Continuity and change: paradigm shifts in neural induction

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ABSTRACT **The problem of "primary embryonic induction" was one of the first areas of developmental biology to become "molecularized." What had been seen as an intractable series of problems became amenable to the techniques of Northern blotting, ectopic RNA insertion, and *in situ* hybridization. These molecular analyses showed that some of the fundamental concepts of primary embryonic induction concluded by experimental embryologists were false. First, primary embryonic induction was not primary. The organizer tissue, itself, was the product of a prior induction. Second, the neural fate of cells was not being induced. Rather, the epidermal fate was induced and the neural state was the default, uninduced, fate of ectodermal tissues. Third, primary embryonic induction was not something unique to vertebrates. Rather, the ventral neural cord of insects formed using the same mechanisms as the dorsal neural tube of vertebrates. Fourth, the brain formed in a matter distinctly different from that the spinal cord. Despite these differences, there has been a clear and strong continuity between the experimental embryological tradition and the molecular genetic tradition, and these new results are seen by many contemporary developmental geneticists as strengthening, rather than destroying, the older science.**

KEY WORDS: *Organizer, Spemann, Mangold, neural induction, molecularization, history.*

Introduction: "Paradigms lost"

The induction of the amphibian central nervous system has long been the model for those cell and tissue interactions that form the vertebrate body axis. The hunt for the molecules active in the organizer in many ways framed the morphogenesis of the field of developmental biology. However, from the late 1930s to the mid-1980s, the "primary induction problem" was considered a graveyard of biologists, a problem so fraught with non-specificity, uninterpretable results, and conflicting data, that a young biologist would be foolish to enter the morass. Joseph Needham (1939) summarized the mood of those scientists who had been working on this problem:

"In conclusion, it may be said that although the progress made in the past ten years in these fields has been very great, we can nevertheless see now that owing to the special difficulties of the subject..., it may be more like fifty years before we can expect to have certain knowledge concerning the chemical nature of the naturally occurring substances involved in embryonic induction."

His fifty years turned out to be a prescient prediction. Near the end of that span of time, other investigators (see Jacobson, 1982) began to question the validity of the enterprise searching for these molecules:

"More than fifty years of effort have failed to reveal the putative inductor substances, nor has any progress been made in discovering the cellular mechanisms of release, transmission, reception, and interpretation of developmental signals that are supposed to result in regional differentiation."

Even Saxén, Toivonen, and Nakamura (1978), three of the few researchers whose laboratories continued to investigate primary embryonic induction during the 1970s, lamented, "Why do the scientists investigating embryonic induction lag behind their brilliant colleagues in many other areas of biology in which the 1960s and 1970s have witnessed many great victories and discoveries of fundamental importance?"

The reasons turn out to be quite simple and quite important. First, developmental biology had pushed its biochemical techniques to the limit. The proteins responsible for induction are often present in very low concentrations, and the embryos are not able to be obtained in enough volume to offset this disadvantage. Second, the amphibian embryo (on which experiments of neural induction were performed) contains large amounts of yolk and lipid which interfere with the purification procedures of traditional biochemistry (Grunz, 1997). It

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would take the tools of a sophisticated brand of molecular biology to find the inducer molecules and to delineate what these factors were doing. However, once the techniques of gene cloning and *in situ* hybridization opened the floodgates, we have been deluged with information about how these processes occur.

In fact, within the past five years, these techniques have occasioned three major paradigm shifts in this area. First, as we shall see, the paradigm for neural induction (since 1924) has been that soluble molecules are secreted by the organizer, a structure that comprises the pharyngeal mesendoderm that underlies the anterior head, the notochord that underlies the dorsalmost ectoderm of the remaining amphibian embryo, and the dorsal blastopore lip which gives rise to the notochord and pharyngeal mesendoderm. These soluble factors have been thought to actively instruct the ectodermal cells above it to become neurons. This was beautifully shown by experiments involving both positive and negative inference. Removing notochord from beneath an area that would otherwise become neural tube causes the ectoderm to become epidermal, while adding notochord to areas that would usually produce epidermis caused these cells to produce a new neural tube. So the ectoderm was seen as having two major fates: neural if underlain by the notochord, and epidermal if it were not underlain by the notochord. The default state was for these ectodermal cells to become epidermis. New information concerning the identity and nature of these factors secreted by the Organizer now causes scientists to think that the default state of the ectoderm is to become *neural*, and that the ectoderm cells are actively induced by the ventral and lateral mesoderm to become *epidermal*. The Organizer is now thought to act by blocking these epidermal-inducing molecules, thereby preventing the induction of the ectoderm above it. In the absence of this induction to epidermis, the dorsal ectoderm above the Organizer becomes committed to a neural fate.

The second paradigm brought about by our knowledge of the factors inducing neural and epidermal specification is that the specification of the insect ventral nerve cord and the vertebrate dorsal neural tube are accomplished by the same set of instructions. The two types of nervous systems develop in very different manners, and prior to 1995, it had been thought that the instructions to form these two types of nervous systems were very different. We now know that the instructions for specifying which region of the ectoderm is to form neural tissue are remarkably similar between these two highly diverged groups of organisms.

The third paradigm has been that this induction caused all the newly induced neural cells to initially assume the fate of forebrain tissue. Only after this initial "activation" would the "transformation" of this fate into midbrain, hindbrain, and spinochordal structures commence. This paradigm has also been called into question by the findings that different inducer molecules are produced by the cells in the region underlying the anterior head and that these molecules have functions distinct from those factors which induce the rest of the neural tube.

Primary embryonic induction

The problem of "primary embryonic induction" can be dissected into three sets of inductive events:
(1) What factors induce the formation of the Organizer? It turns out the cells of the Organizer are themselves induced (by the endodermal cells underlying it).

(2) What are the identities and functions of those molecules which are produced by the Organizer and which neuralize the ectoderm above it, dorsalize the mesoderm adjacent to it, and anteriorize the endoderm beneath it?
(3) How are the regions of the neural tube specified according to their respective anterior-posterior location along the axis?

This article looks at the central problem of these three, the question that historically has been considered as "the induction problem." We will therefore consider (1) how the "soluble inducing factors" have become purified molecules and (2) how knowledge of what these molecules do has changed our traditional ways of thinking about induction. We will focus our attention on the neuralizing property of the organizer, since, until recently, this function was the only one being extensively studied.

In 1924, Hans Spemann and Hilde Mangold revolutionized embryology with their discovery that a particular region of the embryo was responsible for the emergence of the central nervous system. They showed that when this region, the dorsal blastopore lip, was transplanted from one salamander embryo into the future ventral region of another such embryo, it invaginated completely. Moreover, the transplanted dorsal lip tissue (and no other transplant from the early-gastrula-stage embryo) produced a new notochord that caused the formation of a new neural tube and, subsequently, a secondary embryo (Spemann and Mangold, 1924; Hamburger, 1988; Fässler, 1996). They called this region the "Organizer" of the embryo. The Organizer, itself, did not contribute to the neural tissue. Rather, it formed the pharyngeal endoderm and dorsal mesoderm (notochord and somites) that lay beneath the cells that were to become the central nervous system. The cells of the new neural tube were derived primarily from the host ectoderm. Cells that otherwise would have remained epidermal were instructed to become neurons. The use of newts with differently pigmented eggs greatly facilitated their analysis of the five secondary embryos that resulted from their transplants. Of particular interest was transplant Um 132, wherein a dorsal blastopore lip from an advanced *Triturus cristatus* (unpigmented) gastrula was transplanted into the presumptive flank region of a more heavily pigmented, *T. taeniatus gastrula.* Sections taken from the tailbud larval stage showed that the donor cells became part of the notochord and somites of the secondary embryo, while many of the somites and most of the neural tube and other organs were derived from host tissues. Thus, a block of tissue from the dorsal blastopore lip was able to induce the formation of a secondary dorsal axis from host tissue. This induction subsequently initiated a cascade of other inductive events that led to the construction of the embryo (hence the use of the term 'primary embryonic induction' to describe this event).

The published account (Spemann and Mangold, 1924), displaying the terms induction and organizer prominently in its title, was based on only the first two years of research, those encompassing the 1921 and 1922 breeding seasons. This publication refers to only six cases. Sander (1993) has shown that there later were a total of the total 275 transplants, of which fifty-five survived the operation. Twenty-eight of these had prominent secondary neural axes, and eleven of these secondary axes were flanked by somites. Spemann and Mangold coined the term "Organizer" to emphasize the ability of this dorsal blastopore lip tissue to direct the development of the host tissue and to give these redirected cells a coherent, unified, organization.

"A piece of the upper blastoporal lip of an amphibian embryo undergoing gastrulation exerts an organizing effect on its environment in such a way that, if transplanted to an indifferent region of another embryo, it causes there the formation of a secondary embryonic anlage. Such a piece can therefore be designated as an organizer."

This tissue had the ability to invaginate and differentiate autonomously, to induce the neural plate and, by assimilative induction, to organize somites from the lateral plate mesoderm of the host.

This induction of the neural axis by the dorsal blastopore lip and its derivatives has also been demonstrated in *Xenopus laevis*, the amphibian of choice for most molecular studies (Gimlich and Cooke, 1983; Smith and Slack, 1983; Jacobson, 1984; Recanzone and Harris, 1985). The *Xenopus* organizer appears to be similar in many ways to the 'original' newt organizers, despite the difference in mesoderm formation between urodeles and anurans. Like the newt organizers, the *Xenopus* organizer field can regulate to induce secondary axes when split in half (Stewart and Gerhart, 1990).

Neural induction by diffusible molecules

The mechanism of induction was controversial from the start. Basically, the argument centered on whether the inductive signal from the dorsal blastopore lip was sent in a *cis*-fashion, anteriorly and horizontally through the plane of the ectodermal cells, or in a *trans*-fashion, from the dorsal mesoderm vertically to the overlying ectoderm. Spemann (1931; Mangold and Spemann, 1927) was originally in favor of a horizontally transmitted inducing signal that was sent by the dorsal blastopore lip from cell to cell through the ectoderm. However, evidence began pointing towards a diffusible inducer elaborated from the involuting mesoderm. First, Spemann's student Bruno Geinitz (1925) showed that dorsal blastopore lips transplanted into the blastocoel induced excellent secondary neural tubes, and another student, Alfred Marx (1925), showed that pure dorsal mesoderm from a late gastrula could induce the neural plate from the ectoderm, while pure ectoderm could not. This supported the view that signals were transmitted vertically from notochordal mesoderm to ectoderm. The article by Bautzmann *et al.* (1932; where each researcher presents his own rather preliminary set of experiments) demonstrated that dead tissue (including not only dorsal blastopore lip-derived mesoderm, but also dead embryonic intestine and epidermis) could act as organizer and cause the ectoderm to form brain tissue. Moreover, Johannes Holtfreter (op.cit. in Bautzmann *et al.,* 1932) showed that when ectoderm was isolated *in vitro* and wrapped around notochord, the ectoderm would form brain structures. Surprisingly, not only did killed dorsal blastopore lip tissue induce neural tissue from competent ectoderm, but so did killed endoderm, prospective epidermis, and even boiled uncleaved salamander egg. This was confirmed using the 'Einsteck-method' of implanting the potential inducing tissue inside the blastocoel directly beneath the ventral ectoderm. By these experiments, it appeared that the inducer was diffusible and that it could pass from an underlying inducing tissue. Holtfreter (1933) soon followed these observations with his exogastrulation experiments demonstrating that neural tissue failed to form when the mesoderm failed to contact the ectoderm. He also showed that induction was prevented when a sheet of vitelline envelope was placed between the chordamesoderm and the ectoderm. Thus, the inducing signal appears to be transmitted vertically, from the dorsal mesoderm to the ectoderm above it.

The Bautzmann *et al.* (1932) article initiated an enormous expansion of research which attempted to discover the identity of this inducing factor or factors. Løvtrup and his colleagues (1978) remarked 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*", and Harrison (quoted in Twitty, 1966) referred to the amphibian gastrula as a "*new Yukon to which eager miners were now rushing to dig for gold around the blastopore.*" In 1953, Niu and Twitty provided further evidence that diffusible factors from the dorsal mesoderm played a major role in primary induction. They put salamander dorsal mesoderm into a drop of culture medium. After conditioning this medium for several days, the mesodermal tissue was removed, and pieces of ectoderm were placed into the medium. These ectodermal explants became neural cells and pigment cells.

Such diffusible molecules were even more clearly demonstrated in a series of transfilter experiments. In 1961, Lauri Saxén demonstrated that neural induction could occur through a 150 micron thick, 0.8 micron pore size filter, strongly suggesting that the inducer was diffusible. Sulo Toivonen and coworkers (1975, 1976) extended this work by showing that neural induction took place through a Nucleopore filter even though electron microscopy failed to reveal any intercellular contact through the 0.05 um pores. The biochemical purification of this *Induktionsstoffe* had been part of the Finnish laboratory's program, starting with Toivonen's student, Taina Kuusi (1951). It also became the focus of the Tiedemanns' ongoing research program (Tiedemann and Tiedemann, 1964; Tiedemann *et al.*, 1963; 1992) who showed (Born *et al.*, 1989; Janeczek *et al.*, 1992) that neuralizing factors from amphibian embryos may be isolated as large complexes and remain active when complexed to Sephadex beads. This suggests that the factor(s) act by binding to membrane rather than by entering the cell.

The Japanese program for the study of embryonic induction also concentrated efforts in finding inducer substances. Begun by Professors Yo Okada and Osamu Nakamura, this work is being continued by professor Makamoto Asashima, himself a student of Heinz Tiedemann. Eventually, it was Asashima's work on activin which culminated the biochemical search for the organizer molecules. His work linked the Japanese studies to those of the German group by showing that the caudalizing factor isolated in the German laboratory was the same as the activin-like factor found by Japanese researchers (Asashima *et al.*, 1992). Later, activin became more important as a candidate for a factor which induced the organizer to form in the mesoderm (Ariizumi and Asashima, 1994; Gurdon *et al.*, 1994).

Other laboratories, such as the Cambridge-based group of C. H. Waddington and Joseph and Dorothy Needham, attempted to find the inducer by seeing which natural substances could induce neural plate formation when added to competent ectoderm or implanted into the blastocoel (see Abir-Am, 1991). In their work of 1933 and 1935, Waddington and the Needhams showed that ether extracts of adult newts could act as an organizer. Since this activity could turn presumptive epidermis into non-specific neural tissue, Waddington called this activity as the "evocator." (The molecules specifying the type of neural tissue were referred to as the "individuators.") The properties of the evocator fraction suggested

that it was a steroid, and both natural and artificial steroids were found to induce neural plates. A steroid inducer made a great deal of sense (Needham, 1936), since sterols had been found to be the basis for male and female sex hormones, cancer-producing hydrocarbons, cardiac glycosides, and vitamin D. Moreover, such sterol compounds had been found in eggs. It was expected that steroid-like hormones would function in early development just like they did during later development.

The problem of non-specificity

However, sterols were not the only chemicals that induced neural development. One of the reasons for the lack of knowledge about these inducer molecules was the lack of a stringent assay system. It appeared that numerous totally unrelated compounds could induce neural development from the ectoderm of early salamander gastrulae. Following the Bautzmann *et al.* paper, the strategy during the 1930s was simple and straightforward: the normal target tissue, the competent ectoderm, was exposed to various candidate molecules, and the results were monitored as morphologically distinguishable secondary structures after prolonged cultivation. At first, progress was stimulating, and scientists in various laboratories reported successful induction with various purified compounds. The initial reports that natural lipid molecules could induce neural tubes initially caused great excitement, and Waddington and Joseph Needham spent over three years attempting to biochemically characterize the active agent in the ether extracts. However, some of the neural-inducing molecules were so unlike one another that there seemed to be no structural specificity. To further complicate matters, the German workers claimed that acids (oleic, linoleic and nucleic) initiated induction (Wehmeier, 1934), and Barth's experiments (1939) implicated a protein inducer. If this were not confusion enough, Waddington and colleagues (1936) showed that unnatural compounds that did not even resemble naturally occurring molecules were able to induce neural formation in the ectoderm. Even a dye, methylene blue, induced neural tubes. As Waddington and Dorothy Needham end their discussion to one of their articles in 1935:

> "*Dodds has metaphorically spoken of these synthetic substances as skeleton keys, which can unlock several doors... Here the skeleton key is so unlike the householder's latchkey that one wonders whether the house has been entered through the back-door, or, in an even more unorthodox manner, through a window.*"

In 1936, Waddington, Needham, and Jean Brachet hypothesized that the evocator substance might be produced throughout the embryo, but it was just released or activated in one particular region. This fitted well with Holtfreter's (1933) discovery that non-inducing regions of the amphibian gastrula could acquire the ability to induce when they were killed by ethanol treatment. Herrmann [1960] has called this period "*the chemical Odyssey of the 1930s*" and it is recounted in Needham (1959) and in Saxen and Toivonen (1962).

The molecularization of the Organizer

In 1962, Waddington reinterpreted induction and inducers in terms of molecular biology. In particular, he linked embryonic induction to enzymatic induction. Inducible enzymes had been called adaptive enzymes until the early 1950s, and their relationship to development had been proposed by Jacques Monod as early as 1947. Monod saw the phenomenon of enzymatic adaptation as a possible solution to the problem of how identical genomes could synthesize different "specific" molecules. That same year, another researcher in this field, Sol Spiegelman redefined embryonic differentiation as "the controlled production of unique enzyme patterns." He altered the terminology of the adaptive enzyme studies, claiming that such enzymes were *induced*. He thus took the notion of "adaptive enzymes" out of the domain of evolution (where they seemed Lamarckian anyway) and into the domain of embryology. In 1953, the major researchers in this field agreed, signing a joint letter to *Nature* (Cohn *et al.*, 1953). The directed enzymatic synthesis would be known as "enzyme induction" and "any substance thus inducing enzyme synthesis is an enzyme 'inducer'."

When the Jacob and Monod model for the *lac* operon was reported, Waddington immediately saw the importance of enzymatic induction for studies of embryonic induction (1962; see Gilbert, 1996). He even made a diagram based on primary embryonic induction wherein the evocator (inducer) would diffuse from the mesoderm and either act directly on the structural genes or else combine with a regulator substance to make the factor that activated the genes by binding to a promoter region. However, the number, kind, and functions of these possible inducer molecules were totally unknown.

Understanding the biochemical mechanisms of induction would have to wait until the techniques of molecular biology. By the late 1980s, several developmental biologists felt that molecular biology had finally something to offer them. Fred Wilt (1987) urged that the time had come for molecular biology to try explaining development, and John Gurdon (1987) concluded, "*Nucleic acid technology has probably now reached a sufficient level of precision and efficiency of operation to be usefully applied to the analysis of inductive responses...*".

The first of these better techniques was the *Xenopus* assay systems that had been pioneered by Gurdon. Unlike the salamanders and toads used previously, the ectoderm of *Xenopus laevis* fails to respond to non-specific neural inducers (Kintner and Melton, 1987; Ruiz i Altaba, 1992). Thus, the problem of non-specificity was avoided when *Xenopus* was used. For a long while, it did not seem like anything induced neural tube formation in these frogs, and frustration mounted. Indeed, it first appeared that neural induction in *Xenopus* did not take place through the vertical induction system at all. Rather, the evidence from *Xenopus* suggested that induction was through the plane of the ectoderm. In the 1980s and early 1990s, several laboratories had shown that the *Xenopus* ectoderm is heterogeneous with respect to its neural competency, and that this difference is generated both by cell autonomous differences between cleavage-stage blastomeres (Kageura and Yamana, 1983, 1984; Akers *et al.*, 1986; London *et al.*, 1988; Gallagher *et al.*, 1991) and by a signal emanating in a cis-fashion from the newly formed dorsal blastopore lip (Sharpe *et al.*, 1987; Savage and Phillips, 1989). *Xenopus* exogastrulae (of the kind that Holtfreter made with newt embryos) express NCAM, NF3, and Xhox3, three antigens found within induced ectoderm but not in presumptive epidermal tissue (Kintner and Melton, 1987; Dixon and Kintner, 1989; Ruiz i Altaba, 1990). Using a modified sandwich technique that prevented the dorsal mesoderm from vertically

contacting the ectoderm, Doniach and coworkers (1992) and Ruiz i Altaba (1992) both showed that four position-specific neural markers were expressed in the explant ectoderm in the appropriate anterior-posterior sequence. Similarly, Keller and coworkers (1992) demonstrated that planar signals from the early gastrula dorsal blastopore lip are both necessary and sufficient to induce convergent extension and NCAM expression in the presumptive hindbrain-spinal cord ectoderm directly adjacent to it. However, this ectoderm did not roll into a tube or form the dorsal-ventral pattern typical of the normally induced neural tube. These latter functions have been ascribed to the notochord (Holtfreter, 1933; Smith and Schoenwolf, 1989; van Straaten, 1989; Yamada *et al.*, 1991) and probably represent actions of the underlying mesoderm upon the overlying ectoderm.

These experiments were criticized by Gerhart and his colleagues (1989), Sharpe and Gurdon (1990), and by Hemmati-Brivanlou and coworkers (1990). Using different procedures, they halted the migration of the mesoderm into the embryo at various stages of gastrulation. When dorsal mesoderm (notochordal) invagination was stopped at the onset of gastrulation, no dorsal axis was formed. However, when the invagination was halted midway through gastrulation, only the anterior structures were missing. Inhibiting the last movements of gastrulation had little or no effect on axis formation. This suggested that vertical, *trans*, signals from the mesoderm were indeed critical for the development of the dorsal axis. Doniach and her colleagues (1992) hypothesized that while the planar signals might be most important early in gastrulation, the *trans*-inducing signals from the notochord might be essential in reinforcing this pattern and bringing the mesodermal and ectodermal axial patterns into register with one another. So in 1992 it looked like the paradigm of vertical induction from the notochord to the ectoderm had reached an impasse. No soluble factors had been found, and a different source of inductive agency had been proposed.

Solving the soluble

This impasse was broken in 1993. Earlier, Smith and Harland (1991, 1992) had isolated a gene whose product appeared to dorsalize the mesoderm. This gene, *noggin*, was found by constructing a cDNA plasmid library from dorsalized (lithium-treated) gastrulae. RNAs synthesized from sets of these plasmids were injected into the ventralized embryos (having no neural tube) produced by irradiating early embryos with ultraviolet light. Such UV-treated embryos have no dorsal blastopore lip, no notochord, and no organizer activity. Those sets of plasmids whose RNAs rescued the dorsal axis were split into smaller sets, and so on, until single plasmid clones were isolated whose mRNAs were able to restore the dorsal axis in such embryos. One of these clones encoded noggin. Smith and Harland (1992) have shown that newly transcribed (as opposed to maternal) *noggin* mRNA was first localized in the dorsal blastopore lip region and then became expressed in the notochord . Moreover, if the early embryo were treated with lithium chloride (LiCl) so that the entire mesodermal mantle became notochord-like organizer tissue, then *noggin* mRNA was found throughout the mesodermal mantle. Treatment of the early embryo with ultraviolet light inhibited the synthesis of *noggin* mRNA. Injection of *noggin* mRNA into 1-cell, UV-irradiated embryos completely rescued the dorsal axis and allowed the forma-

tion of a complete embryo. The mRNA sequence for the noggin protein suggested strongly that it is a secreted protein.

In 1993, Smith and colleagues found that noggin protein could accomplish two major functions of the organizer: it induced neural tissue from the dorsal ectoderm, and it dorsalized the mesoderm cells that would otherwise contribute to the ventral mesoderm. Moreover, the noggin protein was also able to induce neural tissue in gastrula ectoderm without the presence of any dorsal mesoderm (Lamb *et al.*, 1993). When noggin was added to gastrula (or animal cap) ectoderm, the ectodermal cells are induced to express forebrain-specific neural markers. Moreover, the gene products for notochordal or muscle cells were not induced by the noggin protein.

The second candidate was a protein called chordin. Chordin was also isolated by using dorsalized (lithium-treated) embryos. Here, duplicate filters containing members of a plasmid library constructed from normal dorsal blastopore lip cDNA were hybridized to radioactive probes from either dorsalized or vegetalized embryos. This technique isolated clones whose mRNAs were present in the dorsalized but not in the ventralized embryos. These clones were tested by injecting them into ventral blastomeres and seeing if they induced secondary axes. One of the clones capable of inducing a secondary neural tube contained the chordin gene. The *chordin* mRNA was found to be localized in the dorsal blastopore lip and later in the dorsal mesoderm of the notochord (Sasai *et al.*, 1994).

The third candidate for an inducer molecule was follistatin. This molecule was found through an unexpected result in an experiment designed to determine whether the growth factor activin was critical for mesoderm induction (see Asashima *et al.*, 1992). Ali Hemmati-Brivanlou and Douglas Melton (1992, 1994) constructed a dominant negative activin receptor and injected it into embryos. Remarkably, the ectoderm began to express neural-specific proteins. It appeared that the activin receptor (which also binds other structurally similar molecules such as the bone morphogenetic proteins) normally functioned to bind an inhibitor of neurulation. By blocking its function, all the ectoderm became neural. In 1994, Hemmati-Brivanlou and Melton proposed a "default model of neurulation" whereby the organizer would produce inhibitors of whatever was inhibiting neurulation. This model was supported by and explained the cell dissociation experiments which had produced odd results. Here three laboratories, Grunz and Tacke (1989), Sato and Sargent (1989), and Godsave and Slack (1989) had shown that when whole embryos or their animal caps are dissociated, the isolated ectodermal cells expressed neural markers. This would be explainable if the "default state" was not epidermal, but neural, and that the tissue would have to be induced to have an epidermal phenotype. The organizer, then, would block this epidermalizing induction.

Since mutated activin receptors caused neural tissue to form, it was thought that natural activin inhibitors might be used by the embryo in a similar manner to specify the neural ectoderm. One of these natural inhibitors of activin (and its related compounds such as bone morphogenesis proteins) is follistatin. Using *in situ* hybridization, Hemmati-Brivanlou and Melton (1994) found the mRNA for follistatin in the dorsal blastopore lip and notochord.

So it appeared that there might be a neural default state and an actively induced epidermal fate. This was counter to the neural induction model that had preceded it for 70 years. But what proteins

were inducing the epidermis, and were they really being blocked by the molecules secreted by the organizer?

The leading candidate appeared to be bone morphogenetic protein-4 (BMP4). There appeared to be an antagonistic relationship between BMP4 and the dorsal mesoderm. If the mRNA for BMP4 were injected into 1-cell *Xenopus* eggs, all the mesoderm in the embryo became ventrolateral mesoderm, and no involution occurred at the blastopore lip (Dale *et al.*, 1992; Jones *et al.*, 1992). Moreover, when animal caps from embryos injected with *bmp4* mRNA were isolated and implanted into the blastocoels of young *Xenopus* blastulae, they caused the formation of an extra tail. Conversely, overexpression of a dominant-negative BMP4 receptor (which should block BMP4 reception) resulted in the formation of two dorsal axes (Graff *et al.*, 1994; Maeno *et al.*, 1994). Thus, BMP4 seemed to be able to override the dorsal signals. In 1994, a set of studies headed by Eddy De Robertis discovered that chordin specifically interfered with BMP4 (Sasai *et al.*, 1994; Holley *et al.*, 1995).

BMP4 is initially expressed throughout the ectoderm and mesodermal regions of the late blastula. However, during gastrulation, *bmp4* transcripts are restricted to the ventrolateral marginal zone (Hemmati-Brivanlou and Thomsen, 1995; Northrop *et al.*, 1995). The BMP4 protein induces the expression of several transcription factors (Xvent-1, Vox, Mix.1, Xom) that are key regulators of ventral mesodermal and ectoderm development. These transcription factors induced by BMP4 repress dorsal genes while at the same time activate ventrolateral mesodermal proteins (Gawantka *et al.*, 1995; Hawley *et al.*, 1995; Mead *et al.*, 1996; Schmidt *et al.*, 1996). Wilson and Hemmati-Brivalou (1995) also showed that the addition of BMP4 to dissociated ectoderm cells prevented them from becoming neural. Thus, by 1996, it seemed that BMP4 was the active inducer of ventral ectoderm (epidermis) and ventral mesoderm (blood cells and connective tissue), and that chordin would prevent its function. The Organizer worked by secreting an inhibitor of BMP4, not by directly inducing neurons.

This hypothesis obtained further credence from an unexpected source—the emerging field of evolutionary developmental biology. De Robertis and his laboratory (Holley *et al.*, 1995; De Robertis and Sasai, 1996) found that the same chordin-BMP4 interaction that instructed the formation of the neural tube in vertebrates also formed the neural tube in flies. The dorsal neural tube of the vertebrate and the ventral neural tube of the fly appeared to be generated by the same set of instructions, conserved throughout evolution. De Robertis and Sasai (1996) even resurrected E. Geoffroy St. Hilaire's (1822) discussion (and illustration) of the lobster being but the mouse upside-down and that all animals might be variations upon a common theme. This was the second paradigm shift occasioned by the newly acquired information on the molecular biology of induction.

Several laboratories found that noggin, follistatin, and chordin each prevented the BMP4 proteins from either maturing or binding to the prospective dorsal cells (De Robertis and Sasai, 1996; Piccolo *et al.*, 1996; Sasai *et al.*, 1996; Zimmerman *et al.*, 1996. The Organizer was not so much an inducer as the structure that protected cells from being induced. The neural state was not that which was achieved by induction, but was that fate that was not induced.

Getting a head

The third paradigm concerned the nature of the induced neural tissue. It was thought that all the neural tissue induced by the

organizer was of forebrain specificity and that the Organizer initially used the same activator/evocator molecules throughout its length (Waddington, 1940; Nieuwkoop, 1952; for review, see Gilbert and Saxén, 1993). But the most anterior portion of the Organizer appears to be different from the rest. Whereas most of the dorsal ectoderm is underlain by notochord, the most anterior regions of the head and brain are underlain by anterior pharyngeal endomesoderm. This endomesoderm constitutes the first cells of the dorsal blastopore lip. Recent studies have shown that these cells not only induce the most anterior head structures, but that they do it using a mechanisms distinct from blocking BMP4.

In 1993, Christian and Moon showed that Xwnt8, a member of the Wnt family of growth and differentiation factors, also inhibited neural induction. Xwnt8 was found to be synthesized throughout the marginal mesoderm—except in the region forming the dorsal blastopore lip. Thus, a second anti-neuralizing secreted protein had been found.

In 1996, Bouwmeester and colleagues showed that the induction of the most anterior head structures could be accomplished by a secreted protein called Cerberus. Unlike the other secreted proteins, cerberus promoted the formation of the cement gland, eyes, and olfactory placodes. When *cerberus* mRNA was injected into the vegetal ventral set of *Xenopus* blastomeres at the 32-cell stage, ectopic head structures were formed. These head structures were made from the injected cell as well as from neighboring cells. The *cerberus* gene was found to be expressed in the endomesoderm cells that arise from the deep cells of the early dorsal blastopore lip. It was not found throughout the notochord. Two things this protein did were to bind both BMPs and Xwnt8 (Glinka *et al.*, 1997).

Shortly thereafter, two other proteins, Frzb and Dickkopf, were discovered to be synthesized in the involuting endomesoderm. Frzb ("frisbee") is a small soluble form of the Wnt receptor protein which is capable of binding Wnt proteins in solution (Leyns *et al.*, 1997; Wang *et al.*, 1997). If embryos are made to synthesize excess frzb, the Wnt signaling pathway fails to occur, and the embryos lack ventral posterior structures, becoming solely head. The Dickkopf protein also appears to interact directly with Wnt proteins extracellularly. Injection into the blastocoel of antibodies against Dickkopf protein causes the resulting embryos to have small deformed heads (Glinka *et al.*, 1998).

Glinka and colleagues (1997) have thus proposed a new model for embryonic induction. The induction of trunk structures may be caused by the blockade of BMP signaling from the notochord. However, to produce a head, one needs to block both the BMP signal and the Wnt signal. This blockade comes from the endomesoderm, now considered the most anterior portion of the Organizer. Interestingly, in 1931, Spemann thought that there might be two organizers, one for the head and one for the trunk. After 1933, he did not push this view further.

Layers of complexity: a summary of the organizer

Alfred North Whitehead (a philosopher who had a major influence on the British embryologists investigating the Organizer) wrote in 1920 that the motto of every scientist should be "Seek simplicity and distrust it." Modern studies of the Organizer and its molecules show that this was very good advice. First, "primary embryonic induction" turns out neither to be primary nor an induc-

tion. The presumptive dorsal endoderm forms a region, "the Nieuwkoop Center", which induces the organizer to form in the mesoderm above it. The Nieuwkoop Center, itself, is formed in a complex combinatorial way. First, the cortical rotation accompanying fertilization displaces the Disheveled protein to the future dorsal side of the embryo. This protein then diffuses from the dorsal cortex and stabilizes β-catenin in the dorsal portion of the embryo. A second protein, GBP, also appears to stabilize β-catenin predominantly in the dorsal side of the embryo (Miller *et al.*, 1999; Farr *et al.*, 2000). Thus, β-catenin is able to be a transcriptional co-activator in the dorsal part of the embryo, and there is a gradient of this protein tapering off into the lateral and vegetal regions. Second, VegT or Vg1, two proteins whose messages are found throughout the vegetal cells, interact with β-catenin to induce in the endoderm a gradient of *Xenopus* nodal related (Xnr) proteins. VegT, Vg1, and the Xnrs are all TGF-β family proteins, but the gradient of Xnrs appears to be the critical step in specifying the regional abilities of the mesoderm above it (Agius *et al.*, 2000). The gradient is highest in the endoderm that lies underneath the organizer. Thus, the Nieuwkoop Center is most likely defined by Nodal proteins formed by the intersection of the stabilized β-catenin (marking dorsal) and the TGF-β factors (such as VegT and Vg1) making vegetal.

The Nodal proteins and β-catenin then appear to specify the mesoderm above the Nieuwkoop Center to become the organizer. Here, the paucity of Nodal proteins allows the ventral mesoderm to synthesize BMP4 and XWnt8 (and perhaps other members of these families), while it induces the dorsal mesoderm to activate the Goosecoid (and probably other) genes. Again, there appears to be a combinatorial mode of activation. The β-catenin proteins enable to transcription of the Siamois gene, a gene that produces a transcription factor that binds to the promoter of the goosecoid gene. This protein is critical, but not sufficient to induce goosecoid expression. Another transcription factor appears to be induced by the high levels of Nodal expression. The Siamois and the nodal-derived transcription factors induce the expression of Goosecoid, and the Goosecoid protein and the Goosecoid gene activates the chordin, noggin, and other genes of the organizer (Brannon and Kimelman, 1996; Fan and Sokol, 1997; Kessler, 1997). These studies provide the mechanism behind Pieter Nieuwkoop's (1969) observations that if one rotated the vegetal hemisphere and placed it beneath an animal cap, the polarity of the animal cap mesoderm would similarly be altered.

The BMPs appear to be produced in a gradient inversely corresponding to the gradient of Nodal proteins in the endoderm. High amounts of BMP in the mesoderm produced blood and lateral plate mesoderm, slightly higher levels produce kidney and other intermediate mesoderm, while low levels produce the dorsal mesoderm. In the ectoderm, high BMP levels produce skin, a low level produces neural crest cells, and the very low or non-detectable levels produce the CNS. The BMPs are aided by the products of the Xolloid and BMP1. These two proteins appear to be found throughout the embryo and they will bind and degrade Chordin. Therefore, the gradient of BMP4 from ventral to dorsal is established by the antagonistic interactions of Xolloid and BMP1 (increasing BMP4) and chordin, Noggin, and Follistatin (decreasing BMP4). The same process is also seen in *Drosophila*, where Tolloid degrades Short-gastrulation protein and creates a gradient of DPP (Marqués *et al.*, 1997; Piccolo *et al.*, 1997).

Head formation, as mentioned above, appears to be regulated by the inhibition of both the BMP and the Wnt signaling pathways. But this is not the full story of head induction. There are other factors, such as Anti-dorsalizing morphogenetic protein (ADMP) that is found in the trunk organizer and which seems to prevent the trunk organizer from forming the head. It is inhibited by follistatin, but not by Noggin, Chordin, or Cerberus (Dosch and Niehrs, 2000). Consistent with their roles in head formation, the mRNAs for the Wnt inhibitors cerberus, frzb, and dickkopf are found in the prospective anterior endoderm and prechordal plate, while the BMP inhibitors (as well as ADMP) are found in all the organizer derivatives. The anterior mesendoderm appears to be brought up to the anterior end of the dorsal blastopore lip region by the vegetal rotation of the prospective endoderm cells at the beginning of gastrulation (Winklbauer and Schürfeld, 1999).

The case against planar signals from the dorsal blastopore lip being important in the anterior-posterior neural patterning was made by Chen *et al* (2000) who found that exogastrulae did not form neural tissue outside a confined region where the ectoderm met endomesoderm. However, planar signals from the posterior of the embryo may be critical in specifying neural pattern. These signals include embryonic FGF (which may be induced by the posterior notochord and which may activate caudal gene expression), retinoic acid (which may activate posterior Hox genes), and Wnt3a (Pownall *et al.*, 1996; McGrew *et al.*, 1997; Kolm and Sive, 1997; Taira *et al.*, 1997). Summaries of recent studies on the organizer can be found in Grunz, 1999; Nieto, 1999; and Weinstein and Hemmati-Brivanlou, 1999).

Conclusions

The past five years has seen the overturning of three major paradigms in the field of primary embryonic induction, one of the most central areas of all developmental biology. These paradigms have been replaced by new models that have yet to be fully tested. The research into primary embryonic induction provides a fascinating example of how molecular biology can challenge the core paradigms of a central portion of a science without disrupting that discipline. The field of developmental biology was not shaken to its foundations by the revelations of these molecular approaches. Rather, the molecular biology was seen to be at a deeper level, and it served to explain some of the outlying phenomenon (such as the neuralization of dissociated ectoderm) as well as explaining the critical observations of earlier investigators. Rather than pushing the older research into the dust-bins of history, the molecular researchers cited the older literature whose studies they extended, if not confirmed. The textbooks of developmental biology emphasized the connections between classical embryology and molecular developmental biology, and Viktor Hamburger's (1988) volume, *The Heritage of Experimental Embryology* was written explicitly *"to provide a reliable guide that may be useful to developmental biologists who are willing to accept the challenge of redefining the old problems in the new language of cellular and molecular developmental biology and to address them with the sophisticated methodology at their disposal."* The stability of developmental biology during the paradigm shifts in neural induction has shown how continuity with the past can be maintained while the field undergoes a profound metamorphosis. The "tradition of inquiry" has become an important unit in the development of the discipline.

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