Pieter Nieuwkoop's contributions to the understanding of meso-endoderm induction and neural induction in chordate development

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ABSTRACT **Pieter Nieuwkoop, who died September 18, 1996, at age 79 in Utrecht, The Netherlands, is remembered by developmental biologists for his numerous research contributions and integrative hypotheses over the past 50 years, especially in the areas of neural induction, meso-endoderm induction, and germ cell induction in chordates. Most of his experimentation was done on the embryos of amphibia, the preferred vertebrate embryo of the early years of the 20th century. One of his last publications contains a comparison of the experimental advantages and disadvantages of anuran and urodele amphibians (Nieuwkoop, 1996). The significance of his findings and interpretations for developmental biology can be estimated from the fact that researchers of many laboratories worldwide continue to work on the phenomena he first described and to extend the hypotheses he first formulated. The aim of this article is to review Nieuwkoop's main contributions and to cite the recent extensions by others.**

KEY WORDS: *Nieuwkoop center, Spemann's organizer, meso-endoderm induction, neural induction, germ cell induction*

As described elsewhere in this issue, Pieter Nieuwkoop was born in Enschede, The Netherlands, and began his doctoral studies shortly before WWII at the State University of Utrecht under the supervision of Prof. Chr. P. Raven (who had trained with M.W. Woerdeman, who had trained with H. Spemann). His thesis, written in English after the war and published in 1946, concerned the determination of germ cells and the development of the germinal ridges in urodeles (Nieuwkoop, 1946), a subject to which he returned in later years. For many of us, our first acquaintance with Pieter Nieuwkoop's early work comes with the "*Normal Tables of Xenopus laevis (Daudin)*" published in 1956 with J Faber, an enduring volume now reprinted (Garland Publishing Co.). The book contains not only their original observations of morphogenesis and organogenesis, but also a compilation of the literature on the external and internal anatomy of embryos and tadpoles, and on the breeding and care of frogs. The availability of this book and the great progress made by Pieter Nieuwkoop using *Xenopus*, helped to bring this anuran into widespread laboratory use. During this period of early work, Nieuwkoop and Florschütz (1950) studied *Xenopus* gastrulation in detail and found that most if not all mesoderm precursor cells are located internally in the early gastrula, and that these precursors involute around a previously unrecognized internal blastopore 1-2 h before the surface endoderm cells involute at the visible external blastopore. In urodele embryos and even other anurans, mesoderm precursor cells are largely or wholly located on the surface of the early gastrula and involute around the external blastopore, like the endoderm, during gastrulation. These unusual aspects of *Xenopus* gastrulation were later analyzed in detail by Ray Keller and his colleagues (e.g., see Minsuk and Keller, 1996).

Neural induction

Pieter Nieuwkoop's first major contribution to early development came with his 1952 study of neural induction in urodeles. The subject of neural induction remained a foremost interest of his throughout his career. Over the years, his familiarity with the anatomy of the amphibian nervous system became so great that he was one of the few researchers of recent decades who could write in the methods section of a paper (and have it accepted), "...the authors did not use molecular markers because the first author, having more than 50 years of experience in normal and atypical histology, is perfectly sure of the correct identification of all the definitive larval structures. The reliance on molecular markers *[by others]* has actually given rise to misinterpretations...in several recent studies..." (Nieuwkoop and Koster, 1995).

Nieuwkoop devised in 1952 a novel surgical method for inserting flaps of ectoderm into the dorsal midline of the neural plate of

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an early neurula embryo at different anteroposterior levels (Nieuwkoop *et al.,* 1952). Later he scored the kinds and arrangements of neural tissues formed by the flap which protruded from the then-differentiated neural tube. Anterior neural structures (forebrain) developed at the distal end of the flap, whereas posterior neural structures developed at the base, and matched those of the level of the neural tube at which the implant protruded. This and other experiments (by his student H. Eyal-Giladi) led him to propose that the entirety of inductive neural patterning is accomplished in the anteroposterior dimension by two sequentially acting factors:

1) An activating factor which causes a neuralization of ectoderm. If no other induction follows, the neuralized tissue differentiates only anterior neural structures such as forebrain and midbrain.
2) A transforming (or "posteriorizing" or "caudalizing") factor which can act only on previously neuralized tissue, causing it to develop to more posterior neural parts such as the hindbrain and spinal cord.

His was one of the earliest comprehensive two component hypotheses for neural patterning, made several years before the double gradient model of Saxén and Toivonen (based on neuralizing and mesodermalizing factors). Simultaneous with Nieuwkoop's proposal was that of T. Yamada in 1950 on two agents, one acting inductively and one morphogenetically. Both of these were preceded by the outlining of the possibility of a double gradient by F.E. Lehmann in 1942 and by the discussions of two inducers by Holtfreter and Chuang, who had reported in the mid-1930's that the partial purification of various tissue extracts yielded either a neuralizing inducer or a trunk-tail inducer, but not both, and that dilution or concentration of one did not make it act like the other.

Nieuwkoop's activation/transformation hypothesis has survived to this day and is cited regularly to explain the results of contemporary experiments with pure inducers (such as the noggin and chordin proteins) acting on isolated ectoderm, as discussed later. He soon inquired into the means by which the activating and transforming factors reach responsive ectoderm cells in sufficiently different quantities to give an anteroposterior as well as dorsoventral pattern to the neural plate. While other researchers pursued models of neural patterning which are exclusively spatial, namely, those involving double morphogen gradients, he pursued time-based interpretations involving:

1) inducer release by the dorsal mesoderm during its progressive anterior-ward movement under the ectoderm during gastrulation, and
2) the changing competence of the ectoderm to respond to inducers.

Regarding the movement of mesoderm, he considered that the prospective prechordal mesoderm, which moved near the front of the endo-mesoderm migration, is the strongest source of activating signal and can activate (neuralize) all ectoderm under which it passes. The prechordal mesoderm has long been recognized as a sub-region of the Spemann organizer. It has been called the "head organizer" because under certain conditions it induces forebrain and midbrain parts of the neural tube. Nieuwkoop held it

to be a head organizer simply because it possessed no transforming activity. Behind the prechordal mesoderm moved the chordamesoderm, which is often called the trunk-tail organizer. Along its entire length it releases the transforming agent, and the posterior end of the chordamesoderm is the strongest source. It is a poor source of activating agent. It transforms (posteriorizes) the neuralized ectoderm under which it passes, to an extent related to the duration of contact and intensity of the source. Ectoderm nearest the blastopore forms the most posterior neural structures because chordamesoderm passes under it for the longest time and brings under it the strongest source. Activated ectoderm near the animal pole, on the other hand, is never reached by the chordamesoderm or its poorly diffusing signal. It forms only fore- and midbrain. Intermediate neural plate levels experience intermediate durations of exposure to the transforming factor. Thus, according to the hypothesis, the neural plate gains its anteroposterior organization. As discussed later, additional temporal provisions are needed for the dorsoventral dimension of patterning.

Signals from the prechordal and chordamesoderm reach the overlying ectoderm first by a vertical path, inducing the midline of the neural plate, which later becomes the floor plate. Then, according to Nieuwkoop, the signals spread laterally (in the dorsoventral dimension) and anteriorly by a propagation mechanism (a homeogenetic spreading) in the plane of the neural plate. He distinguished this planar propagation of signals from "planar induction", which to him meant signals passed in a plane between two *different* tissues. A further interesting proposal of his, based on his experimental observations, is that the midbrain is not formed by these initial activations and transformations but secondarily by an interaction of forebrain and hindbrain rudiments, once they have formed (Nieuwkoop, 1991). Recent research reveals the midbrain to be an interesting region with local organizer-like effects due to its secretion of Shh, FGF8, and FGF4 (Ye *et al.,* 1998).

Then, he suggested that the dorsoventral dimension of the neural plate, and especially its boundaries, is patterned by the decline and eventual cessation of the ectoderm's competence at stage 12 to respond to activating signals slowly propagating in the tissue plane. Thus, the dorsoventral pattern was not set by a morphogen gradient but by the time of exposure of the ectoderm to the activating and transforming signals. The later the signal arrives in the competence period, the more ventral is the ectoderm's response. Neural crest and placodes arise where competence has all but disappeared when the activating signal arrives. Such signals, he thought, continue to pass through the ectoderm even after stage 12, despite its non-responsiveness. B. Albers (1987), in her published thesis work done under Pieter's direction, supported this conclusion by grafting stage 10 gastrula ectoderm onto the lateral edge of the neural plate at stage 12 (late gastrula) and showing that neuralizing signals still reached it. Nieuwkoop and Albers (1990) then showed that although the competence toward activators is over by stage 12, the competence to respond to propagated transforming signals continues until stage 16 (late neurula). This analysis involved transplantation of prospective forebrain regions to posterior positions in the neural plate and assessment of their extent of posteriorization.

In his last experimental publication, Nieuwkoop and Koster (1995) concluded that neural induction can only start by way of an activating signal transmitted by a vertical path from prechordal mesoderm to overlying ectoderm, and not by a planar path from

posterior chordamesoderm to ectoderm at the blastopore. He was not against the planar path for propagation of activating signals within the ectoderm, once the initial activation signal had been received vertically. Part of his argument against a planar path of initial activation of ectoderm by chordamesoderm was that posterior chordamesoderm, although a rich source of transforming signal, has very little activating signal to transmit by a planar path. He concluded that in *Xenopus* there is a very early vertical neural induction at the internal blastopore, well before researchers get around to making exogastrulae and "Keller sandwiches" of mesoderm and ectoderm (Nieuwkoop, 1997), the test materials used by them to demonstrate planar activation. Although this issue remains to be analyzed further in *Xenopus,* where conflicting results obtain, such a requirement for vertical activation has been long accepted by researchers of urodele neural induction, ever since Holtfreter's 1933 demonstration that exogastrulae fail to accomplish neural induction.

What is the current interpretation of neural induction in light of the recent identification of specific inducers? Evidence of the past decade has reinforced many of Nieuwkoop's activation/transformation proposals. Abundant evidence points to a basic difference of the anterior and posterior neural regions. The former, which is the domain of activation but not transformation, is the domain of expression of the *emx/otx* genes but not the *Hox* genes. The latter, which is the activated and transformed domain, is the domain of *Hox* gene expression but not *emx/otx*. (A similar segregation of domains is found in the arthropod nervous system, e.g. in *Drosophila*). Furthermore, as thought for many years, ectoderm of the early gastrula has an inherent dual competence to develop as either epidermis or anterior neural tissue. Recent work shows that the ectoderm self-suppresses its neural option and sustains its epidermal option by a process of intercellular signaling involving BMP2 and/or 4, both of which are TGFβ family members (reviewed by Harland and Gerhart, 1997). That is, the ectoderm cells release these ligands and then bind them to their own transmembrane receptors to maintain neural suppression. Neural development is a default pathway taken by ectoderm when the self-suppression fails.

Spemann's organizer releases neural inducers, several of which turn out to be antagonists of BMP signals. Two of these, noggin and chordin, bind directly to BMP ligands and prevent them from binding to receptors (Piccolo *et al.,* 1996; Zimmerman *et al.,* 1996). Xnr3, a nodal-related secreted protein, is also an antagonist, perhaps by way of blocking the receptor (Hansen *et al.,* 1997). When intercellular self-suppression is antagonized, ectoderm cells switch to the neural option and suppress the epidermal option. This is the activation step of the Nieuwkoop model.

The inducer, it is now appreciated, provides very little information. It does not inform the ectoderm about neural development. It simply releases the ectoderm's inherent competence for neural development. The ectoderm's self-suppression of the neural option and the antagonism by neuralizing signals was suspected from the time of L. Barth and J. Holtfreter in the late 1940's when they shocked gastrula ectoderm from newts or frogs briefly with high or low salt or extreme pH and found it to develop as neural tissue, including brain vesicles and eyes or nasal pits. Holtfreter (1948) proposed that the ectoderm self-suppressed its own neural development by an intracellular, not intercellular, means, which inducers antagonized. Thus, he was very close to the current intercellular

self-suppression picture. Nieuwkoop (1963) also studied neural differentiation by salt-shocked ectoderm and was struck by its great capacity to self-organize local neural structures such as individual brain vesicles and placodes despite the incoherence of the inductive signal. He considered this self-organization capacity a major research problem for future experimental attention. "Autoneuralization" as studied by Barth, Holtfreter, and Nieuwkoop is now thought to be the outcome of the explanted ectoderm's failure to maintain its BMP-mediated suppression of the neural option under culture conditions. This outcome can be prevented by the presence of a low level of BMP2/4 in the culture medium with the explanted tissue (Wilson and Hemmati-Brivanlou, 1995). Hence, activation occurs when self-suppression fails.

But there may be more to activation than just the single element of BMP antagonism. The largest and most complete heads are induced in *Xenopus* when a BMP antagonist is accompanied by a Wnt antagonist such as Frzb (Leyns *et al.,* 1997) or the Cerberus protein, both secreted by the Spemann organizer, or by a dominant negative Wnt receptor, introduced experimentally (Glinka *et al.,* 1997). Wnt ligands may also serve as agents of neural suppression. This effect is not fully understood. Ectoderm near the marginal zone, as well as mesoderm of the marginal zone, seem to produce and respond to Wnt ligands. These may operate in the dorsoventral dimension of patterning whereas the BMP ligands may operate more in the anteroposterior dimension. Even anterior neural tissue has a dorsoventral pattern (Knecht *et al.,* 1995). If true, both Wnt and BMP ligands must be eliminated to obtain pure activation and hence dorsal anterior head structures, those most readily scored as forebrain. Activation may have two components.

Transformation is less well understood in contemporary terms. Various results implicate Wnt ligands also as transforming inducers, acting on previously neuralized tissue. These experiments involve the co-expression of noggin (a BMP antagonist) and Wnt3a in ectoderm to get posterior neural tissue of the hindbrain and spinal cord levels (McGrew and Moon, 1995) whereas noggin alone leads only to anterior neural tissue. Instead of Wnt3a, β-catenin can be experimentally expressed in cells as a Wnt pathway intermediate to produce the same posteriorizing effect. Also, FGF treatment has posteriorizing effects under certain conditions (Lamb and Harland, 1995), and deserves further investigation since it is suspected of both activating and transforming ectoderm, out of keeping with the activation/transformation model (though perhaps a single kind of molecule can act in both of the two roles). As an extension of the Nieuwkoop-Albers experiments, Cox and Hemmati-Brivanlou (1995) removed anterior neural place tissue, which had undergone activation *in vivo*, and exposed it to FGF *in vitro* and observed transformation. Concerning the source of transforming inducers, recent authors have suggested that transforming signals arise not just from the chordamesoderm of the midline, but also from somite mesoderm (Bonstein *et al.,*1998).

Finally, the activation/transformation model suggests that, just as there might be regions of the embryo with anti-activation systems in place (such as BMP signaling in the ectoderm), there might be other regions with anti-transforming signals. Such regions would be unique in differentiating no neural tissue on their own, but being particularly prone to producing anterior neural tissue (head parts) when activating signals are released nearby. They would be capable of antagonizing transformation. The recent findings of the anterior visceral extraembryonic endoderm (the AVE) in mouse

embryos (Beddington and Robertson, 1999) and in *Xenopus* (Jones *et al.,* 1999) may favor this possibility. This region releases signals (Cerberus among them) which do not induce neural tissue in ectoderm on their own, but if they are absent, the nearby ectoderm fails to form anterior neural tissue when the prechordal mesoderm and node approach. More posterior neural tissue is formed instead, and the embryo is partially or wholly headless.

In summary, Pieter Nieuwkoop's contributions to studies of neural induction have been fundamental and lasting. Substantial current work is built upon his findings. The temporal aspects of his proposals have still not been explored extensively by others.

Meso-endoderm induction

In 1969 Nieuwkoop made his second major contribution, the discovery and analysis of endo-mesoderm induction in the amphibian blastula. Prior to this time, mesoderm was assumed, as one of the three fundamental germ layers, to originate from a unique cytoplasmic region of the egg, just as ectoderm and endoderm do. It was not anticipated that mesoderm would form by an induction. He found this induction first in urodele embryos by surgically recombining vegetal hemisphere cells with animal hemisphere cells of the 2000 cell blastula, after eliminating all regions of prospective mesoderm including the territory of Spemann's organizer. Neither the animal cap nor vegetal cells alone differentiated mesoderm or pharyngeal endoderm. However, recombinates made these tissues and in some cases developed "embryoids" with good axial organization and a nervous system, a clear indication that Spemann's organizer had been restored. He alone (1969a,b) and with G. Ubbels (Nieuwkoop and Ubbels, 1972) showed by several means that the mesoderm, pharyngeal endoderm, and some of the gut roof endoderm, derived from animal cap cells responding to inducers that the vegetal cells produced. The vegetal cells developed very few tissues, mostly yolky endoderm. Boterenbrood and Nieuwkoop (1973) then showed that the inductive cells of the vegetal hemisphere are of two kinds:

1) the lateroventral members, constituting about 270° of the circumference, induce nearby animal cap cells to form ventral meso-endoderm, which eventually develops to somites, lateral plate, kidney, heart, blood cells and gut roof.

2) the dorsal members, constituting about 90° of the circumference, induce nearby cap cells to form dorsal meso-endoderm, including none other than Spemann's organizer consisting of prospective pharyngeal endoderm, prechordal mesoderm and chordamesoderm.

The dorsoventral pattern of the blastula vegetal hemisphere is inductively imprinted on the cells of the animal hemisphere, generating a dorsoventral pattern of meso-endoderm in the marginal zone. By means of induction, the dorsal vegetal cells are "the organizer of the organizer". There may be yet another part of the organizer in *Xenopus*, namely, a deep anterior endoderm portion (Bouwmeester *et al.,* 1996; Glinka *et al.,* 1998) which secretes the Cerberus and Dickkopf (Dkk) proteins and induces very anterior neural plate, adhesive organ, and other anterior endoderm. Little is known at present about the formation of this region.

Once Sudarwati and Nieuwkoop (1971) found meso-endoderm induction in the anuran *Xenopus* as well as in several urodeles, this induction was seen as general to amphibia and probably to most if not all chordates. In the 1980's the "endo-" aspect of meso-endoderm induction tended to be dropped by other researchers in their enthusiasm to study the formation of mesoderm (especially muscle) from ectoderm that had been treated with purified protein growth factors. However, Nieuwkoop had emphasized from the beginning that pharyngeal endoderm is also induced (and also the gut roof), and hence "meso-endoderm induction" is the appropriate term. So great has been the influence of Nieuwkoop's work on current studies of meso-endoderm inducers, regional gene expression, and organizer formation, that it seems appropriate to call the dorsal vegetal cells, which are the organizer of the organizer, the "Nieuwkoop Center" (although he himself disliked this term).

Upon finding that mesoderm and pharyngeal endoderm are derived exclusively from the animal cap ectoderm, Nieuwkoop concluded that an induction is at work, and not a regulation of an animal-vegetal double gradient as favored by Ogi, Nakamura, and their colleagues in their interpretation of their simultaneous similar studies of recombinates. At first, Nieuwkoop thought that ventral and dorsal vegetal cells differed quantitatively in their release of a single meso-endoderm inducer, the latter releasing more. Although he was well aware that Spemann's organizer later released inducers with mesoderm patterning effects (an induction now called "dorsalization" of the mesoderm), he thought that the marginal zone mesoderm had already gained extensive patterning (e.g. chordamesoderm, somites, lateral plate) before the organizer acted during gastrulation, due to the multipotent responsiveness of animal hemisphere cells of the late blastula to a gradient of meso-endoderm inducers from vegetal cells (Weijer *et al.,* 1977). Later J. Slack (Smith and Slack, 1983; Dale and Slack, 1987) proposed a "three signal model" for mesoderm patterning to integrate meso-endoderm induction at the blastula stage and the organizer's inductions at the gastrula stage. He proposed that the two parts of the vegetal hemisphere differ qualitatively in the kind of inducers they release, and that the marginal zone mesoderm of the late blastula/early gastrula gains only a two part pattern by this induction. One part is the dorsal meso-endoderm, which is the organizer, and the other part, the lateral-ventral meso-endoderm, which has the competence to develop to a wide variety of mesodermal tissues. The rest of the pattern of the lateral-ventral meso-endoderm (e.g. heart, somites, kidney, lateral plate, blood cells) is subsequently built up in gastrulation by the organizer's inductions (the third signal). The proposals of Kimelman *et al.* (1992) have introduced a further distinction about the two kinds of meso-endoderm inducers. Namely, one is a general meso-endoderm inducer which is secreted by all cells of the blastula vegetal hemisphere, sufficient to induce a ventral type of meso-endoderm in the responsive animal hemisphere cells. The second is a competence modifier secreted only in the vegetal dorsal sector. This modifier lacks effect on its own but acts in concert with the general meso-endoderm inducer to lead to dorsal meso-endoderm (rather as the transforming agent of neural induction acts only on tissue that has previously received the activating agent). According to this proposal, the Nieuwkoop Center would be that region of the vegetal hemisphere secreting both the general meso-endoderm inducer and the competence modifier. The organizer would form from animal hemisphere cells receiving both signals.

How well do these proposals fit with current findings about meso-endoderm inducers? Several lines of evidence now identify the competence modifier of the dorsal vegetal cells as connected

in some way to β–catenin. This interesting multifunctional protein not only resides at the periphery of the cell in complexes with cadherins and actin filaments (in adherens junctions), but it also enters the nucleus and complexes with a transcription factor, Tcf/Lef1, to modify this factor's interaction with specific promoters, thereby activating or repressing the expression of various genes. In this second role, it serves as an intermediate of the Wnt signal transduction pathway which is used widely in animal development.

The first evidence for the involvement of β-catenin was obtained when McMahon and Moon (1989) found that Wnt1 or Wnt8 mRNA, if injected into the prospective ventral side of early *Xenopus* embryos, leads frequently to twinning, that is, to the formation of a secondary axis as complete as any obtained from a Spemann organizer graft. Injection of β-catenin mRNA itself is fully as effective as is the injection of other intermediates of the Wnt signaling pathway that stabilize β-catenin and thereby favor its accumulation (Miller and Moon, 1996). Members of the Hubrecht laboratory contributed to the analysis of the β-catenin/Tcf effects (Molenaar *et al.,* 1996). Furthermore, when the maternal mRNA for β-catenin is eliminated from *Xenopus* oocytes, the eggs strikingly fail in dorsal development (Heasman *et al.,* 1994), as if the Nieuwkoop center and Spemann's organizer cannot form. The embryos succeed only in ventral/posterior development, as if the general meso-endoderm inducer operates independently of β-catenin. Thus, β-catenin is taken to be a necessary ingredient of the center, though not sufficient for its formation, as we will see. β-catenin normally accumulates on the prospective dorsal side as early as the end of the first cell cycle (1 cell stage), and it persists there until new gene expression begins at the 4000 cell stage (the midblastula transition) (Schneider *et al.,* 1996; Larabell *et al.,* 1997). The domain of accumulation of β-catenin extends from the dorsal vegetal region to the dorsal animal region, almost 120° of the blastula's animal-vegetal circumference. This domain is larger than that of the Nieuwkoop center which, as mentioned before, is thought to be a region of overlap of cells secreting not only β-catenin but also the general meso-endoderm inducer.

β-catenin is a surprising ingredient because it is not a secreted maternal protein, as one would expect for an early-acting inducer. It is a transcription cofactor and therefore cannot function until the mid- to late blastula stage (>4000 cells) when gene expression begins. Although Boterenbrood and Nieuwkoop (1973) assessed the time at which meso-endoderm induction ends (just before gastrulation), they did not assess the time of its onset. Jones and Woodland (1987) recombined animal and vegetal fragments of different ages and concluded that meso-endoderm induction begins at the 32-64 cell stage, but other researchers report conflicting results (Wylie *et al.,* 1996). Blastomeres at the 32-64 cell stage can be transplanted from the dorsal vegetal position of one embryo to the ventral vegetal position of another, and the grafted host embryo develops twin axes (Gimlich and Gerhart, 1984). However, this result does not provide evidence that meso-endoderm induction occurs at the 32-64 cell stage, but only that the graft blastomeres at this stage carry the ingredients for eventual induction. In light of the strong evidence for a role for β-catenin, it seems plausible that its complex with Tcf activates genes such as *siamois* (Carnac *et al.,* 1996) and *twin* (Laurent *et al.,* 1997), and these in turn activate genes for secreted proteins such as the *Xnr3* gene encoding a nodal protein, a TGFβ family member (Smith *et al.,* 1995). Thus, the dorsal meso-endoderm induction by cells of the Nieuwkoop center

might be in part or wholly zygotic, and not maternal, though localized to the dorsal vegetal region by maternal means.

The general meso-endoderm inducer presents similar problems of interpretation. This, too, is expected to be a maternal secreted protein inducing lateral-ventral mesoderm. Various experimental results have implicated a TGFβ family member such as Vg1 or activin, or RTK ligands such as FGF, as the maternal inducer. Indeed, injection of mRNA for these can lead to at least some axis duplication. Furthermore, Vg1 mRNA is located in the vegetal hemisphere of oocytes and is translated in late oogenesis (Weeks and Melton, 1987). However, mRNA knockouts have not been reported as critical evidence of Vg1's indispensability. From a separate line of inquiry, the mRNA for VegT, a T-box transcription factor (Lustig *et al.,* 1996; Stennard *et al.,* 1996; Zhang and King, 1996), has been found essential for meso-endoderm formation. It is localized at the vegetal pole and translated after fertilization. When this is eliminated from oocytes, the subsequent egg develops into an embryo with no mesoderm or endoderm (except for a minor amount of mesoderm at the vegetal pole), as if meso-endoderm induction had widely failed. Also, it has no dorsal structures (Zhang *et al.,* 1998). The implication of this result is that the T-box factor activates the transcription of genes encoding secreted zygotic proteins used in the general meso-endoderm induction. Hence both the general inducer as well as the dorsally-restricted competence modifier may be zygotic proteins secreted in the mid- to late blastula from locations determined by the prior location of maternal transcription factors. Kimelman and Griffin (1998) have suggested a two-fold interpretation, that low levels of maternal inducers (e.g. FGF, Vg1) may be present in the early and mid-blastula, sufficient to give some meso-endoderm induction, and that localized maternal transcription factors, both vegetally and dorsally, activate zygotic genes at the mid- to late blastula stage. The secreted proteins encoded by these genes then give strong meso-endoderm induction.

Regardless of the time and mode of induction, the Nieuwkoop center remains that region of the embryo from which two kinds of inducers are released, leading to the induction of Spemann's organizer. Nieuwkoop concluded that the organizer can only form by an inductive mode, and indeed there seems to be no wholly intracellular path of organizer formation, that is, by way of lineages of cells receiving cytoplasmic localizations such as β-catenin and VegT, and never needing intercellular signaling. For example, when TGFβ signaling is blocked by a dominant negative activin receptor, the cells are healthy but meso-endoderm does not form, including the organizer (Hemmati-Brivanlou and Melton 1992). If the Nieuwkoop Center is composed of cells releasing both inducers, which cells of the animal and vegetal hemispheres are able to respond to these inducers and form the organizer? Organizer cells derive from both hemispheres (Vodicka and Gerhart, 1995). The responding cells could be, it would seem, ones secreting the widespread inducer, or the competence modifier, or both, or neither. As mentioned above, some β-catenin accumulating cells are present even in the animal hemisphere. These probably do not release the general meso-endoderm inducer, but they are in a location to receive that inducer as well as the competence modifier they produce. The last-mentioned category of cells, producing neither inducer, would receive both. This last-mentioned population is well known to exist from the results of Nieuwkoop's own animal-vegetal recombinates (Nieuwkoop, 1969a,b), in which ani-

mal cap cells formed an organizer and dorsal meso-endoderm. As a further demonstration, cells can be grafted from the animal pole of one blastula to the dorsal marginal zone of another, and they form part of the organizer and eventually differentiate as notochord (R. Gimlich and J. Gerhart, unpublished).

As Pieter Nieuwkoop fully appreciated, organizer formation is not a single step process, and may not be entirely attributable to meso-endoderm induction (Nieuwkoop, 1997; see his many attributions to the work of Hama, Okada, Kaneda and Suzuki). The prospective pharyngeal endoderm and pre-chordal mesoderm of the organizer may be the parts induced by meso-endoderm induction in the late blastula, and these in turn may induce the prospective chordamesoderm portion of the organizer during gastrulation, perhaps by entirely zygotic means. Suzuki *et al.* (1984) proposed such a sequence for the newt organizer, with the former parts interacting vertically with the latter (i.e. across apposed planes of tissue) once they had involuted at the blastopore. The interaction was seen as reciprocal, and needed for the prechordal mesoderm to gain head inducing character. These interactions have been less well investigated in *Xenopus*. In this anuran it is known that the chordamesoderm part of the organizer can be formed by animal cap cells grafted into the dorsal marginal zone in the late blastula when meso-endoderm induction has declined (Stewart and Gerhart, 1991), and that small grafts of ectoderm cells are incorporated into the chordamesoderm even in the mid-gastrula stage (Domingo and Keller, 1995). In the normal embryo, the recruitment of marginal zone cells into the posterior chordamesoderm may continue throughout gastrulation, long after the Nieuwkoop Center loses activity. Embryos defective in FGF signaling, due to the presence of a dominant negative FGF receptor (Amaya et al., 1991), can nonetheless form the head organizer portion (prechordal mesoderm plus pharyngeal endoderm) of Spemann's organizer, but not the chordamesoderm portion. The secreted signal which is needed to maintain the chordamesoderm and recruit new cells to it during early gastrulation is probably eFGF (Isaacs *et al.,* 1994). Recent work has revealed yet another part of the organizer, the deep prospective anterior endoderm which secretes Dkk and Cerberus, antagonists of certain TGFβ and Wnt ligands (Bouwmeester *et al.,* 1996; Glinka *et al.,* 1998). This region induces anterior neural plate, adhesive organ, and anterior endoderm. There is little known about the formation of this region, whether by meso-endoderm induction or by a lineage-restricted localization.

Finally, why is the Nieuwkoop Center formed only on the prospective dorsal side of the amphibian egg? My own work on this question was inspired by Pieter Nieuwkoop's insights into meso-endoderm induction. When Marc Kirschner and I visited the Hubrecht Laboratory for experimental work in 1977, Palacek, Ubbels, and Rzehak (1978) had just identified the sperm entry point of the *Xenopus* egg. The site of sperm entry is random in the animal hemisphere, but the Nieuwkoop Center and organizer are formed at an equatorial position approximately opposite it, as shown by the final location of dorsal midline of the body axis. The cylindrically symmetric egg has the capacity before fertilization to form the Nieuwkoop Center and organizer at any meridian but settles on one meridian depending on events following sperm entry. Knowing about the sperm entry point, Geert Ubbels, Koki Hara, and we sought artificial conditions to dissociate the relationship of the positions of it and the Nieuwkoop Center. We found that tipping the egg out of gravitational equilibrium readily did this, and the direction

of movement of the yolky egg contents during the tipping period predicts the new site of the Nieuwkoop Center and organizer with great accuracy (Gerhart *et al.,* 1981). It was eventually found in my laboratory (Gerhart *et al.,* 1989) and in Richard Elinson's laboratory (Elinson, 1995) that the normal egg undergoes a cortical rotation in the first cell cycle after fertilization. The cortex moves relative to the deep contents along a parallel, polarized array of microtubules, which its movement helps to align (Elinson and Rowning, 1988). Along this array, materials from the vegetal pole move up to the equator and even into the animal hemisphere (Rowning *et al.,* 1997). Tipping of the egg causes movement of these materials by means of gravity, which the *Xenopus* egg does not normally rely upon. These materials adhere strongly to the cortex at the vegetal pole (Kageura, 1997), and resemble intermediates of the Wnt pathway in their effects on animal hemisphere cells when injected into them (Marikawa *et al.,* 1997). As mentioned before, β-catenin accumulates along the dorsal side of the egg after rotation (Rowning *et al.,* 1997). Since this protein is degraded rapidly and is continuously replaced by translation from a uniformly distributed maternal mRNA, the accumulation of β-catenin protein on the dorsal side probably results from the localization there of an agent *stabilizing* it against degradation. This agent is presumably translocated unidirectionally on microtubules during cortical rotation, or by gravity during the artificial tipping procedure. This agent has not yet been identified.

Not only is the direction of cortical rotation precisely related to the eventual location of the Nieuwkoop Center and Spemann organizer, but also the extent of movement of materials during rotation is related to the inductive strength of the eventual Center and organizer. When rotation is blocked by inhibitors of microtubule polymerization, materials never leave the vegetal pole, and the egg remains cylindrically symmetrical. It fully develops ventral-posterior meso-endoderm (red blood cells, coelomic mesoderm, and posterior gut), indicating that the general meso-endoderm induction has no dependence on cortical rotation. It lacks a Nieuwkoop center and an organizer, and forms no dorsal-anterior parts (Gerhart *et al.,* 1989). Over the years, conditions have been found to disturb the egg's early development so that it establishes any amount of Nieuwkoop Center, from no Center at all to a Center around the entire periphery (the latter by the treatment of eggs with D_2O or of blastulae with lithium ion). In their final morphology these embryos range from purely posterior-ventral forms to purely dorsal-anterior ones. The smaller the Center, the smaller the organizer, and the less complete is the eventual anteroposterior axis, progressively lacking parts from the anterior end (Stewart and Gerhart, 1990; Elinson, 1995). All the data fit the interpretation that cortical rotation is needed for the dorsal-ward translocation of the competence modifier but not the general meso-endoderm inducer, and that the less is the amount of translocated competence modifier, the less complete is dorsal anterior development. When I first described these headless embryos to Pieter Nieuwkoop, he suggested that they were diminished in the quantity of their activating inducer and in their capacity to move involuted tissues anteriorward during gastrulation. Hence the transforming agent converts all neuralized tissue (potential head) to posterior neural parts (P. Nieuwkoop, personal communication). This remains an apt explanation.

In summary, Nieuwkoop's discovery of meso-endoderm induction at the blastula stage, which is the embryo's earliest induction,

has opened a large and rewarding area of developmental biology. Laboratories worldwide have pursued molecular analyses of inducers and responses. An abundance of new ideas has emerged about the early steps of axis formation, and early attempts have been made to assess the universality of these steps among all chordates, from ascidians to mammals.

Germ cell induction in urodeles

With his colleagues Nieuwkoop continued these studies which he had begun in his doctoral thesis research (Sutasurya and Nieuwkoop, 1974). Urodele germ cells are formed by ventral marginal zone cells exposed to the ventral meso-endoderm inducer but not the competence modifier or dorsalizing signals from the organizer. Although an inductive mode of germ cell formation was familiar to researchers studying amniote germ cells, it came as a surprise to *Xenopus* and *Rana* researchers. Anuran eggs contain at the vegetal pole a collection of germ plasm granules remarkably like the well-studied polar granules at the posterior pole of the insect egg (Wylie, 1999). In anurans, germ cells arise only from a cell lineage harboring these granules, a compelling example of a cytoplasmic localization mechanism. There is no evidence for germ cell induction in anurans. This basic difference of urodeles and anurans led Nieuwkoop to favor the notion that amphibia are di-phyletic. In recent years, a few of the components of the anuran germ plasm have been identified (Wylie, 1999), following the leads from *Drosophila* researchers. However, there has been little progress with germ cell induction in urodeles.

Finally, in less well known work, Nieuwkoop undertook in the 1980's the study of turtle development (at the Institute of Technology, Bandung, Indonesia), feeling that reptilian development was a neglected area, and that turtles represent a particularly unmodified order of reptiles. He noted the egg's soft shell, thin albumen solution, and great uptake of water as intermediate characters in the evolution of the cleidoic egg, one of the most important of the land adaptations of vertebrates (Nieuwkoop and Sutasurya, 1983).

He wrote three books of lasting value to developmental biologists and comparative embryologists. These include "*Primordial Germ Cells in the Chordates: Embryogenesis and Phylogenesis*" (P.D. Nieuwkoop and L.A. Sutasurya, Cambridge University Press, 1979) *and "Primordial Germ Cells in the Invertebrates*" (P.D. Nieuwkoop and L.A. Sutasurya, Cambridge University Press, 1981). These grew from his lifelong studies of germ cells, and his evidence for a di-phyletic origin of amphibia. His third book was *the "The Epigenetic Nature of Early Chordate Development*" (P.D. Nieuwkoop, A.G. Johnen and B. Albers, Cambridge University Press, 1985), in which he explored the possible universality of meso-endoderm induction in chordates, and the central role of this induction in organizing the chordate body plan. He suggested that studies of meso-endoderm induction in *Amphioxus* should be done to probe the evolutionary origins of this induction. For his synthesis of amphibian development, several reviews are well worth reading (Nieuwkoop, 1973, 1977), in which he emphasizes the amphibian oocyte's two part organization (the animal and vegetal hemispheres), and the stepwise build up of complexity in the early embryo by way of repeated and ever more local inductive interactions among ever more parts. Throughout his career he believed strongly in the importance of inductive interactions across compartment boundaries for chordate pattern formation, and this appreciation has certainly proved to be correct.

Pieter Nieuwkoop was a Professor of Zoology at the University of Utrecht from 1956-1984 and was the Director of the Hubrecht Laboratorium (a semi-governmental institution under the supervision of the Royal Netherlands Academy of Arts and Sciences) from 1953 until 1980. As described elsewhere in this issue, the laboratory moved in 1964 from a city location at the University of Utrecht to a new building on the city outskirts while he was Director. He assembled a group of staff searchers studying the development of frogs, urodeles, chicks, mouse, *Dictyostelium*, and *Drosophila*, by a variety of techniques. This selection reflected his very broad interests in development, and made this laboratory the world's only national laboratory of developmental biology at the time. Among his doctoral students and postdoctoral colleagues are J. Faber, H. Eyal-Giladi, K. Hara, G.A. Ubbels, L. Sutasurya, E. Boterenbrood, R. Rao, and S. de Laat, who has been the Director of the Laboratory until recently. Many researchers, including myself and Marc Kirschner, visited the laboratory for sabbatical research and discussions with Pieter and staff members, and for an introduction to *Xenopus*. We all found that Pieter had an enormous store of unpublished observations and ideas he was delighted to share, in his quietly intent manner, with those who asked. Some of his broad views of, and deep interest in, chordate development can be found in an article based on an interview I had the privilege to conduct at the time of his 70th birthday (Gerhart, 1987). For many years, an international course on developmental biology and techniques was offered at the laboratory, in which Pieter participated. Students of many countries benefited from this introduction to the subject and contact with him and other laboratory members. It is with deep appreciation that we remember Pieter Nieuwkoop's numerous contributions to the understanding of early chordate development, contributions that still vitalize our studies.

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