punctate pattern. At later stages these cells express alpha tubulin and become ciliated. The expression pattern of this gene suggests that its transcription is regulated by *Notch-Delta* signaling. Preliminary observations from Chris Kintner's laboratory support this suggestion.

A second region of expression of *XFKH5* is a ring of cells around the equator of blastula stage embryos. During gastrulation these cells form the limit of involution. These cells appear to converge but do not extend during gastrulation (Ray Keller, preliminary observations) and they possibly represent a previously undescribed cell type. Expression of *XFKH5* increases during gastrulation and turns off abruptly when gastrulation is finished. Our preliminary experiments indicate that expression of *XFKH5* interferes with induction or differentiation of neural and mesodermal tissue.

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Regulation of Spemann's Organizer Formation and Function by Homeobox Factors

In *Xenopus*, maternal factors establish dorsoventral pattern in the cleavage embryo and result in the formation of Spemann's organizer at the gastrula stage. Maternal dorsal determinants, localized to the vegetal pole at fertilization, are displaced by cortical rotation to the future dorsal domain of the cleavage embryo. An early response to these determinants is the nuclear accumulation, in dorsal blastomeres, of βcatenin, a component of the Wnt pathway required for dorsal development. The identified components of the Wnt pathway are maternally expressed and a strong candidate for a zygotic effector of this maternal pathway is Siamois, a Wnt-inducible homeodomain factor.

Our results show that Siamois, as an effector of maternal Wnt signaling, is required for organizer formation and organizer gene expression. Fusion of defined transcriptional regulatory domains to the Siamois homeodomain demonstrated that Siamois activates transcription and that this function is required for organizer formation, organizer gene expression, and subsequent axial development. Consistent with a direct role in organizer formation, Siamois activates transcription of Goosecoid, an organizer-specific gene, by binding to conserved regulatory sites within a previously identified Wnt-responsive element. These consensus homeodomain binding sites were sufficient and necessary for transcriptional activation by Siamois, Xwnt8, and endogenous dorsal signals. This role for Siamois in Goosecoid transcription is supported by the presence of identical regulatory elements in the fish and mouse Goosecoid genes. In addition, the interaction of the Siamois-response element with an adjacent TGFβ-response element was examined. Low levels of Activin and Xwnt8, neither sufficient to activate transcription, induced a strong response when combined, similar to the synergy of these pathways in inducing endogenous Goosecoid. The results suggest that the Wnt and TGFβ signaling pathways converge at the Goosecoid promoter and result in synergistic activation of transcription in organizer tissue. In conjunction with the observed synergy, the presence of vegetal TGFβ signals and dorsal Wnt signals in the embryo suggests an overlap model in which the combined action of these signaling pathways regulates the appropriate spatial control of organizer gene transcription. Ongoing analyses have identified conserved TGFβ- and Wnt-responsive elements in the promoters of two additional organizer genes, supporting the overlap model.

The function of Goosecoid in organizer function was assessed using chimeras containing the VP16 activation or Engrailed repression domains fused to the Goosecoid homeodomain. The results indicate that Goosecoid controls anterior development by directly repressing zygotic transcription of Xwnt8, thus excluding Xwnt8 expression from the organizer. Therefore, during early *Xenopus* development maternal signals induce a cascade of homeobox factors that activate and repress transcription and ultimately result in formation of the functional organizer.

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CHARACTERISATION OF HOMEOBOX GENES FROM ECHINODERMS

Homeobox-containing genes are a major class of transcription factors regulating many aspects of development. Genes with homeoboxes have been found in all metazoans thus far examined. On the basis of several criteria, including sequence identity, organisation into gene clusters, association with other sequence motifs and position of introns, homeodomain sequences can be subdivided into, at least, 20 different classes. The three best characterised families of homeobox genes are: the HOM/Hox class of clustered homeobox genes; the engrailed class; and the paired class.

Across different phyla, members belonging to particular classes show a high degree of sequence similarity, and, in some cases, a striking degree of conservation in expression domains and functions. These features bestow homeobox genes with unique advantages for studying the evolution of development and the origin and diversification of body plans. Particular attention has been given to the class of HOM/Hox genes. These genes act along the antero-posterior axis of an embryo providing cells with positional information according to their location in that axis. Remarkably, their domains of activity in the embryo are correlated with their position in the cluster. The experimental correlation has been only established for three groups of triploblastic metazoans: the chordates, the arthropods and the nematodes.

Because it seems that patterns of homeobox gene expression are sometimes more conserved than morphology it has been suggested that an experimental approach to study the evolution of body plans can make use of the expression domains of these genes, in particular HOM/Hox, as markers with which can serve for relating anatomy in close-related groups of animals.

In this context I started, as a postdoctoral student in the laboratory of Dr. Eric H. Davidson, the characterisation of a group of genes belonging to the above mentioned HOM/Hox, engrailed and paired classes in echinoderms using as a model the sea urchin Strongylocentrotus purpuratus, a classical model of development for which we have accumulated a great deal of molecular and embryological data during the last 20 years. From an evolutionary point of view, the key position of echinoderms as a sister group of all the other deuterostomes make them an ideal choice to study the evolution of body plans and the origin of the chordates.

Ten Hox genes have been fully characterised. I have demonstrated that they belong to a chromosomal cluster spanning a total of 500 kb. A contig in bacterial artificial chromosome fragments allowed us to establish the specific order of these genes in the sea urchin genome.

Functions of *Hesx1* and *Hex* in developmental patterning of the rostral brain

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Hesx1 is a member of the paired-like class of homeobox genes and is expressed in the anterior visceral endoderm (AVE) at the onset of gastrulation. As gastrulation progresses, *Hesx1* transcripts are detected in the underlying anterior neural ectoderm (ANE) which is destined to give rise to the forebrain. We have generated mice lacking *Hesx1* and found that they exhibit a variable degree of forebrain truncations, pituitary hypoplasia and midline brain commissural defects. Highly chimeric embryos, composed of wild type neuroectoderm developing within mutant AVE, show no defects in the forebrain. This data indicates that *Hesx1* is not required in the AVE for normal brain development and that its primary requirement is in the ANE.

The divergent homeobox gene *Hex* is expressed in the AVE at prestreak and streak stages and is the first indicator of anterior asymmetry. A null mutation has been introduced into this gene in mice resulting in deletions of the forebrain region which are very similar to those found in the *Hesx1* mutant mouse. Since *Hex* is never expressed in neuroectodermal tissues, these results provide evidence for a crucial function of the AVE in patterning the anterior brain.

A GRADIENT MODEL OF NEURAL CREST INDUCTION

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During vertebrate neurulation, the ectoderm is divided into three sets of cells: the neural plate, which will form the brain and the spinal chord, the epidermis which will form the skin and the neural crest which will generate several cell types including peripheral neurons, glia, the pigment cells of the skin, etc. The neural crest originates at the border between the neural plate and the surrounding epidermis.

The process of neurulation is initiated by neural induction, where signals coming from the dorsal mesoderm (the organizer) induce and organize a correctly patterned nervous system in the neighboring dorsal ectoderm. In the absence of this induction, ectoderm develops as epidermis, which requires the activity of BMPs, secreted by ectodermal cells. Recent evidence has shown that neural induction is caused by soluble neuralizing factors (noggin, chordin, follistatin, etc.), that are expressed in the organizer and some of them bind to BMPs, preventing them from interacting with their receptors. Therefore, these molecules inhibit epidermal induction by directly inhibiting BMP signaling, unmasking the default neural fate of the dorsal ectoderm. This model explains how the ectoderm is divided into neural plate and epidermis, but it does not explain how the neural crest tissue is induced at the border of the neural plate.

We have used the expression of several neural crest markers to analyze the role of mesoderm and different molecules in the induction of the neural crest cells. We found that neural crest markers can be induced in competent ectoderm at varying distances from the mesodermal-inducing tissue, depending on the dorsolateral origin of the mesoderm: dorsal tissue induces crest at a distance whereas dorsolateral tissue induces crest directly adjacent to itself. Theses results can be interpreted as different mesodermal tissues having different amount of an inducer, with high levels in dorsal mesoderm and lower levels in dorsolateral mesoderm. The inducer diffuses from the mesoderm to generate a gradient and, at a distance from the source of inducer, a neural crest threshold is reached. We explored the possible role of BMPs and noggin in the generation of this hypothetical gradient. We found that : (1) progressively higher levels of BMP activity are sufficient for specification of neural plate, neural crest and non neural cells, in that order; (2) in directional conjugates, progressively higher levels of noggin are able to induce neural crest at greater distances from the source of inducer; (3) by modifying the level of BMP activity, we were able to induce neural crest in the absence of neural plate, suggesting these tissues are induced independently. These results suggest a model in which a gradient of BMP activity is established in the ectoderm. Neural crest is induced when BMP activity in the ectoderm is between two threshold levels. This gradient is established by interactions between BMP from the ectoderm and BMP-binding molecules arising from the mesoderm. Low BMP activity induces neural plate whereas high levels of BMP induce epidermis. Therefore BMP can be considered as an ectoderm-patterning morphogene.

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Left-right asymetry: role of *Pitx2*

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The body plan of most vertebrates presents a bilateral symmetry. However, the shape and position of several internal organs show a clear handed asymmetry. Left-right (L/R) asymmetries are established very early during embryonic development and are under the strict control of a molecular pathway. Although the establishment of L/R morphological asymmetry is evolutionary conserved, the genetic cascade that controls it shows both, common features and interspecific differences. Nodal, a member of the TGFb superfamily, is a conserved element in this cascade and is transiently expressed in the left lateral plate mesoderm of mouse, chick and frog. *Pitx2*, a bicoid-related homeobox-containing gene, was recently identified as a downstream gene of *Nodal*. *Pitx2* has three known isoforms and is, at present, the most downstream component of the laterality pathway. We have investigated the possible differential expression of these isoforms during early embryonic development. In addition, we have analyzed late patters of *Pitx2* expression during development of the heart in mouse and chick.

CYCLOPS SIGNALLING IN ZEBRAFISH AXES DETERMINATION

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The zebrafish *cyclops* gene encodes a Transforming Growth Factor ß-related factor, which is required for patterning the ventral neural tube, specification of dorsal mesendoderm, separation of the eye field, and for left-right asymmetry of the visceral organs. Cyclops transcripts are expressed in various cell types during gastrulation and later, asymmetrically in the left side of the embryo. In addition, *cyclops* also has a maternal component, the role of which is being determined.

Signalling by proteins of the TGFß superfamily involves the phosphorylation and activation of the cytoplasmic proteins, Smads. Upon TGF-ß receptor activation, effector Smads are translocated to the nucleus, where they bind DNA-binding factors or directly to specific DNA sequences, and activate transcription of target genes. The effector Smad that functions downstream of *cyclops* has been identified. Results from these experiments will be presented.

DOWNSTREAM GENES OF NEURAL INDUCTION: REQUIREMENT OF SOX2 SIGNALING IN NEURAL DEVELOPMENT

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Vertebrate neurogenesis is initiated by the organizer factors that inhibit antineuralizing activities of BMPs in the ectoderm. Here I will decribe a candidate mediator of neuralization, Sox-D. Expression of Sox-D starts at late blastula stages widely in the prospective ectoderm and becomes restricted to the dorsal ectoderm by mid-gastrula stages. Sox-D expression is enhanced by the neural inducer Chordin and is suppressed by BMP-4 and its downstream genes. Microinjection of Sox-D mRNA causes ectopic formation of neural tissues in vivo and induces neural and neuronal markers in the isolated animal cap. Injection of a dominant-negative form of Sox-D mRNA can block neuralization of ectoderm caused by attenuation of BMP signals and can strongly suppress formation of anterior neural tissues in vivo. These data show that Sox-D functions as an essential mediator of downstream signaling of neural induction.

In addition, I will present two other types of transciprion factors, Zic-related and Sox-2. Both factors are expressed in ealry neuroectoderm. Zic factors can promote neurogenesis when overexpressed in vivo and in the animal cap. By contrast, Sox-2 does not initiate neural differentiation when acting alone, but can enhance responsiveness of the ectoderm to FGF neuralizing signals. By using dominant-negative forms of Sox-2, loss-of-function phenotypes were studied. Inhibition of Sox-2 signaling resulted in suppression of NCAM expression as well as expression of anterior and posterior neural markers. Early neural markers such as Zic were resistant to dominant-negative Sox-2 expression. I will discuss the role of Sox-related factors in the view of instructive and permissive roles.

Use of the GAL4-UAS technique for targeted gene expression in the zebrafish

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The aim of this poster is to report on the use of the GAL4-UAS system for targeted gene expression in the zebrafish. To achieve that goal various activator lines have been generated which express GAL4 in a stage and tissue specific manner. Besides a number of activators with heterologous promoters, transgenic lines with zebrafish promoters have been established. Thus, in one of these lines, GAL4 is under control of a zebrafish-heatshock promoter, which makes it possible to turn on GAL4 expression at any time of development. Another fish promoter used is the *deltaD* promoter, characterized in our laboratory by Stephan Hans.

Transgenic lines which carry stably integrated effector genes which are under the control of the DNA binding motif for GAL4 (UAS_G) have also been generated. Besides the reporter gene *gfp*, we are using different variants of genes which are expressed in the developing neural plate. Crosses of these transgenic effector lines to different activator lines have demonstrated the efficacy of the GAL4-UAS system in the zebrafish.

Data on the transgenic lines, as well as on the phenotypic effects of overexpression of the different effector genes, will be presented.

GENETIC ANALYSIS OF THE ZEBRAFISH GASTRULA ORGANIZER

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We are studying genetic pathways involved in the formation and function of the dorsal gastrula organizer in zebrafish. The zygotic and maternal activities of the *bozozok* (*boz*) locus are required in the blastula and throughout gastrulation for expression of organizer-specific genes and for development of dorso-anterior embryonic structures, including notochord, prechordal plate and ventral and anterior neuroectoderm. *boz* mutations disrupt the homeobox gene *dharma* expressed predominantly in the dorsal aspect of the extraembryonic yolk syncytial layer (YSL). Overexpression of *boz* in the YSL of mutant embryos is sufficient for normal development of the overlying blastoderm, revealing an involvement of extraembryonic structures in anterior patterning in fish similarly to murine embryos. Epistatic analyses indicate that *boz* acts downstream of β-catenin and upstream to TGF- β signaling or in a parallel pathway (Fekany et al., 1999).

To identify the genetic pathways by which *boz* regulates different aspects of embryonic axis formation we analyzed expression patterns of region and cell type specific markers during development of mutant embryos. Expression of *chordin* (*din*), encoding a BMP2/4 antagonist is missing or strongly reduced in *boz* mutants at the blastula and early gastrula stages, but its expression is affected to a lesser extent at the end of gastrulation. Accordingly, the expression domains of genes encoding the ventral morphogen BMP2/4, the ventralizing factor Wnt8 and ventro-lateral markers *eve1* and *tbx6* are expanded to the dorsal side, with expression of the *bmp4* gene returning to normal levels at the end of gastrulation. While axial and adaxial mesoderm never forms in *boz* mutants, expression domains of genes marking lateral and ventral mesoderm, including the pronephros and blood are surprisingly normal in *boz* mutants during segmentation. Within the ectoderm of *boz* mutants, expression domains of forebrain and midbrain markers (*opl*, *otx1*, *otx2* and *anf*) are reduced, while expression domains of non-neural ectodermal markers, such as *gata2* and *gta3* are expanded dorsally. The reduction of prospective nueroectoderm is less pronounced at the end of gastrulation.

Additional analyses of gene expression patterns and fate mapping experiments indicate that the loss of forebrain in *boz* mutants is due to decreased neural induction as well as due to posteriorization of neuroectoderm. Ectopic expression of *din* in *boz* mutants suppresses the neural induction defect, however it fails to rescue the forebrain and notochord. In contrast, inhibition of Wnt signaling in *boz* mutants by overexpression of dominant negative Wnt8 mutant suppresses forebrain reduction and lack of notochord. We hypothesize that the homeodomain Bozozok protein leads to specification of dorso-anterior embryonic structures by negativly regulating BMP2/4 and Wnt signaling at the blastula and gastrula stages of development.

To determine the role of the residual expression of *din* in *boz* mutants and to reveal possible functional interactions between *boz* and *din* loci, *boz din* double mutants were generated. *din* mutant embryos exhibit an excess of ventral fates including multiple ventral fin folds, but relatively normal dorsal structures. Two classes of *boz din* double mutants were identified. The weaker class of double mutants shows an exacerbated loss of dorso-anterior structures characteristic for *boz* and an increase of ventral fates typical of *din*. The stronger double mutants exhibit a radially symmetric tail-like structures with head and trunk missing completely. Neuroectoderm is severely reduced or absent and anterior somites do not form, suggesting that *boz din* mutants fail to develop dorsal and anterior fates in both germ layers. These and other analyses indicate that *boz* and *din* loci functionally interact during organizer formation and axis specification in zebrafish.

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Herbert Steinbeisser

Axis formation and gene activity in early *Xenopus laevis* development

In the early stages of development, a dorsoventral and an anteroposterior axis is established in the embryo. Though the embryonic axes form only during gastrulation, their foundations are laid down much earlier. A crucial step in this developmental program is the dorsoventral patterning of the prospective mesoderm. In the South African clawed toad *Xenopus laevis,* this is achieved by the antagonistic activities of genes that are expressed dorsally (in the Spemann organizer) and genes expressed in the ventral mesoderm. Our goal is to analyze how mesoderm-specific genes are regulated and how they function in the developing embryo. In order to assess this problem, we have identified several previously unknown genes that are expressed in the developing mesoderm and we are in the process of characterizing their function. At the moment we are focusing on the analysis of *ETS*-type transcription factors and transmembrane receptors of the *frizzled*-family.

Another problem under study is through what mechanisms genes such as the organizer-specific genes get activated in a spatially restricted manner. It is assumed that early events which occur before zygotic transcription is initiated program the temporal and spatial activation of genes. We therefore study the effect of early patternig processes such as cortical rotation and mesoderm induction on the expression of dorsal- and ventral-specific genes. Our results indicate that determinants transported by cortical rotation from the vegetal pole of the egg to the marginal cortex induce the localized entry of ß-catenin into dorsal nuclei. This process is reflected in localized zygotic gene expression. When cortical rotation is prevented in the fertilized egg, the determinants remain in their original position and induce a transient activity of dorsal-specific genes at the vegetal pole of the pregastrula embryo. The effect of cortical rotation on the spatially correct activation of dorsal-specific genes has been confirmed using a blastomere explant assay.

POSSIBLE TARGET GENES FOR THE LIM HOMEODOMAIN PROTEIN XLIM-1 IN THE SPEMANN ORGANIZER

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Xlim-1 is implicated on the basis of RNA injection experiments in the functions of the Spemann organizer as assayed by secondary axis formation in whole emoryos, and in neural induction in animal caps. To clarify the molecular mechanisms of Xlim-1 functions in the organizer, it is necessary to identify direct target genes for Xlim-1, since homeodomain proteins are believed to be transcription factors. We first examined which known organizer specific genes are activated in animal caps by an activated form of Xlim-1 (Xlim-1/3m). We next screened a subtraction cDNA library which was constructed using Xlim-1/3m expressing and control animal caps to look for new target genes. As results, we found that Xlim-1/3m activated goosecoid (gsc), Otx2, chordin, and cerberus genes, as well as several novel genes which were found to be expressed in the organizer region. Dominant negative constructs of Xlim-1, in which the activation domain was replaced with the engrailed repressor domain, partially or completely repressed expression of gsc, Otx2, chordin, and cerberus in whole embryos.

Among these candidate target genes, we examined the regulation of the gsc promoter by Xlim-1 in animal caps using gsc/luciferase reporter constructs. We showed that a 492-bp upstream region of the gsc gene responded to Xlim-1/3m. Footprint and electrophoretic mobility shift assays revealed that the Xlim-1 homeodomain recognized several TAAT core elements in the 492-bp upstream sequence. Thus gsc is very likely to be a direct target gene of Xlim-1. We also found that wildtype Xlim-1, Ldb1 (LIM domain binding protein 1), and Otx2 (a homeodomain protein) synergistically activated gsc reporters and that the Otx2 homeodomain bound to the gsc promoter. Since gsc encodes a homeodomain protein which has been shown to dorsalize ventral mesoderm, Xlim-1 and Otx2 appear to be important components of the transcriptional regulatory network in the organizer to maintain expression of organizer-specific genes such as gsc.

The role of *Xmsx-1* in the ventralization of early Xenopus embryo

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Xmsx-1 is homeobox gene responding to bone morphogenetic protein, BMP. Overexpression of Xmsx-1 in early Xenopus embryo mimics the gain-of-function of BMP signal, suggesting that Xmsx-1 mediates BMP signals as a BMP target gene. To understand the molecular basis of ventralization triggered by BMP, we examined the role of its target gene Xmsx-1. First, we examined the effect of eve-Xmsx-1 or VP16-Xmsx-1 in early Xenopus embryo. Ventral overexpression of VP16-Xmsx-1 induced a secondary body axis phenocopying the effect of a dominant negative BMP receptor BMPRIA (ALK3), while similar overexpression of eve-Xmsx-1 had similar effects as wild type Xmsx-1. The dorsalizing effect was canceled by co-overexpression of Xmsx-1. These results suggested that Xmsx-1 is a transcriptional repressor and VP16-Xmsx-1 inhibited the *in vivo* function of Xmsx-1. Animal cap assay of VP16-Xmsx-1 expressing embryo indicated that ventral mesoderm and ectoderm markers were down-regulated and conversely, dorsal marker genes specific to organizers such as *goosecoid* and *chordin* were up regulated, which, in turn suggests that Xmsx-1 suppresses organizer genes in normal embryo. Taken together, these results suggest that organizer specific genes are not only positively regulated by dorsal signals by activin/Vg1/nodal but also negatively regulated by Xmsx-1.

Next we tested whether Xmsx-1 is required for the ventralization caused by BMP by coinjecting VP16-Xmsx-1 mRNA into ventralized embryo by BMP signal. Interestingly, none of ventralized embryos caused by a BMP ligand, BMP-4, a constitutively active form of a BMP receptor, ActRIB (ALK2) or an intracellular signaling component of BMP, Smad1 was rescued by coinjection of VP16-Xmsx-1 mRNA. Thus we propose that Xmsx-1 is sufficient for ventralization of embryo but not required for the ventralization by BMP signal.

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