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The impact of Spemann's concepts on molecular embryology

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In 1927 Hans Spemann wrote in his article *New work on the organizer:* "What has been achieved is but the first step; we still stand in the presence of riddles, but not without hope of solving them. And riddles with the hope of solution-what more can a scientist desire?" The problem referred to, which to date still waits for a solution, arose from an experiment that Hans Spemann and Hilde Mangold performed in 1924 where they transplanted the dorsal lip of a *Triturus* gastrula to the ventral side of a host gastrula (Spemann and Mangold, 1924). This manipulation resulted in the induction of a secondary body axis in the recipient embryo. Analysis of the secondary axis revealed that the histological composition of the induced structures was "chimaerisch", meaning composed of both graft and host tissue. The grafted material differentiated into notochord, portions of the somites and neural tube. Muscle and neural tissue consisted of both host and graft material. This observation lead to the conclusion that the dorsal lip is able to induce and organize mesodermal and neural tissue in the host.

The ability of the "Urmundregion" (blastopore lip) to act as an organizing center for cell differentiation had been observed earlier by Spemann, when he separated the cells of a 2-cell newt embryo using the ligature technique (Spemann, 1919). Only those blastomeres which contained the future "Urmund" developed an axis whereas the other blastomeres became a "Bauchstück" (bellypiece) without axis structures. To explain these results, one has to postulate an unequal distribution of maternal components in the fertilized egg and the early embryo. The questions that arose from these experiments were: What are the determinants responsible for the organizer activity? What activity induces the organizer?

Attempts to solve the organizer mystery can be grouped into 3 types of approaches: (1) the embryological, (2) the biochemical and (3) the molecular approach.

This article will focus mainly on the results that have been obtained by the use of molecular methods, which represent the most recent attempts to study embryonic induction and axis formation in vertebrates.

The embryological approach

Spemann and his colleagues focused in their work on the description of properties of tissues with organizing (axis-forming) activities. The thinking was based on the concept of morphogenetic fields that had been established by Gurwitsch (1923). One of the properties of the "Urmundregion" described by Spemann was that its inducing activity changed over time (Mangold and Spemann, 1927). Transplanted early dorsal lips from salamander embryos were able to induce secondary heads whereas late dorsal lips induced tails in the host. O. Mangold (1933) argued that the regionalization of the body axis (consisting of mesodermal and neural structures) is dependent on the regional position of the dorsal mesoderm derived from the dorsal lip. Based on these results, Spemann postulated two inducers: a head and a trunk-tail organizer. These inducers, present in the mesoderm, should be responsible for a regionally specified neural induction. Several groups tried to analyze the driving forces of what was called "morphogenetic fields" and many researchers postulated gradient models to explain their results, including Dalcq and Pasteels (1937) who brought forward the idea of a "cortical gradient" in the amphibian egg. Spemann was very

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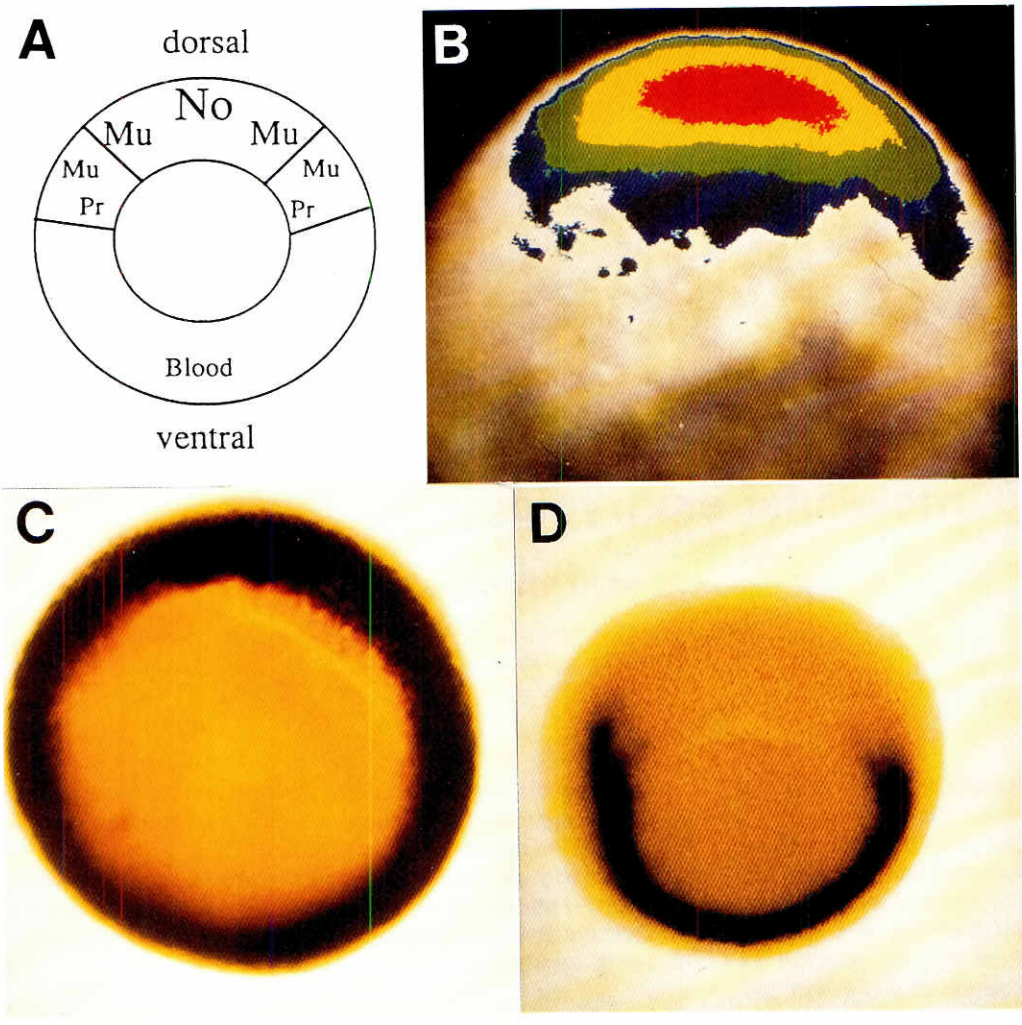

Fig. 1. Expression of *goosecoid, brachyury* and *Xwnt-8* mRNA in the *Xenopus* gastrula. (A) *Specification map of the early Xenopus gastrula as determined by explantation experiments (Dale and Slack, 1987). No, notochord; Mu, muscle; Pr, pronephros.* **(B)** *Graded distribution of gsc mRNA in the dorsal marginal zone of the stage 10 gastrula analyzed by whole-mount in situ hybridization. In this image red indicates the highest concentration of gsc mRNA.* **(C and D)** *Whole-mount in situ hybridization for Xbra* **(C)** *and Xwnt-8* **(D)** *in stage 10.5 Xenopus gastrulae.*

sceptical of the gradient theories and indeed, none of the models is without problems. The controversy went on until Nieuwkoop (1969) was able to demonstrate that Spemann's organizer is induced by an activity located in the dorso-vegetal region (endoderm) of the early embryo, a discovery that forms the basis for research on mesoderm and neural induction in recent years.

When members of Spemann's group tried to define more clearly the nature of the inducing activities, they made a quite surprising discovery. Dead tissue from various sources were able to induce organized axis structures when implanted into the blastocoel of a newt embryo (Bautzmann *et al.*, 1932). The article by Bautzmann spurred research to identify the inducing factor and led to what often has been called the 'chemical odyssey of the 30's' (Gilbert and Saxén, 1993).

The biochemical approach

The strategy used to characterize inducing factors biochemically was straightforward. Various candidate substances could be tested for their mesoderm and neural tissue-inducing activity by applying them to competent ectoderm. Løvtrup wrote in 1978 that: "few compounds other than the philosopher's stone have been searched for more intensely than the presumed agent of primary induction in the amphibian embryo" (Løvtrup *et al.*, 1978).

The experiments of Holtfreter (1933) had pointed towards a diffusible inducer and Needham and Waddington (1934) proposed lipids to be the active inducing component. Wehmeier (1934) claimed that oleic and nucleic acids were inducing substances whereas Barth and Graff (1938) brought forth the idea of inducing proteins. Another group of scientists explained the organizer activity as due to an increased metabolic rate in this region (Child, 1941). Based on the 'Einsteckexperiment' using boiled neural tissue, Holtfreter (1934) postulated that a "masked neural inducer or evocator" would be present in competent ectoderm while Waddington argued that an uniformly expressed evocator is activated locally in the embryo (Waddington *et al.*, 1936). The essence of the research in these years was a model involving two morphogenetic gradients as the basis for morphogenesis. This model involves overlapping mesoderm and neural inducing gradient fields creating regional specificity along the dorso-ventral and anterior-posterior axes (Toivonen and Saxén, 1955).

The goal of H. Tiedemann in Berlin, was to purify the mesoderm inducing and neuralizing factor who was able to isolate a

mesoderm inducing protein (vegetalizing factor) from chicken embryos which turned out to be activin (Tiedemann *et al.*, 1992). Based on the experiments of Nieuwkoop in *Xenopus* showing that dorsal vegetal blastomeres are able to induce mesodermal tissue in naive ectoderm, Grunz and Tacke (1986) demonstrated that this induction is caused by a diffusible factor(s) by placing a small pore sized filter between the signaling and responding tissue. Candidates for mesoderm inducing factors in *Xenopus* are TGF-β and FGF-like growth factors (reviewed by Kessler and Melton, 1994). Despite all these efforts, the entire process of mesoderm induction *in vivo* has not yet been untangled.

The molecular approach

Spemannn and many embryologists of his time were extremely sceptical of the genetic approach introduced by Thomas Morgan to answer ontogenetic questions. In his book written in 1938, Spemann avoided carefully the word "gene" but used terms like "Potenzschatz "or "Erbschatz" (inherited potential), bringing this controversy to an almost semantic level. The rapidly development of molecular biology techniques during the 1970s and 1980s provided a new set of useful tools for embryological research.

Cloning by sequence homology

This strategy takes advantage of conserved sequences between genes relevant for embryonic development that are shared among different species. The progress that has been made in *Drosophila* embryology to identify regulator genes for early development using genetic and molecular techniques has had a particularly fruitful influence on the study of amphibian embryogenesis. The discovery of homeobox genes in the frog was an important step in this direction (Carrasco *et al.*, 1984). In 1983 Sargent and Dawid reported a set of genes specifically expressed during gastrulation in *Xenopus,* and the group of J. Gurdon pioneered the use of molecular markers indicative of mesoderm induction (Sargent and Dawid, 1983; Gurdon *et al.*, 1984). To date, many genes have been cloned on the basis of their sequence homology to *Drosophila* genes, including homeobox genes and genes of the *Wnt* family, which have been demonstrated to play a important role in vertebrate development (Moon, 1993).

Cloning of genes with localized transcripts in eggs and embryos

During oogenesis, mRNA can be stored in a localized fashion in the egg, which can be the basis of embryonic axis formation as is the case in *Drosophila.* In 1987, Weeks and Melton reported the localization of *Vg1* mRNA in the vegetal pole of *Xenopus* oocytes using a differential screening procedure. cDNAs from animal and vegetal portions of oocytes were used to screen an egg cDNA library, a procedure which allows one to identify localized mRNAs in the egg (Weeks and Melton, 1987). Another approach is the construction of cDNA libraries from excised embryonic regions and the screening for genes preferentially expressed there. Using this technique, Blumberg *et al.* (1990) were able to identify the dorsal lip-specific homeobox gene *goosecoid* (gsc).

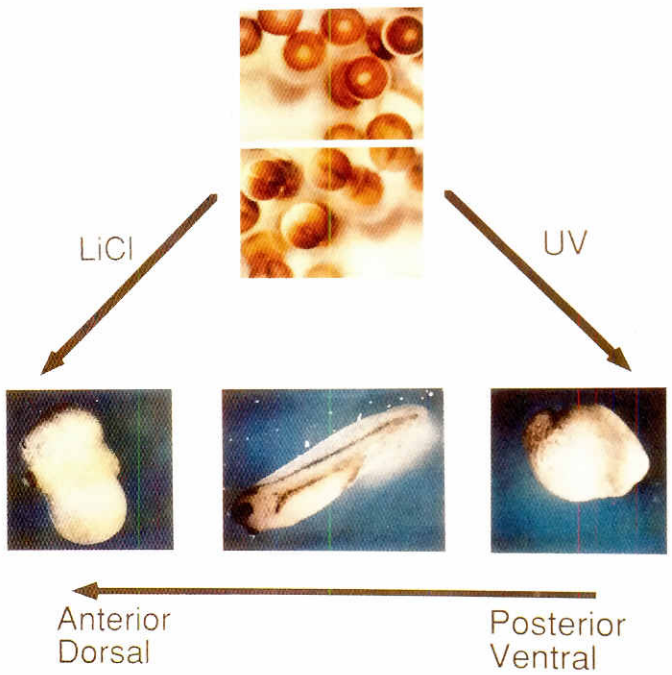

Fig. 2. Manipulation of the body axis in *Xenopus*. *Embryos can be dorsalized by exposure to LiCl or ventralized by irradiation of the fertilized egg. Dorsalization results in a loss of trunk- and tail-structures. Ventralization abolishes all axis-structures resulting in a 'bellypiece'.*

Subtractive and functional screening assay

This technique is designed to find differentially expressed genes that can compensate for developmental defects in mutant embryos. The *Xenopus* system permits one to manipulate embryonic axis formation experimentally (Fig. 2). Commonly used procedures include UV irradiation of fertilized eggs, which abolishes dorsal development (Scharf and Gerhart, 1983), and LiCl treatment of the early embryo, causing a hyperdorsalization (Kao *et al.*, 1986).

Harland and coworkers successfully used pools of mRNA from LiCl-treated embryos to rescue axis formation in embryos ventralized by UV, thereby identifying dorsalizing genes. In this screen, the dorsalizing secreted protein *noggin* as well as *Xwnt-8* were isolated (Smith and Harland, 1991, 1992). Sasai *et al.* (1994) cloned *chordin*, a secreted factor with dorsalizing activity, using a subtractive screening protocol in which dorsalizing genes enriched in LiCl-treated embryos were isolated. Refinements and modification of the techniques outlined above are a means by which to find other genes involved in early embryonic patterning.

Analysis of gene expression and function

The cloning of developmentally important genes is only the first step towards their functional analysis. A combination of embryological experiments developed by researchers like Spemann and molecular biological techniques developed more recently represent a new chapter in developmental biology. Three techniques in particular have proven immensely useful for studying gene function on a molecular level. First, mRNA can be microinjected into *Xenopus embryos* to obtain 'gain of function' or partial 'loss of function' phenotypes (Krieg and Melton, 1984).

Second, the non radioactive whole mount *in situ* hybridization technique developed by Harland and coworkers enables researchers to map gene expression within the embryo (Harland, 1991) (Fig. 1). Third, the availability of marker genes for embryonic tissue types or body regions allow an ultra-sensitive analysis of gene expression by state-of-the-art methods such as PCR (Rupp and Weintraub, 1991).

In the following section I will briefly summarize some molecular embryology data from *Xenopus*. The focus will be on (i) mesoderm induction and (ii) the role of genes expressed in the marginal zone involved in dorsal-ventral patterning of the mesoderm.

Mesoderm induction in *Xenopus*

Growth factors of the TGF-β and FGF families are involved in mesoderm formation which is induced in the equatorial region of the embryo (marginal zone) by the underlying vegetal cells. *Vg1*, a TGF-β type growth factor, is localized in the vegetal pole of the egg and the protein is abundantly expressed in the embryo (Weeks and Melton, 1987). To become an active inducer of dorsal mesoderm, the *Vg1* precursor protein has to be proteolytically processed to generate the mature, active peptide (Thomsen and Melton, 1993). Because of his localization and activity, *Vg1* is a strong candidate for the Nieuwkoop center activity which induces Spemann's organizer in the dorsal marginal zone. In order for this model to work, one has to postulate a localized protease activity which activates *Vg1* protein only on the dorsal side of the embryo. Such a factor has not yet been described. Activin, another TGF-β like growth factor, and its receptors are present in the egg and early embryo (Asashima *et al.*, 1991). Its role in mesoderm induction was demonstrated by injection of a dominant negative activin type 2 receptor (Hemmati-Brivanlou and Melton, 1992). In embryos overexpressing a mutant receptor which lacks the intracellular kinase domain, mesodermal tissue fails to develop. A similar approach involving the overexpression of a dominant negative FGF receptor mutant demonstrated the important *in vivo* role of FGF in mesoderm formation (Amaya *et al.*, 1991). More recent experiments have shown, that the activin signal requires a functional FGF signaling cascade (Cornell and Kimelman, 1994; La Bonne and Whitman, 1994). The mesoderm-inducing signals provided in the early embryo lead to the expression of a set of zygotic genes in the prospective mesodermal tissue. As a result, the mesoderm acquires dorso-ventral patterning, leading to the formation of dorsal tissues like notochord and muscle as well as ventral tissue such as blood. Using the whole-mount *in situ* method to detect mRNA expression of genes in specific regions of the developing mesoderm, one can produce a map of different types of mesoderm in the marginal zone and intensive research is underway to study the function and interaction of these genes.

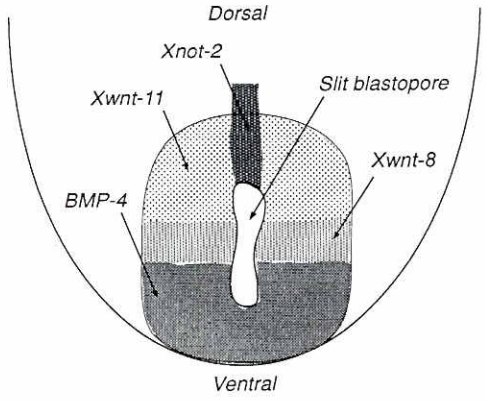

Fig. 4. Patterning of the slit blastopore marginal zone in *Xenopus*. *Diagrammatic representation of the main sites of expression of genes expressed around the slit blastopore (late gastrula). The different localization of these gene transcripts raises the possibility of their role in patterning of the mesoderm at this stage. The localization for Xnot-2 is from Gont et al. (1993), for Xwnt-11 from Ku and Melton (1993), for Xwnt-8 and BMP-4 from Fainsod et al. (1994).*

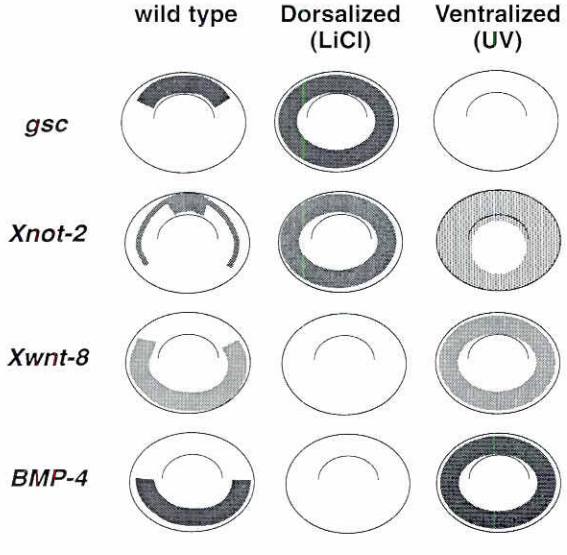

Fig. 3. Effect of LiCl- and UV-treatment on genes expressed in the marginal zone of the *Xenopus* gastrula. *Genes expressed dorsally such as gsc and Xnot are expanded in embryos treated with LiCl (dorsalized state) and repressed in UV-irradiated embryos (ventralized state). The field of expression of genes expressed ventro-laterally such as Xwnt-8 and BMP-4 is expanded towards the dorsal side in UV- and reduced in LiCl-embryos.*

Genes expressed in the marginal zone

A number of genes from different families are known to be expressed in Spemann's organizer at the early gastrula stage. Those genes are likely to be involved in the specification of dorsal mesoderm and neural tissue. They include the nuclear factors *goosecoid* (Cho *et al.*, 1991), *Xlim* (Taira and Dawid, 1992), *Xfdh* (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992), *pintallavis* (Ruiz i Altaba and Jessel, 1992), *Xnot-1* and *Xnot-2* (Gont *et al.*, 1993; von Dassov *et al.*, 1993), the cell adhesion molecule integrin α (Whitacker and De Somone, 1993), and the secreted factors *noggin* (Smith and Harland, 1992), *Xwnt 11* (Ku and Melton, 1993), *follistatin* (Hemmati-Brivanlou *et al.*, 1994) and *chordin* (Sasai *et al.*, 1994). Genes with a complementary expression pattern in the ventral marginal zone, which promote the formation of ventral mesodermal derivatives, are *Xwnt-8* and

BMP-4 (Jones *et al.,* 1991; Köster *et al.,* 1991; Dale *et al.,* 1992; Christian and Moon, 1993; Fainsod *et al.,* 1994). *Xbra* (Smith *et al.,* 1991) and *Xcad-3* (Northrop and Kimelman, 1993) are expressed in the entire marginal zone demarcating the mesoderm. One way to study the function of these genes is to analyze their expression pattern in embryos experimentally dorsalized or ventralized (Fig. 3). The organizer-specific genes with dorsalizing activity such as *gsc, noggin* or *chordin* are expanded in dorsalized and repressed in ventralized embryos (Cho *et al.,* 1991; Smith and Harland, 1991; Sasai *et al.,* 1994). Ventro-laterally expressed genes with ventralizing activity, such as *Xwnt-8* or *BMP-4,* show an expanded expression pattern in ventralized embryos and are repressed by hyperdorsalization (Christan and Moon, 1993; Fainsod *et al.,* 1994). A second approach to understand gene function is to overexpress genes ectopically by injecting synthetic mRNA or DNA constructs into defined regions of the developing embryo and analyze the expression pattern of putative target genes indicative of a change in dorso-ventral patterning of the embryo. From these type of experiments, the following scenario arises. First, the inducing factors define the prospective mesoderm before gastrulation. Second, at the beginning of gastrulation, *gsc, noggin* and *chordin* are expressed first in the dorsal region, thereby excluding the ventro-lateral genes from being expressed in the organizer and permitting the dorsal mesoderm to be formed. The activation of ventro-lateral genes then restricts the organizer from expanding so that ventral mesoderm can be formed. Key players in these interactions might be the secreted proteins such as *noggin, chordin,* members of the *Wnt* and *BMP* families. Strong evidence to support this assumption comes from experiments in which a dominant negative *BMP-4* receptor mutant was overexpressed in the ventral marginal zone that allowed the development of dorsal structures on the ventral side (Graff *et al.,* 1994; Suzuki *et al.,* 1994). The formation of a dorsal-ventral polarity in the mesoderm, therefore, is very likely the result of the interaction between a dorsalizing and a ventralizing center, an 'Organizer' and an 'Antiorganizer' (Sive, 1993; Fainsod *et al.,* 1994).

Finally, the availability of molecular markers that define the marginal zone enables us to study the phenomenon of 'head' and 'tail' organizer that has been proposed by Spemann. Using a combination of cell lineage tracing methods and marker gene expression in the late blastopore lip, the mechanism of tail formation is now better understood (Fig. 4). Gont *et al.* (1993) defined distinct populations of cells in the *Xenopus* tailbud that derived from the late blastopore, based on the expression of *Xnot 2* and *Xbra,* supporting the hypothesis that tailbud formation is a continuation of gastrulation.

Conclusions

The progress that has been made in the analysis of the Spemann organizer phenomenon in amphibia, and especially the molecular characterization of its activities, has had a strong influence on other vertebrate systems. Cloning and functional analysis of homologs of organizer-specific genes in zebrafish, chicken and mouse opened new doors in vertebrate embryology (De Robertis *et al.,* 1994). Despite all efforts, some of the questions raised by Spemann's experiments in the 1920 and 1930s remain unsolved and the amphibian gastrula is still, in Harrison's words, "a new Yukon to which eager miners were now rushing to dig for gold around the blastopore" (Gilbert and Saxén, 1993).

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