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Peptide growth factors in amphibian embryogenesis: intersection of modern molecular approaches with traditional *inductive interaction* paradigms

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ABSTRACT Recent discoveries of the role peptide growth factors (PGFs) play in regulating embryonic patterning and differentiation have profoundly influenced research on the molecular biology of early amphibian embryogenesis. Several PGFs have been recognized to be present as endogenous components of amphibian eggs and early embryos, while other PGFs – which are known from heterologous systems (e.g., *Drosophila*) – exert remarkable effects when injected as either protein or mRNA into eggs/embryos or when added to cultured embryonic tissue. For a variety of reasons (reviewed herein) optimism abounds that an understanding in molecular terms of the classical Spemann and Nieuwkoop tissue interactions which are generally believed to drive embryonic patterning is within reach. A critical assessment of the interpretations of some of the contemporary data on PGFs (included herein) should, however, temper some of that optimism. Likely, multiple rather than single PGFs act in a combinatorial fashion to contribute to individual patterning events. As well, substantial redundancy in PGF regulatory circuits probably exists, so the heavy reliance on tissue culture assays and overexpression studies which characterize much recent research needs to be circumvented. Potential experimental approaches for "next generation" experiments are discussed.

KEY WORDS: *peptide growth factors, amphibian inductive interactions, primary embryonic organizer, early embryonic patterning, Spemann induction*

Introductory remarks

History

Almost a dozen years have passed since reports began appearing (e.g., Kimelman and Kirschner, 1987; Slack *et al.*, 1987; Asashima *et al.*, 1989) that biochemically well-defined peptide growth factors (PGFs) play important roles in early amphibian embryonic inductive events such as mesoderm and neural tissue formation. Earlier reports had indicated that proteins were likely active in promoting inductive interactions (e.g., Toivonen and Saxen, 1955; Yamada and Takata, 1961; see also Tiedemann *et al.,* 1996 for a brief historical review), thereby setting the stage for the newer discoveries. These recent reports succeeded in identifying *specific* proteins as likely candidates for regulatory roles. Indeed, the search for the exact molecular components which comprise the putative inducers of dorsal and ventral, anterior and posterior patterning in the early embryo, sparked by these molecular-era reports, has become the preoccupation of a substantial proportion of contemporary amphibian developmental biologists.

Happily, PGF discoveries have rejuvenated a research area which was experiencing the “law of diminishing returns.” By the early 1980’s much traditional experimental amphibian embryology appeared to have run its course. Descriptive cell-lineage studies and tissue-level manipulations dominated the literature, with little experimental evidence accumulating to support hypothetical models of molecular mechanisms such as the original two-signal model for neural induction (reviewed in Doniach, 1995) and the more recent three-signal model for mesoderm formation (reviewed in Smith, 1993). Indeed, for many research issues in embryogenesis the spotlight had begun to shift to the genetically more amenable experimental models, including *Drosophila* and *C. elegans* . Thus, searches which turned up PGF effects (usually assayed in cultured “animal cap” tissue explants), regardless of

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whether evidence for a natural or *in vivo* role was provided, generated substantial amounts of excitement for amphibian embryologists: at long last, well-defined molecules were identified as plausible candidates for the *Nieuwkoop mesodermalizing center* and the *Spemann primary embryonic organizer!*

What appears to be emerging from the most recent PGF research is "sets" of overlapping regulatory circuits with both positive and negative control elements guiding specific cells of the embryo through the classical tissue interactions so well described by Spemann and Mangold (1924), Townes and Holtfreter (1965) and Nieuwkoop (1973). The pace with which specific components, including various PGFs as well as several of their presumed target genes (which code for putative transcription factors), is being researched is truly frenetic. Review of the literature reveals, however, that in the ensuing commotion many authors – perhaps inadvertently – have promoted only that function of the PGF which comprises the focal point of their own research project. In many instances, the role a single PGF plays has often been emphasized without acknowledging the known roles of other PGFs. In some cases the possibility that this or that PGF plays a role which is subordinate to that of other PGFs even appears to have been deliberately ignored.

In spite of that human failing, optimism that molecules which comprise the Nieuwkoop and Spemann tissue patterners will soon be discovered abounds. However, what is actually being elucidated is a labyrinth of interconnected signal transduction pathways and hierarchies of regulatory circuits (e.g., Moon *et al.*, 1997a). It appears increasingly unlikely that mesoderm and/or primary embryonic induction will – in the final analysis – be demonstrated to be the product of the action of one or even a few PGFs as was the original, naive expectation (e.g., Tiedemann, 1961).

Pause for assessment

The purpose of this review is to assess progress made 'during the past dozen years' on the role PGFs and PGF binding proteins play in early amphibian embryogenesis. Reasons for favoring an optimistic outlook for this research area are first noted. Included are summaries of the roles PGFs have been proposed to play in various inductive events. Then, unfavorable circumstances which likely will limit the rate at which further progress is made are also delineated. Next, a divergent view on the timing of PGF action is briefly summarized. Finally, comments which might facilitate a return to the optimism of the early days of PGF discoveries are offered.

Rather than attempt to generate a historical record of the discovery and elucidation of the action of various PGFs, or to assign discovery credit to this or that researcher, we have cited data and reviews mostly from the last half-dozen years. For a more comprehensive treatment of various discoveries, and a balanced historical perspective, several earlier reviews, including those of Kimelman *et al.* (1992), Smith, (1993), Asashima (1994), Slack, (1994) and Tiedemann *et al.* (1996) can be consulted.

Reasons for optimism

It is easy to compile a list of factors which have contributed to the sense of accomplishment enjoyed by the many contributors to the emerging PGF story:

1. Many PGFs have been demonstrated to be present in the early embryo or even earlier, as a maternally inherited component of the egg cytoplasm – e.g., activin (Fukui *et al.*, 1994); Wnt (Cui *et al.*, 1995), noggin (Smith and Harland, 1992), etc. They have also been demonstrated to be present at the "right place" at the "right time" [e.g., BMP-4 (Thomsen, 1997); chordin (Sasai *et al.*, 1994); nodal (Jones *et al.,* 1995)] and thereby – in principle – are capable of facilitating inductive interactions. Figure 1 summarizes some of those data.

2. Several PGFs, including chordin, noggin, and Wnt contain bona fide signal sequences, a fact which is fully consistent with the traditional view that embryonic inductions involve short distance cell-cell signaling.

3. Most PGFs have been demonstrated to act on responding tissues in a dose-dependent manner (e.g., Ariizumi *et al.*, 1991a,b; Jones *et al.*, 1995), in support of popular morphogen gradient paradigms.

4. PGFs believed to be active in amphibian embryos are legitimate candidates for similar roles in other vertebrate embryos (e.g., mouse, zebrafish, and chick). Activin (Mitrani *et al.*, 1990), BMP (Winnier *et al*., 1995), nodal (Jones *et al.*, 1995) etc., are notable examples. Indeed, homologs exist even in invertebrates (e.g., insects and nematodes), and provide similar (supporting) data. For example, amphibian "chordin" and *Drosophila* "short gastrulation" genes code for homologous proteins, and both are apparently antagonized by similar gene products [chordin vs. BMP in *Xenopus*; short gastrulation vs. decapentaplegic in *Drosophila* (Holley *et al.,* 1995)]. Even synergistic interactions between PGFs are likely conserved among species (e.g., Watabe *et al.*, 1995).

5. Several of the mouse gene knockout experiments have verified the role certain PGFs play in controlling early embryonic patterning/differentiation. Likewise, introduction into early embryos of dominant-negative receptor proteins, which interfere with PGF action, has in a few cases been very informative (reviewed by Harland, 1994). Even injection of putative, PGF-specific inhibitors such as the protein follistatin into eggs can be demonstrated to inhibit mesoderm formation (Marchant *et al*., 1998).

6. The list of PGFs and PGF binding proteins (which may play critical roles in patterning) which have been demonstrated to alter early pattern formation by either bathing (animal cap) tissue in excess PGF or injecting their mRNAs into eggs or single, early blastomeres is impressive. Table 1 summarizes recent discoveries.

7. A variety of downstream signal molecules coupled to PGF action have been elucidated. Several of them represent components of well-known signal cascades. Others are genes which have known patterning effects in other organisms [esp. homologs of *Drosophila* regulatory proteins – e.g., *Xenopus Mad* proteins act downstream of TGF-β proteins (Graff *et al.*, 1996)]. Yet others clearly represent so-called transcription factors. Table 2 contains a sampling of components which come into play downstream of PGF action.

The above reasons for optimism can be briefly summarized as follows: a variety of PGFs have – in the past decade – been discovered to possess the "logistics" expected of signal molecules which are responsible for early embryonic patterning. Furthermore, several PGFs have been demonstrated to stimulate the expression of specific, known downstream patterning genes.

Complicating factors present challenges

To what extent should that optimism be allowed to prognosticate the future for this research area? Are we being propelled towards realistic expectations by those initial successes? Or, do various factors such as those listed below complicate the prospects for resolving the molecular circuitry involved in *Nieuwkoopian mesoderm formation* and *Spemannian primary embryonic induction*?

1. Each so-called *peptide growth factor* actually consists of a family of closely related peptides, rather than a single peptide. Not all members of a family should be considered to have exactly the same biological activity. For example, the Wnt-related genes comprise a multigene family consisting of more than a dozen members. For FGF, a truly complex picture is likely to emerge, because although some FGFs lack a secretory sequence, at least one family member (XeFGF) has one (Isaacs, 1997). Thus, each family member probably is regulated differently.

2. Antagonists exist in some cells; thus the *active* concentration of any given PGF in a cell or region of an embryo is virtually impossible to measure with present technology.

For example, for BMP, presumed to play a role in inducing ventral mesoderm (blood and ventral mesenchyme), at least three antagonists have been identified, including noggin (Zimmerman *et al*., 1996), chordin (Piccolo *et al*., 1996), and follistatin (Fainsod *et al*., 1997). As well, evidence exists that ventral mesoderm-inducing signals such as BMP may normally overpower endogenous dorsalizing influences, such that if the BMP signal is blocked, dorsal mesoderm forms (Graff *et al*., 1994). For activin (which has a presumed role in inducing dorsal mesoderm components) follistatin is a known antagonist (Asashima *et al*., 1991). For Wnt, a protein which binds it (Frzb) is expressed in the organizer region of the gastrula (Leyns *et al*., 1997; Wang *et al*., 1997) and probably neutralizes some of Wnt's effects. Smad7 has been reported to "inhibit both" activin and BMP pathways (Casellas and Brivanlou, 1998).

Such antagonistic interactions among PGFs also likely drive ectoderm, mesoderm, and endoderm differentiation (reviewed by Thomsen, 1997).

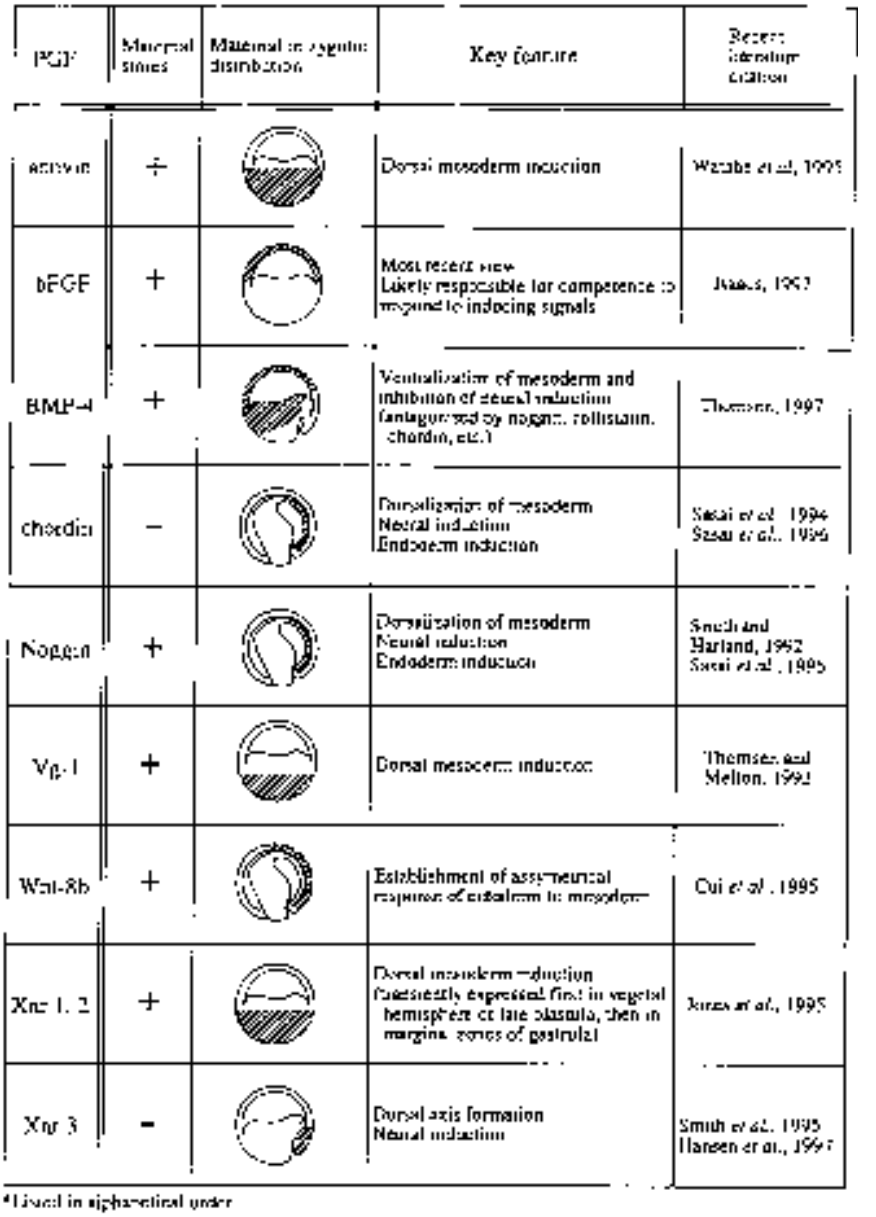

Fig. 1. Examples of diverse features of PGF distribution and mechanisms of action in early amphibian embryogenesis.

3. Combinatorial and/or additive action exists for many growth factors. That is, effects observed when a single growth factor is applied to, say, an animal cap bioassay system, can be very different when a cocktail consisting of more than one PGF is applied.

For example, Wnt action in mesoderm induction *in vitro* is facilitated by the presence of FGF-β and activin (Christian *et al*., 1992; Sokol and Melton, 1992), while its action in neural induction is enhanced by noggin (McGrew *et al*., 1995). Chordin alone does not induce mesodermal structures, but in the presence of bFGF both notochord and neural tissues are induced (again, in the animal cap assay) (Sasai *et al*., 1994). Likewise, *Brachyury* alone will not promote dorsal mesodermal differentiation in animal cap explants, but when co-expressed with noggin and Wnt-8 it does (Cunliffe and Smith, 1994).

The concept of combinatorial and/or synergistic modes of action of PGFs has been championed by Kimelman *et al.* (1992). Many authors have, however, tended to neglect this important concept. Instead an "ultra-reductionist" approach often appears to be favored in which a single "causal" PGF is the object of a search. Nevertheless, data continues to accumulate from diverse approaches which points to combinatorial action. For example, activin can be deemed *necessary* for mesoderm differentiation based upon the inhibition of patterning observed when a defective activin receptor is injected into the egg (Dyson and Gurdon, 1997). However, injection of another type of receptor which ignores both activin and BMP and presumably binds an as yet-to-be-identified TGF-β PGF also inhibits mesoderm differentiation (Mahony *et al.*, 1998). Thus, although activin appears to be *necessary* for mesoderm development it cannot be considered to be *sufficient*, as well. Combinatorial action is therefore most likely. In other instances it is proposed that a combination of gene expression patterns downstream of PGF action are *necessary* for mesoderm patterning, and furthermore that several different PGFs or related proteins are capable of triggering those gene expression patterns (Crease *et al.*, 1998; Onichtchouk *et al.*, 1998).

4. Heavy reliance on the animal cap explant assay [a bioassay which applies exogenous PGFs to cultured blastula animal hemisphere tissue – described in Asashima (1994), Slack (1994), and Doniach (1995)] yields an incomplete picture. Gradients of both PGFs and their antagonists, as well as their receptors, which likely exist in the whole, intact embryo, are unlikely to be easily reproduced in a simple animal cap explant. Nevertheless, the animal cap assay – despite those limitations – is very useful because of its inherent simplicity and ease of use.

Activin treatment of animal caps, for example, yields different results depending on the source (dorsal vs. ventral) of the animal explant tissue (Sokol and Melton, 1991; Kinoshita *et al.*, 1993; Kinoshita and Asashima, 1995). The size of the explanted tissue can also affect the final result (Ariizumi and Asashima, unpublished data). As well, various PGFs can elicit similar responses, depending on the embryological origin of the target tissue used in the explant assay (De Robertis and Sasai, 1996). That chaos can be further compounded when different laboratories introduce even minor modifications to the methods used to prepare animal cap explants [Doniach, 1995 (see esp. Figure 3)] and attempt to make sweeping generalizations which usually exclude consideration of competing data. As Doniach (1995) points out, that sort of confusion "harks back to the nightmare of neuralizing conditions seen with newt ectoderm that once drove away most sensible embryologists."

5. Morphologies as well as biochemical markers employed to measure the effects of PGF action most often represent classical *end point indicators*, rather than *immediate downstream* gene expression indices. Indeed, diverse PGFs, including chordin, Wnt, noggin, and Vg1, yield a similar end point (rescue of axial structures) when injected into ultraviolet-irradiated eggs, or twinning of axial structures when injected into normal eggs. Such observations, while generating dramatic photographs, are usually scored in an "all or none" manner. Little opportunity is often available for interpreting negative data, which might – because intermediate effects may be present – offer potentially very informative data.

6. The intrinsic complexity of regulatory circuits and cell signaling pathways, in which *redundancy* is common, will require more than simple *gain of function* or *loss of function* tests to discover additional components. Chordin and noggin, for example, most likely represent parallel axial structure induction systems (see Sasai *et al.*, 1994), which complicate attempts to research the intricate details of their regulatory circuitry.

7. Genetic analyses, which have provided definitive evidence in many other model systems, have so far been lacking for amphibian inductive-interaction research (e.g., see comments in Jones *et al.*, 1995). Consequently, an inordinate amount of significance is attached to a PGF when it is found to be located "in the right place, at the right time." That is, when its distribution corresponds to a tissue site (e.g., blastopore lip) involved in a dramatic morphogenetic event, researchers are often quick to celebrate its candidacy as a natural inducer (e.g., Ninomiya, Ariizumi, and Asashima, 1998). As well, the extent to which the "connecting features" of various amphibian regulatory circuits can be elucidated without high resolution genetics deserves mention. Conceivably, the best which can be expected from amphibian studies is to merely identify which PGF and/or other regulatory molecules [e.g., transcription factors – see Zhang *et al.* (1998)] can be scored as *present and accounted for* in early embryos using various micro injection and animal-cap bioassays. Generating the type of data required to establish circuit diagrams may simply not be practical with amphibia, considering that an alternative – zebrafish developmental genetic systems (see below) – is quickly becoming available.

8. Simple PGF *receptor deletion* and other so called «dominant-negative» exercises are informative, but not necessarily definitive. While some experiments have indeed generated definitive data (e.g., Graff *et al.*, 1994, for BMP), others have led to confusion about the specificity of ligand/receptor interactions (e.g., Hemmati-Brivanlou and Melton, 1994 vs. Schulte-Merker, *et al.,* 1994, re. the activin receptor). Likewise, the role of Wnt in embryonic axis formation, despite its dramatic ability to induce a secondary axis when injected ectopically, can be questioned since it has been reported that dominant-negative Wnt does not prevent natural axis formation (Hoopler *et al.*, 1996).

9. Some of the gene knockout experiments in mice provide data which confuses interpretation of PGF roles elucidated in amphibia. Although many mouse gene knockout experiments have validated hypotheses developed with amphibian embryos (e.g., Winnier *et al.*, 1995, for BMP; McMahon and Bradley, 1990, for Wnt), other results, such as for the activin gene knockouts in mice, have generated negative data for the presumed role of activin in early embryogenesis (Vassalli *et al.,* 1994; Matzuk *et al.*, 1995; reviewed by Cooke *et al.*, 1997). Similarly, an FGF(6) gene knockout in mice has no effect on embryonic viability (Fiore *et al.*, 1997).

In place of bona fide gene knockouts, the expression of a defective receptor which is highly specific for activin has been employed to test activin's role in amphibia. This so called «dominant-negative» truncated receptor does indeed interfere with normal *Xenopus* patterning (Dyson and Gurdon, 1997), which of course conflicts with the murine gene knockout data just mentioned.

10. The traditional view of the *Spemann primary organizer* as the 'dominant paradigm' behind contemporary data interpretation is itself subject to challenge. Although liable to charges of heresy from the point of view of *classical* embryologists, various authors have broached the subject (e.g., Bard and Lehtonen, 1996) in a constructive fashion. Rather than consider *induction* as a "cause" (of patterning), it might be time to consider it an "effect" (of cumulative regulatory circuit action, metabolic pathway functions, and signal transductions). In this latter context no single PGF would be conceptualized to play a dominant role.

For example, BMP (a ventral patterning PGF) can erase the positive influences of the classical Spemann primary organizer activity. Thomsen (1997) therefore calls for a modified concept in which the organizer is imagined as playing an antagonistic or defensive role towards ventral inducing substances.

11. Apparent multiplicity of action of various PGFs complicates development of simple schematic models. Follistatin, for example, has been shown to induce a different patterning response with different tissue samples (at various stages) (e.g., dorsalization of ventral mesoderm at late blastulation (Sasai *et al*., 1995) and induction of neural tissue at a later stage (Hemmati-Brivanlou *et al*., 1994)**.** Activin displays both concentration differences and stage-specific tissue response differences (Ariizumi and Asashima, 1995a,b; Asashima *et al*., 1997; Miyanaga *et al*., 1998; Yokota *et al*, 1998).

12. Finally, there is the issue of "competence to respond to PGFs" which has not yet been adequately addressed. It is likely that responsiveness involves more than simply the acquisition of a receptor molecule. Yet the manner in which cells and tissues develop the ability to respond to stimulation by specific growth factors is a difficult research area which has not yet been analyzed. Thus, achieving a holistic view of PGF action must await data collection on this topic (see Kinoshita and Asashima, 1995 and Yokota *et al*., 1998).

Persistence of the Nieuwkoop and Spemann paradigms

One might ask, why have those paradigms hung on for so long? First and foremost, they have provided a convenient conceptual framework for data interpretation. Even beginning students of developmental biology can quickly grasp the notion that one cluster of cells – in an anthropomorphic metaphorical fashion – instruct another group of cells. The "linear thinking" which those old paradigms promote is seductive in its simplicity. Combinatorial thought processes, which could more realistically represent the action of PGFs are, needless to say, more difficult to conceptualize.

Second, amphibian experimental embryology largely leap-frogged the cellular analysis stage which has been so fruitfully exploited with *C. elegans.* Exceptions exist, of course (e.g., Carnac and Gurdon, 1997). But the regulative nature of early amphibian cells has largely precluded conceptualization of amphibian embryogenesis in the kinds of cellular terms which lend themselves to

TABLE 1

INVENTORY OF PRESUMED PEPTIDE GROWTH FACTORS (PGFS) ACTIVE IN EARLY *XENOPUS* EMBRYONIC PATTERNING AND/OR DIFFERENTIATION

Peptide Growth Factor (with known receptors)		Suggested major role*	Sample reference
FGF			
	βFGF	mesoderm induction neural induction	Amaya *et al.,* 1993 Launay *et al.,* 1996
	XeFGF	A-P neural pattern / mesoderm induction	Isaacs *et al.,* 1992
TGF-β			
	activin A, B, D	dorsal mesoderm induction	Asashima, 1994; Oda *et al.,* 1995
	Vg-1	dorsal mesoderm induction	Thomsen and Melton, 1993
	BMP 2, 4, 7	ventral mesoderm induction	Harland, 1994
	Xnr-1, 2	organizer formation / mesoderm induction	Jones *et al.,* 1995
	Xnr-3	organizer formation / neural induction Hansen et *al.,* 1997	Smith *et al.,* 1995
Wnt			
	Xwnt-11	contribute dorsal axis	Ku and Melton, 1993
	Xwnt-8	ventral mesoderm formation	Christian *et al.,* 1991
	Xwnt-8b	possibly induce dorsal axis	Cui *et al.,* 1995
		PGF binding proteins**	
noggin (to BMP)		organizer formation / neural induction	Zimmerman *et al.,* 1996 Smith and Harland, 1992
chordin (to BMP)		organizer formation / neural induction	Piccolo *et al.,* 1996 Sasai *et al*., 1994
follistatin (to activin and BMP)		organizer formation / neural induction	Nakamura *et. al.,* 1990 Hemmati-Brivanlou *et al .,* 1994 Fainsod *et al.,* 1997
Xfrezzled (to Wnt)		organizer formation	Leyns *et al.,* 1997; Wang *et al., 1997*
Sizzled (to Wnt)		most ventral region formation	Salic *et al.,* 1997
		Other PGFs	
lunatic Fringe		mesoderm induction	Wu *et al.,* 1996
cerberus		head induction	Bouwmeester *et al.,* 1996

*based on our interpretation of various analyses, whole embryo, animal cap overexpression studies and natural expression pattern.
**no known receptors and uncertain binding affinities for other PGFs.

the design of molecular biological approaches. Recall, as much as 50% of the 8-cell embryo (two animal, one dorso-vegetal, and one ventro-vegetal cell) can be deleted without negating the formation of mesodermal and neural tissues (Kageura and Yamana, 1984).

The lack, until very recently, of genetic tools in amphibia has constrained the imagination of embryologists. Clearly, the combinatorial actions of PGFs will require clever genetics to unravel. Too often regulatory circuits have been demonstrated to be comprised of components which were – to the research biologist – counterintuitive. The discovery that nitric oxide gas serves as a neurotransmitter (Bredt and Snyder, 1992) represents an excellent example of an truly unexpected discovery.

A very recent divergent view

Following the traditional paradigm that "cytoplasmic localizations" in the early amphibian embryo control patterning, PGFs have been searched for in oocytes with the expectation that some of them will be recognized as being maternally inherited (either as protein or mRNA). They will thus be candidates for regulating zygotic gene expression from blastulation onwards. In some instances (e.g., activin) maternal stores have indeed been recognized (Oda *et al.*, 1995).

Recent experiments by Zhang *et al.* (1998) in which maternally inherited mRNA for a transcription factor (Veg T) was deleted by micro injection of an antisense oligonucleotide into oocytes have, however, yielded fascinating data on early patterning which could possibly lead to a diminution of the importance of that traditional paradigm. The $VegT^-$ embryos lacked well-defined endoderm and displayed changes in fate of various regions. It appears, therefore, that germ layer formation is dramatically altered when this maternal transcription factor (VegT) is inactivated. Mesoderm formation – generally believed to be induced by one or another PGF – was delayed, and occurred in vegetal regions of the embryo rather than in the equatorial region. In fact, vegetal cells depleted of their maternal store of VegT lack the ability to induce – in tissue recombination experiments – animal caps to express mesoderm markers. Thus, it was proposed that this transcription factor acts by regulating gene expression during late blastulation, and that those genes which are thus expressed at that relatively late stage control mesoderm formation. A role for endogenous, maternally inherited PGF in mesoderm formation can easily be obviated by that proposal. In other words, this view could be used to *reverse* the schedule for mesoderm induction, for it intimates that it is transcription factors which are maternally inherited and *PGF* genes which are zygotically expressed, rather than the other way around as generally assumed in conventional pattern specification models.

As that experimental approach is extended, clarification of the significance of maternally inherited, vegetally-localized transcription factors in early patterning, and their role *vis a vis* PGFs will hopefully be forthcoming. Modifications in some of the induction paradigms in which PGFs play key roles may eventually be called for.

Overcoming the challenges

First and foremost, the following suggestion is offered: view early amphibian patterning more in terms of a series of partially overlapping signal pathways (as emphasized by Kimmelman *et al.*, 1992) rather than in terms of discrete events or steps. Alternatively, patterning could be viewed as a long metabolic pathway consisting of complex regulatory circuits which involve ornate signaling intersections and series of subtle, oftentimes redundant, cues (e.g., see Alberts *et al.,* 1994- page 82, for a hypothetical example of such a complex regulatory circuit system). Indeed, recent data indicates that FGF activity in the early amphibian embryo is probably regulated autocatalytically through the action of *brachyury* gene expression (Isaacs, 1997). FGF is easily, therefore, fitted into this more modern approach to conceptualizing PGF action.

In this fashion various components can be assigned “locations” in the pathway, and thus fruitless searches for “single engines” or “definitive inducers” can be avoided. For example, activin can induce goosecoid expression, which in turn can activate chordin expression. Each of those PGFs have been demonstrated by themselves to possess some sort of inducing activity either in animal cap assays or when overloaded in ectopic expression experiments, although in the intact embryo their functions are likely to be interconnected. It is our view that in the intact embryo it is unlikely that a single PGF is *sufficient* to drive a morphogenetic process (e.g., primary axis formation). Rather, several PGFs are most likely actually *necessary* for any given morphogenetic event to occur on time and in the proper place. Some of them may act as “relay” factors, transferring a signal, or re-locating the site of action of a regulatory event (see Slack, 1994).

Fortunately, such an approach is underway for TGF-β PGFs. Signaling by TGF-β components in *Xenopus* can now be imaged as comprising a complex regulatory cascade, akin to an automobile expressway, complete with intersection points and «on/off» ramps. Signaling components which are downstream from the initial interaction of a TGF-β PGF with its cognate receptor, the so-called "Smads", have been identified as transcription activators.

TABLE 2

SELECTED EXAMPLES* OF INTRACELLULAR REGULATORS AND/OR EXPRESSED GENES ASSOCIATED (USUALLY DOWNSTREAM) WITH PGF ACTION IN EARLY AMPHIBIAN EMBRYOS

PGF cascade	signal molecule	target gene	sample reference
activin / Vg-1	Smad 2 + Smad 4 + Fast 1	*goosecoid, Mix 2*	Chen *et al.,* 1996
BMP	Smad 1+Smad4+?	*Mix 1, Xvent 1, 2*	Graff *et al.,* 1996
FGF	ras MAP kinas	*Xbra*	Kremer *et al.,* 1991
Wnt	gsk-3 kinase β- catenin + XTcf-3	*Xnr 3, siamois*	He *et al.,* 1995 Pierce and Kimelman, 1996

Experimental introduction of Smads into embryos serves to mimic the action of various TGF-β PGFs (reviewed by Massague *et al.*, 1997).

One experimental approach which could be expanded *vis a vis* the "regulatory circuitry" concept would involve the administration of various combinations of PGFs (e.g., Cunliffe and Smith, 1994) or "matrices" of mixed concentrations of different PGFs to various test systems (e.g., Yokota *et al.*, 1998).

A second suggestion would involve refining the "embryo/tissue" paradigm. Instead of viewing the action of PGFs in terms of interactions which carry historical tissue designations (e.g., ectoderm/mesoderm/endoderm), view their action in terms of cellular models. This has of course been done very successfully in the case of vulval development in *C. elegans* (e.g., Sulston and Horvitz, 1977). Nematode development follows a strict *cell-lineage program*, as opposed to the *regulative nature* of amphibian embryogenesis, so it will be important to avoid oversimplification. Nevertheless, in our opinion, a great deal could be accomplished by deleting from the vocabulary of amphibian embryology such generic terms as *mesoderm, ectoderm* and *endoderm* and replacing them with terms which more accurately provide information on the address or function of specific cells. For example, the cell layer, quadrant of the embryo, or activity (e.g., "involuting cells", or "archenteron roof tissue") would be more accurate and appropriate designations. By doing so, experimentalists would be encouraged to refine their analyses in more specific cellular (vs. tissue) terms.

A third suggestion is to pay more attention to the role inhibitors of various PGFs might play in regulatory circuits. Smad7, mentioned above, appears to inhibit activin and BMP signaling as well as act as a neural inducer (Casellas and Brivanlou, 1998; Nakayama *et al.,* 1998). The classical embryonic induction models of course emphasize the role of substances which *drive* patterning. Modern mechanistic molecular models however give substantial weight to inhibitors (e.g., Nakao *et al.,* 1997). Although perhaps not regarded as so *fancy* as discoveries go (due to historical considerations), the identification of inhibitors of PGF action should be considered to have an intellectual merit equivalent to the discovery of novel PGFs.

A fourth suggestion would be to broaden the scope of experimental organisms to include other amphibia. The almost obsessive use of *Xenopus*, mainly because of its attributes as a laboratory organism rather than its merits as a model embryological system (reviewed by Nieuwkoop, 1996), should be put into question. Perhaps it is time to extend some of the studies which have generated either conflicting data or "dead ends" in *Xenopus* to urodeles, where most of the original tissue interactions were originally discovered and where gastrulation movements are more easily followed (Nieuwkoop and Koster, 1995).

Fifth, of course, would be the refinement of amphibian transgenesis methods (e.g., Kroll and Amaya, 1996). Although technically very difficult, the use of the "restriction endonuclease mediated incorporation" (i.e., REMI) method has recently been independently validated in *Xenopus* gene expression studies (Knox *et al.*, 1998). In addition, an "inverted terminal repeat sequences" (i.e., ITRs) method, which employs direct injection of foreign genes into amphibian eggs, has recently been developed (Fu, Wang, and Evans, 1998). This method adds ITRs from adeno-associated virus to a transgenic plasmid, followed by direct injection into fertilized eggs (reviewed by Sheets, 1998).

For unraveling complex regulatory circuits of the type which employ the combinatorial action of multiple PGFs developing a method for routine transgenesis will not, however, be enough. Promoter sequences which function in amphibia will need to be generated so that inserted genes can be expressed in particular cells and tissues at designated times in embryogenesis.

The lesson to be learned from *Drosophila* developmental genetic analyses is, however, clear: "spaghetti tangle" types of regulatory circuit models are emerging. The use of an ever increasing collection of mutant alleles and transgene constructs continues to reveal subtle features which expose the inherent complexity of embryonic patterning mechanisms. Cell polarity and Wnt signaling pathways, for example, have recently been demonstrated to exhibit extraordinarily intricate associations whereby separate domains of a single regulatory protein act on those different processes (Axelrod *et al.*, 1998). The following question should be asked: is there any reason to expect that amphibian regulatory circuits will be less complex?

For understanding the complexity of those regulatory circuits in vertebrates zebrafish developmental genetics shows great promise. Filling the gap between *Drosophila/C. elegans* and mammals may prove to be more practical with zebrafish mutants than with amphibian micro injection/animal cap bioassays.

Mutants which code for PGF-related proteins such as *nodal* have been recognized. Indeed, the combinatorial action of the nodal-related mutant genes *sqt* and *cyc* has been studied in double mutants: Feldman *et al.* (1998) have reported that those two genes cooperate to establish the embryonic organizer and mesoendoderm. Zebrafish mutant phenotype studies are also elucidating interactions between other gene products, which will soon lead to the formulation of complex regulatory circuit models of the type which are familiar to *Drosophila* researchers (e.g., Sampath *et al.*, 1998).

Concluding comment

Progress during the past decade has been impressive. Numerous specific PGFs, their receptors, and their cognate downstream target genes have been identified. Those factors/genes most likely act in combinatorial ways, rather than in the strictly linear order predicted by traditional "stepwise" tissue-interaction models for inductive interactions. Now that various molecules have been identified, integrating them into the complex regulatory circuitry which generates differentiation (the next logical research step) will likely represent a challenge. Paradigm shifts and the use of transgenic technology will be needed to achieve this uphill task. Nevertheless, "success breeds success", so once amphibian transgenic methods start to become routine laboratory practice and alterations in classical paradigms set in we can expect a burst of "cause effect" experiments which might – optimistically – even exceed the last dozen year's flood of PGF discoveries!

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