

# 1998 Warkany Lecture: Signaling Pathways in Development

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**ABSTRACT** Cell-cell signaling pervades all aspects of development, not just in vertebrates, but in all animals (metazoa). It is a typifying characteristic of the major multicellular life forms, animals, plants, and fungi, which diverged about 1.2 billion years ago from a common ancestor descended from a lineage of unicellular life forms. In metazoa, at least 17 kinds of signal transduction pathways operate, each distinguished by its transduction intermediates. Five kinds predominate in early embryonic development, namely, the Wnt, TGF- $\beta$ , Hedgehog, RTK, and Notch pathways. Five more are used in late development, and seven more in the functions of differentiated cells. The pathways must have evolved and become conserved in pre-Cambrian times before the divergence of basal members of most of the modern phyla. In metazoan development and physiology, the responses of cells to intercellular signals include cell proliferation, secretion, motility, and transcription. These responses tend to be conserved among metazoa and shared with unicellular eukaryotes and in some cases even with unicellular prokaryotes. Protein components of the responses date back 2 billion years to ancestral eukaryotes or 3 billion to ancestral prokaryotes. Each metazoan developmental process consists of a network of signals and responses, and many of these networks are conserved among metazoa, for example, by insects and mammals. The study of model organisms, even of nonvertebrate groups, is expected to continue to contribute greatly to the understanding of mammalian development and to offer opportunities to analyze the effects of toxicants on development, as well as opportunities to devise incisive assays for toxicants. *Teratology* 60:226–239, 1999. © 1999 Wiley-Liss, Inc.

The crucial role of cell-cell interactions in vertebrate development was revealed in the early 1900s when Spemann discovered the induction of the lens by the optic cup in 1908, and more strikingly, when he and Hilde Mangold discovered the organizer in 1924. Working with the amphibian embryo, which was the favorite experimental material of the time, Spemann and Mangold ('24) surgically removed the dorsal lip of the blastopore from one early gastrula and grafted it into the opposite side of another embryo of the same age, providing that host with a second dorsal lip (Fig. 1). As is well known, the grafted embryo developed two neural

plates at the neurula stage and then two body axes. In the best cases a mirror-image twin was formed, joined across the belly. One axis was located at the expected position where the resident dorsal lip had been, and the second was located at the site of the graft, which had been a presumptive ventral site in the host. The second axis, like the first, contained a well-organized central nervous system, but this system had developed from cells that would otherwise have developed into ventral skin in an unoperated embryo. The second axis, like the first, contained somite derivatives such as axial muscle, but these derivatives developed from cells that would have formed ventral coelomic and flank mesoderm in an unoperated embryo. In the midline of the secondary axis, the graft itself differentiated mostly as notochord. The dorsal lip was called the organizer because it was the only tissue fragment of the gastrula embryo capable of massively redirecting the development of nearby cells when transplanted into their midst. The resident lip was assumed to have equivalent effects on its neighbors in a normal embryo. The organizer was imagined to release inductive signals that caused nearby cells to pursue paths of development different from those they would take without the signal.

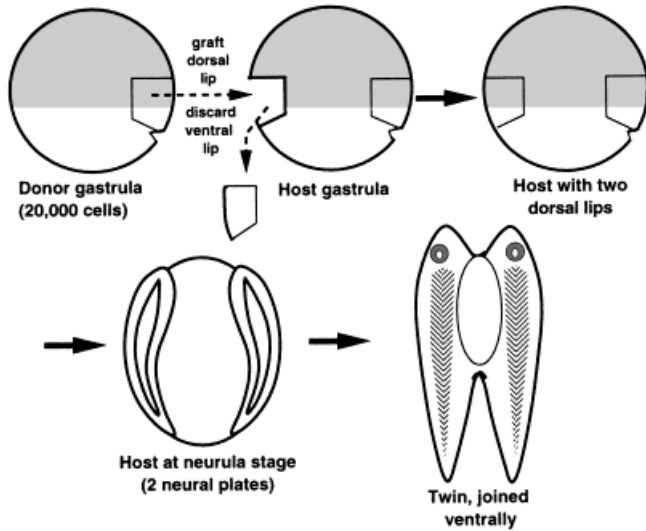
Questions immediately arose about the level of information conveyed by the inducers. Some researchers felt the signals must be very instructive, acting on naive and indifferent surrounding tissues to direct the most minute details of their subsequent development. A few researchers even suggested in the 1930s that inducers are virus-like particles capable of shuttling genes to nearby cells. The term "organizer" seemed to connote the inducer's role as a source of detailed instructions. Others, though, felt that the signals were permissive, merely releasing surrounding tissues to undertake paths of development for which they were inherently capable but repressed.

In the 40-year period after 1924, many embryonic stages were surveyed in a wide variety of vertebrates to assess the generality of inductive signaling in development. Wherever two or more embryonic tissues interacted, and this included all cases of organogenesis, an

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**Fig. 1.** The Spemann-Mangold experiment of 1924. Newt embryos at the early gastrula stage were used (20,000 cells). The cellularized pigmented hemisphere is shown uppermost, derived from the animal hemisphere of the egg. The blastopore lip has just begun to form on the right side, which is the prospective dorsal side as drawn. From a donor embryo, a block of cells is cut from this position, using simple tools such as an eyebrow hair mounted in a glass handle. This is the graft piece. From a recipient embryo (the host) of the same age, a similar sized block of cells is removed from the opposite side of the embryo and discarded. The graft piece is inserted in the hole left in the host. It soon heals into place. The host continues to develop. At the neurula stage there are two neural plates, at roughly opposite sites on the surface. Later, a twin is formed, with two body axes each containing a notochord, nerve cord, bilateral somites, heart, and a gut tube. The ventral gut region is shared. In the best cases, anteroposterior development is nearly complete, with two eyes and a mouth in each head. See text for further description and for references.

inducer was found to be released by one partner tissue and responded to by the other. Vertebrate development proceeded by a succession of inductions and responses. Signaling was everywhere. However, it proved impossible in the period from 1930–1980 to learn about the inductive signaling molecules themselves or to discern at a molecular level the responses of cells to these signals. Molecular genetic methods were not yet available to isolate genes and mRNAs, or to generate proteins by *in vitro* translation of synthetic mRNAs, or to do *in situ* hybridization to identify the time and place of a specific gene's expression in the embryo. Furthermore, cell biology was not advanced enough to provide basic insights about cell signaling or about the responses of induced cells, *i.e.*, about their *cdk/cyclin*-based cell cycle, their cytoskeleton and motor molecules, their steps of secretion, or their transcriptional control.

When developmental toxicologists of this period sought to understand the action of toxicant molecules on embryos, they gained little satisfaction. Basic information about development just did not exist, especially since they sought molecular explanations for the action of toxicant molecules. For example, despite the wide-

spread appreciation of induction in development, Wilson ('73) in his masterly *Environment and Birth Defects* could only write:

It has long been accepted that cell interactions (induction) are an important part of normal embryogenesis, despite the fact that specific 'inducer substances' have not been identified. [Failures] . . . of normal interactions, which may lead to deviations in development, include, for example, lack of usual contact or proximity, as of optic vesicle with presumptive lens ectoderm; or the incompetence of target tissue to be activated in spite of its usual relationship with activator tissue, as in certain mutant limb defects; or the inappropriate timing of the interrelation, even though all parts are potentially competent. That the nature of cell-to-cell contacts and the manner of their adhesion are important determinants in both normal and abnormal development has been demonstrated. . . Insufficient or inappropriate cellular interactions usually result in arrested or deviant development in the tissue ordinarily induced or activated by the interaction.

After the long hiatus, research of the past 15 years has yielded significant insights about cell-cell signaling in development. Signaling indeed pervades almost every event of vertebrate development, from the earliest steps of axis specification, then through the diverse kinds of morphogenesis, organogenesis and cytodifferentiation in the embryo, then through the growth and sexual maturation of the juvenile, and finally to the tissue replacement of the adult (*e.g.*, skin, gut, blood cells, muscle satellite cells) and the adult's ongoing physiology. If one considers all this signaling, the variety of signaling pathways and basic cell responses to signals appears to be surprisingly limited and comprehensible, though not simple. Defects of signaling are turning up in a variety of heritable diseases and in a variety of kinds of cancer. In this latter respect, numerous oncogenes (those genes modified in the cells of tumors) have been found to encode altered signals and intermediates of signal transduction pathways. The altered product is often spontaneously active or is produced in an unregulated way (Capobianco *et al.*, '97; Hata *et al.*, '98; Ming *et al.*, '98; Porter and Vaillancourt, '98).

This article is meant to serve as a review of the multiple uses of the major signaling pathways at different stages of embryonic development, and as a review of the evolution of signaling within the metazoa (the animal kingdom). The relevance of this information to developmental toxicology is found in the expectation that, since major signaling pathways are widely conserved among metazoa and crucial to almost all steps of development, they are plausible targets of toxicants and attractive candidates for toxicological investigation. Since signals, receptors, and signal-modifying agents reside in the intercellular space, signaling is vulnerable to disruption by toxicants that cannot cross the cell's plasma membrane to act inside the cell, as well as those that can. Temporary disruption

of signaling is likely to have lasting developmental consequences because, as is well known, development builds successively on stage-specific decisions.

### THE MAJOR SIGNALING PATHWAYS

Seventeen signal transduction pathways are currently recognized (Table 1). Each is distinguished by its set of signal-transducing components. Five of these pathways are used repeatedly in the early development of complex metazoa such as vertebrates, arthropods, and nematodes. Five more are used repeatedly in later development, that is, in organogenesis and cytodifferentiation. The remaining seven are used almost entirely in the physiological functioning of the fetus, juvenile, and adult, which is accomplished by differentiated cells.

As information transfer pathways, these signaling pathways are basically different from metabolic pathways, even though both are called "pathways." In a biosynthetic metabolic pathway, a carbon compound passes through a series of enzymatic steps, with appropriate energy inputs, undergoing modifications until it emerges as an end product ready for incorporation into a macromolecule or complex lipid. But in a signal transduction pathway, carbon atoms and energy are not passed along. Only an impulse is relayed by way of successive reversible changes of state of switch-like intermediates. At the end of the pathway, the transduced signal activates or inhibits some target protein which is a component of a cellular process such as transcription, secretion, motility, proliferation, or apoptotic cell death. In development, the most frequent target of signaling is transcription, and some pathways affect only transcription. Different pathways activate or repress different genes at different times and places in the embryo.

What do the 17 signaling pathways have in common? Each entails an extracellular signal released by some cell of the organism. The signal is called a ligand since it eventually binds ("ligates") to a specific receptor protein of another cell, or sometimes the same cell. Many of these signal ligands are complex proteins, although some are small molecules (e.g. steroids). Some require several steps of processing in the extracellular space before they can be bound by a receptor, and some require the presence of extracellular matrix (ECM) components before they act as signals. As shown in the generalized pathway of Figure 2, most pathways involve a transmembrane receptor protein that binds the signal ligand, which cannot cross the plasma membrane on its own. The two or three exceptions are those pathways with hydrophobic ligands such as steroid hormones, nitric oxide, and prostaglandins. These pass through the membrane but nonetheless are bound by receptors inside the cell. When a ligand binds to a transmembrane receptor, the receptor changes state and transduces a signal from its cytoplasmic tail inside the cell. This sets off the successive changes of state of each of a series of switch-like intermediates, each going

**TABLE 1. The 17 intercellular signaling pathways\***

Early development and later
1. Wnt pathway
2. Receptor serine/threonine kinase (TGF $\beta$ ) pathway
3. Hedgehog pathway
4. Receptor tyrosine kinase (small G proteins) pathway
5. Notch/Delta pathway
Mid-development and later
6. Cytokine receptor (cytoplasmic tyrosine kinases) pathway
7. IL1/Toll NF $\kappa$ B pathway
8. Nuclear hormone receptor pathway
9. Apoptosis pathway
10. Receptor phosphotyrosine phosphatase pathway
Larval/adult physiology
11. Receptor guanylate cyclase pathway
12. Nitric oxide receptor pathway
13. G-protein coupled receptor (large G proteins) pathway
14. Integrin pathway
15. Cadherin pathway
16. Gap junction pathway
17. Ligand-gated cation channel pathway

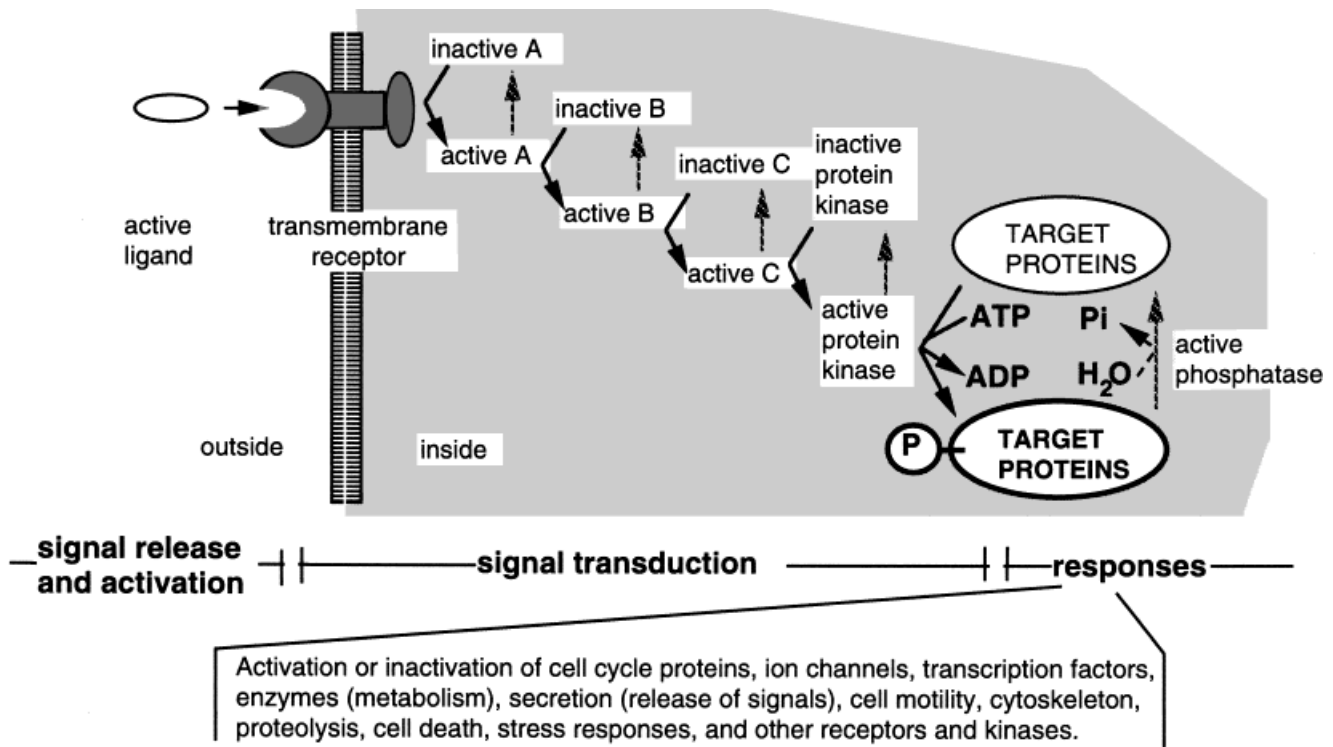
\*These are shared by most animals (metazoa). Note the five pathways used heavily in the early development of most animals, that is, before organogenesis begins. Along with these, five more are used in later development after organogenesis begins. Seven pathways are used, mostly in the function of differentiated cells, much of which also involves signaling. Each pathway is identified by the particular transduction intermediates it contains. Most pathways are unique to metazoa, although components of each are often found in single-celled eukaryotes in other signaling roles, such as pathways of checkpoint control, stress response, infection response, mating, or feeding.

from inactivity to activity, or vice versa. The first intermediate affects the second which affects the third, and so on. However, this change of state is only transient, and by the time step 3, for instance, is active, the receptor and step 1 may be inactive again and need another input of ligand for activation anew. In many cases the intermediates are protein kinases. Each phosphorylates the next kinase, thereby activating it. These activated kinases are soon dephosphorylated by low-level phosphatases and return to inactivity until a new input of signal arrives. Finally at the end of the pathway, an activated protein kinase phosphorylates a target protein, leading to the activation or inhibition of a particular cellular process, depending on the particular function of the target. The human genome is estimated to contain approximately 80,000 genes; 2,000 of these may encode protein kinases, and 1,000 may encode phosphoprotein phosphatases. The genome of the animal *Caenorhabditis elegans*, a nematode, has been completely sequenced, and at least 11% of the 19,000 genes encode signaling components (Chervitz et al., '98). This abundance is a measure of the metazoan organism's reliance on signaling.

### THE EVOLUTION OF SIGNALING PATHWAYS

In the past decade, a universal tree of living organisms has been deduced from the comparison of DNA sequences of a variety of prokaryotic and eukaryotic





**Fig. 2.** Generalized pathway for signal transduction (information transfer via a series of on/off switches). From left to right, a protein signal (the ligand) in the intercellular space binds to a cell's transmembrane receptor on its extracellular domain. Upon binding, the receptor undergoes a transient modification of its cytoplasmic domain, e.g., association with another receptor molecule. This triggers a transient modification of the first of a series of proteins in the cell, each an intermediate in the signal transduction pathway. Each intermediate modifies the next. Often this is done by each phosphorylating the next, using ATP as the phosphate source, and thereby activating the kinase

activity of the next. Phosphatases slowly remove the phosphates, inactivating the intermediates until they are rephosphorylated following the next input of signaling. The last pathway component modifies a target protein, usually by phosphorylation or limited proteolysis. This target protein is a component of a cellular process such as transcription, the cell cycle, motility, or secretion. Modification of the target leads to activation or repression of the process. This is the cell's response to the signal. A circled P indicates a phosphate group covalently added to a target protein.

organisms (Woese, '87; Lake, '90). If we look backwards in time, we see that animals shared a last common ancestor with plants and fungi 1.2 billion years ago. Some very special single-celled or simple multicellular eukaryotic organism gave rise to the three major multicellular kingdoms. If we look in the period from 1.2–2.2 billion years back, this ancestor of the multicellular forms shared ancestors with all the single-celled eukaryotic forms of life such as amoebae, flagellates, and ciliates. Then, approximately 2.2 billion years ago, there was an ancestor of all the living eukaryotic life forms, single-celled and multicelled, and this was also the ancestor of all the extant archaeobacteria, which are prokaryotic organisms. Since the ancestor was presumably a prokaryote, all eukaryotic features must have evolved more recently than 2.2 billion years ago. This prokaryotic ancestor, in turn, is thought to have shared a last common ancestor with the extant eubacteria approximately 3.2 billion years ago. This prokaryotic ancestor of ancestors would be the progenitor of all extant life forms.

The sequence information confirms and extends proposals from biochemists, geneticists, and cell biologists

that most basic cellular processes, which are shared by all life forms, must have evolved billions of years ago and must have been conserved to the present. For example, replication, transcription, translation, biosynthetic metabolism, degradative metabolism, energy metabolism, processes of transport of materials across membranes, and the basic structure of proteins, nucleic acids, and membranes, were already present 3.2 billion years ago in the ancestor of ancestors. This is borne out by the commonality of DNA sequences, protein sequences, and biochemical functions in all life forms. Furthermore, the basic organization and functions shared by all eukaryotic cells, but not prokaryotes, must have been present at least 2 billion years ago, before single-celled eukaryotes diverged. This conservation would include their large size ( $>1,000\times$  the volume of the prokaryotic cell), their dynamic membranes capable of endocytosis and exocytosis, their membrane-bounded organelles (most prominently the nucleus), mitosis and meiosis, sexual reproduction by cell fusion, a cdk/cyclin-based cell cycle, actin- and tubulin-based dynamic cytoskeletons, cilia and flagella, and histone/DNA chromatin complexes. These ancient processes,

which evolved in the single-celled prokaryotes and early eukaryotes long before metazoa, constitute the core biochemical, genetic, and cell biological processes of metazoa. These ancient processes will be referred to as "conserved core processes." They turn out to constitute most of the responses metazoan cells make to intercellular signals.

One can ask, then, what have metazoa been doing for the past 1.2 billion years? They, as well as plants and fungi, have uniquely evolved in the dimension of multicellularity. This is an informational dimension in which the conserved core processes are activated and inhibited (regulated) at specific times and places in the organism during its life cycle. In the evolution of multicellular organisms, the activities of all these core processes, even the cell cycle, have become contingent on signals passing from cell to cell, whereas in single-celled organisms these same processes have no such dependence. Through variation and selection, contingency has been imposed secondarily on the old core processes. To this end, metazoa have evolved a wide variety of extracellular signals, cell-cell signaling pathways, and elaborate circuits and networks of response. This informational dimension, which is sometimes referred to as "regulatory biology," is different from the material/energy dimension perfected by the prokaryotes and single-celled eukaryotes. Inhibitions (not doing something) have as much efficacy as activations (doing something) in regulatory pathways, whereas in the material/energy realm, only activity counts. The embryonic development of metazoa is a vast regulation of conserved processes, unfolded in time and multicellular space with the proliferation of cells in the embryo. As many authors have noted (Wolpert, '69), metazoa from flatworms to mice have rather similar differentiated cell types and differ mostly in their arrangement of these cell types. These cell types carry out the ancient conserved core processes. Development generates the arrangements.

Recent genome comparisons have also illuminated the evolution of signaling pathways in metazoa. It is generally agreed that the Cambrian period (543–505 million years ago) was a watershed in metazoan evolution. By the mid-Cambrian, members of all the extant phyla were already present, including chordates. Each phylum is distinguished by a body plan, which is a unique anatomical configuration of secreted signals and regionally expressed selector genes, encoding transcription factors. The signals and factors loom large in late development, as discussed later. In any event, all these body plans were already present by the mid-Cambrian and have been conserved ever since. All signaling pathways evolved in pre-Cambrian times before the diversification of body plans. This would explain why diverse phyla such as chordates, arthropods, and nematodes share so many pathways. In their distinguishing sequences of transduction intermediates, the pathways have not changed since pre-Cambrian times.

In the pre-Cambrian evolution of intercellular signaling, metazoa borrowed heavily from the limited pathways of single-celled eukaryotes, those used for feeding, mating, pathogen response, and stress response. Yeast, for example, is a single-celled fungus sharing a last common ancestor (perhaps a single-celled ancestor) with metazoa 1.2 billion years ago. The yeast genome has been completely sequenced (Mewes et al., '97). Yeast and metazoa share many signaling components, e.g., those of cell cycle checkpoint pathways, DNA damage pathways, and stress pathways such as that for hyperosmotic shock (Toone and Jones, '98). These involve several protein kinases very similar to those used in metazoan intercellular signaling. Another stress response pathway senses and responds to temperature extremes, alcohol, or anaerobiosis. Known as the "unfolded protein response," this pathway in metazoa is shared, not just with yeast, but with eubacteria, and it must date back 3.2 billion years. Single-celled eukaryotes have added an unfolded protein response for prospective secreted proteins in the ER/Golgi involving a transmembrane receptor serine/threonine kinase (Sidrauski et al., '98). Related transmembrane kinases turn up in intercellular signaling pathways (see the TGF- $\beta$  pathway, below).

The IL1/Toll/NF $\kappa$ B signaling pathway is used in plants and metazoa for nonadaptive detection and response to bacterial infection (O'Neill and Greene, '98). Hence it must date back at least 1.2 billion years. It is used prominently in *Drosophila* early development. Yeast mating involves specific cell-cell recognition of mating partners, the arrest of the cell cycle at the G1 phase, and pronounced changes of transcription. It makes use of numerous components (e.g., 7-pass transmembrane receptors, large and small G proteins) found in metazoa in intercellular signaling. Finally, some crown eukaryotes such as the cellular slime molds (e.g., *Dictyostelium*), whose ancestors branched from the metazoan line shortly before plants and fungi, spend part of one part of the life cycle feeding as single-celled predators and spend the other part as nonfeeding multicellular slugs and spore-dispersing bodies with at least two differentiated cell types. These cells have cell-cell signaling involving several components shared by metazoa, such as cAMP, G-protein linked receptors, a variety of protein kinases, and JAK/STAT transcriptional control. From their unicellular past, early metazoa had a lot to draw upon in the evolution of intercellular signaling.

Though made of many borrowed components, the metazoan signal transduction pathways are largely unique. The receptor tyrosine kinase pathway is found in all metazoa, even sponges, but not outside metazoa. The uniquely metazoan Wnt and TGF- $\beta$  pathways are shared by arthropods, chordates, and nematodes, whereas the Hedgehog pathway is shared by chordates and arthropods but seems to be missing from nematodes. Early metazoa also evolved cell junctions, the extracellular matrix, and epithelial organization. Some

of the cell-cell signaling components resemble matrix and junctional components.

Although most signaling pathways were already present before the pre-Cambrian divergence of the metazoan phyla, the different phyla made different use of these pathways in the evolution of their distinctive body plans. As mentioned above, each phylum is defined by a characteristic body plan constituting a spatial arrangement of compartments of expressed selector genes (encoding transcription factors) and secreted signals (Gerhart and Kirschner, '97). Among the selector genes, the Hox genes are the best known, but there are many more (Gellon and McGinnis, '98). Body plans differ in their particular variety and arrangement of these signals and factors. These plans have been conserved from the Cambrian to now. Still, within each phylum, evolution has continued. Although signaling pathways are basically unchanged in their transduction components, the variety of ligands and receptors has increased, and the variety of targets of signaling has increased, as has the sophistication of regulatory networks and circuits. Presumably in the pre-Cambrian, when modern body plans first arose, early development of these animals led directly to that body plan, which then served directly as the platform for late development of a limited variety of definitive cell types and cell arrangements, yielding a rather simple adult compared to modern forms. Since then, many steps of organogenesis have been inserted in the developmental sequence after the body plan has formed but before cytodifferentiation occurs. In vertebrates, for example, neural crest derivatives have been added, as have the bony skeleton, fins, and limbs. Also, early development has become more complex. In the steps from the egg to the body plan, various reproductive specializations have been inserted such as more yolk, more protective layers, more extraembryonic tissues (especially in the evolution of the reptilian cleidic egg in the Permian), and eventually placentation (in the Cretaceous mammalian line after lactation was accomplished).

## SIGNALING IN EARLY DEVELOPMENT

Five pathways are used repeatedly in early development, namely, the Wnt, TGF- $\beta$ , Hedgehog, receptor tyrosine kinase, and Notch/Delta pathways. When active, these mostly affect gene expression and do so rather directly. These five plus an additional five pathways are used repeatedly in later development, i.e., in organogenesis and cell differentiation. The molecular genetic analysis of *Drosophila* development has been essential in the elucidation of the roles and components of the pathways. Mutants which were blocked in early development at specific times and places were collected in large numbers and analyzed genetically and by gene sequence (initially by C. Nüsslein-Volhard and E. Wieschaus, who received the Nobel Prize in 1996 for this work). To everyone's surprise, when similar sequences were later sought in vertebrates, these were found in

**TABLE 2. Similarities of arthropods and chordates\***

1. Anteroposterior organization: HOX gene complex: similar order of genes in the cluster, and similar order of expression domains in the posterior head and trunk (thorax/abdomen). *Drosophila* and mouse, recognized 1987–1992.
2. Anterior organization: *Ems/otd* (*emx/otx*) selector genes: similar nesting of expression domains in the anterior head. *Drosophila* and mouse, recognized 1992–1995.
3. Dorsoventral organization: *Sog/dpp/tolloid* (*chordin/BMP2, 4/xolloid*): similar gene expression domains (inverted). *Drosophila* and *Xenopus*, recognized 1995–1997.
4. Segmentation: *Engrailed* and Hh/Shh expression domains similar in posterior half of segment or somite. *Hairy* gene expression in alternate segments or somites. *Drosophila*, amphioxus, and zebra fish, recognized 1996–present.
5. Appendage/limb patterning: based on Wg/Hh/Dpp (Wnt/Shh/BMP) signaling and *en*, *Ap* (*En*, *Lmx*) selector genes. *Drosophila*, chick, and mouse, recognized 1994–1997.
6. Eye specification: based on *eyeless/Pax6* selector genes. *Drosophila*, mouse, and human, recognized 1994–1997.

\*The organisms of these two phyla seem very different, e.g., insects and crustaceans vs. fish and mammals. Most similarities were unsuspected 10 years ago. These similarities serve as evidence that the pre-Cambrian common ancestor of chordates and arthropods was already complex in its organization and perhaps segmented. The chordate line then underwent a dorsoventral inversion. Many aspects of cytodifferentiation are also similar, e.g., the use of MyoD in muscle and Achaete/Scute in nerve cells.

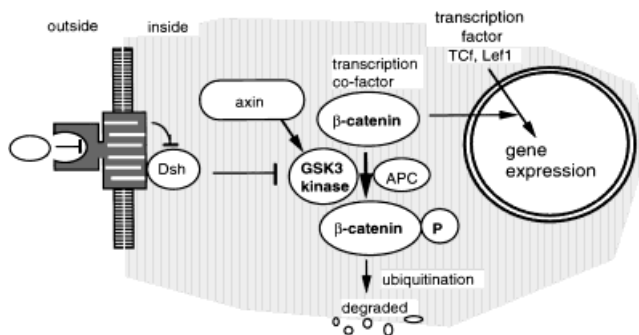
large numbers, and the encoded proteins were often used in developmental roles similar to those in *Drosophila*. The similarities of arthropod and chordate development, which are outlined in Table 2, exceeded everyone's expectations. The similarity of gene order and region-specific expression of the Hox genes and *emx/otx* genes of fruit flies and mice is now well-known, but 15 years ago the Hox genes were widely considered an arthropod specialization. As a more recent example, informed thoughtful experts of chordate evolution had long thought that the body segmentation of arthropods and the somite segmentation of chordates have nothing in common, being independently evolved in two separate lines of animals. However, in the past 3 years, the development of vertebrate somites and insect segments has been found to involve the expression of many similar genes in similar spatial and temporal patterns of expression, several of these for signaling components. Now there are discussions of the possibility that the common ancestor of chordates and arthropods was in fact already segmented (Christ et al., '98).

To review the roles of the five pathways of early development, they will be taken one at a time.

### The Wnt pathway

The name is a contraction of Wingless and Int, referring to *wingless*, a *Drosophila* mutant lacking the signal, and to *int*, a transformed and malignant mouse cell line in which Maloney mammary tumor virus had integrated next to a gene encoding the Wnt signal. As shown in Figure 3,  $\beta$ -catenin is the key intermediate of this complex pathway (Wodarz and Nusse, '98). This





**Fig. 3.** The Wnt pathway.  $\beta$ -catenin is the key intermediate protein. It is phosphorylated by the GSK3 kinase, making it susceptible to degradation by proteolysis. The Wnt signal leads to inhibition of the kinase by way of the Dsh (dishevelled) protein.  $\beta$ -catenin is not degraded and it accumulates. It binds to a transcription factor, enters the nucleus, and alters the transcription of specific genes. A circled P indicates phosphate covalently added to a protein. See text for details.

interesting protein is also used in all metazoa in adherens junctions and septate junctions or desmosomes, where it complexes to cadherins and actin microfilaments. When the protein is phosphorylated by a specific kinase, it is destroyed rapidly by proteolytic degradation through a ubiquitin pathway. It is rapidly replaced by translation. When the Wnt signal binds to the transmembrane receptor, signal transduction via several intracellular steps leads to the inhibition of the specific kinase, so that  $\beta$ -catenin is not phosphorylated and hence not degraded. It accumulates and complexes with the transcription factor Tcf/Lef. The complex enters the nucleus and activates transcription of specific genes. When the Wnt signal disappears, the phosphorylation of  $\beta$ -catenin resumes and it is degraded again. Specific transcription stops.

The ligand, which sticks to extracellular matrix components, hardly diffuses from its source and acts mostly on nearby cells. In *Drosophila*, there are genes for at least 3 receptors, 2 ligands, and 1 of each intermediate. The pathway is used in this organism in the development of the segments of the head, thorax, and trunk, in the development of appendages such as legs and antennae, in the development of the wings and eye, and in aspects of oogenesis and neurogenesis.

In vertebrates, there are genes for at least 11 ligands, 3 receptors, and several of each intermediate (e.g., 3  $\beta$ -catenin genes in mouse). In general, vertebrates contain many more genes for each step of a pathway than do invertebrates, a point we will return to later. A list of the many developmental processes involving Wnt signaling in vertebrates is given in Table 3. More processes are added every month. These include steps of early axis specification, the posteriorization of the nervous system, hair follicle development, and the development of the female genital tract.

### The TGF- $\beta$ pathway

The transmembrane receptor consists of two proteins (receptor types I and II), and the cytoplasmic tails of

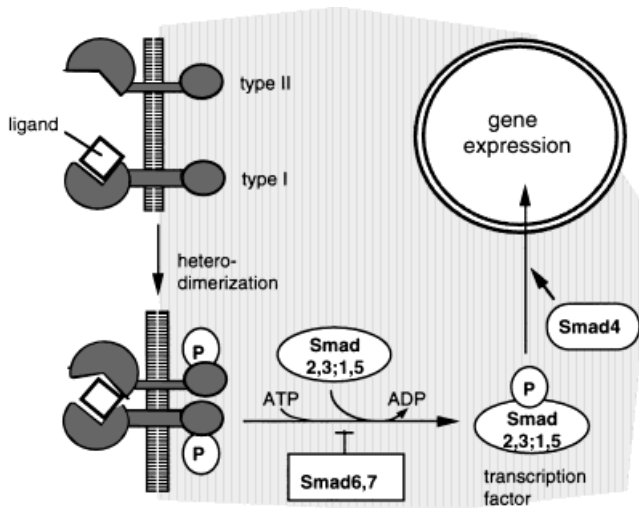
**TABLE 3. Various uses of signaling pathways in vertebrate development\***

1. Wnt pathway (Wnt 1-11)
  - Dorsalization of body (fish, frogs), induction of organizer or node
  - Posteriorization of neural plate, midbrain development
  - Dermamytome induction, somite dorsoventral organization
  - Dorsalization of fin or limb
  - Female reproductive development, kidney development
  - Dorsoventral differences of limb, hematopoiesis
2. TGF- $\beta$  pathway (e.g., BMP, TGF- $\beta$ , GDF, VEGF, Nodal, Activin, Dorsalin)
  - Mesoderm induction
  - Induction of organizer, left-right asymmetry
  - Ventralization of mesoderm and ectoderm
  - Neural crest development to neurons
  - Chondrogenesis of limb, bone development
  - Digit-web spacing, tooth, heart
3. Hedgehog pathway (e.g., Sonic, Indian, Desert)
  - Notochord induction of floor plate of neural tube
  - Notochord and floor plate induction of sclerotome of somite
  - Prechordal mesoderm induction of prosencephalon
  - Left-right asymmetry, somitogenesis, lung
  - Fin/limb development: ZPA induction of anteroposterior axis
  - Gut/visceral mesoderm, hair follicle, skin, tooth, spermatogenesis
4. Receptor tyrosine kinase pathway (e.g., EGF, FGF, PDGF, Eph)
  - Mesoderm maintenance
  - Limb (AER), vasculogenesis
  - Hair follicle, inner ear, retinotectal projection
  - Astrocyte differentiation, branchial arch signal to neural crest, heart, lung, tooth
5. Notch/Delta pathway (e.g., Delta, Serrate, Jagged)
  - Several steps of neurogenesis
  - Oligodendrocyte differentiation, retina development
  - Somitogenesis, inner ear development
  - Feather bud development
  - Blood cell development (e.g., thymocytes)

\*These pathways are used repeatedly in early development. The particular uses in developmental processes are well-conserved across vertebrates.

both are serine/threonine kinases (Fig. 4). Upon ligand binding, they form a heterodimer in which the type II kinase phosphorylates the type I tail, which then phosphorylates specific pathway intermediates, the Smad 2 and 4 proteins. These form a complex and translocate to the nucleus to activate the transcription of certain genes, some of which encode inhibitory Smad proteins (Smad 6, 7) which antagonize the Smad2, 4 complex (Whitman, '98). Some cancer cells produce spontaneously active mutated Smad proteins (Hata et al., '98) and seem to remain in a "signaled" state. TGF- $\beta$  ligands are generally produced as protein precursors requiring cleavage by a protease in the Golgi apparatus, yielding two or more forms differing in their diffusability from the source.

In *Drosophila* there are at least three secreted protein ligands (Dpp, Gbb, and Screw). In early development, the pathway is used in this organism in the establishment of dorsoventral compartments (espe-



**Fig. 4.** The TGF- $\beta$  pathway. The receptor is a serine/threonine kinase. When it binds its signal ligand, it becomes active due to dimer formation. It phosphorylates a cytoplasmic protein, a Smad protein. This enters the nucleus and alters specific gene expression. A circled P designates a phosphate group covalently added to a protein. See text for details.

cially the amnioserosa and dorsal ectoderm) and in the development of appendages, eyes, wings, and the gut. Signals are distributed in gradients over a large field of cells in the wing and the amnioserosa. The mechanism of gradient formation was recently deciphered in *Drosophila* with the help of mutants. The diffusing Screw ligand meets a secreted antagonist, the Sog protein, which binds to it and prevents its binding to its receptor. When the Sog protein is complexed with Screw, it is in turn susceptible to degradation by an extracellular metalloproteinase (Tolloid), leading to Screw release. Depending on the locations of cells secreting these three agents, a gradient of active Screw protein is set up. When it binds to its receptor, it increases the intensity of signaling within that cell from yet another TGF- $\beta$  receptor, which binds the Dpp protein (Neul and Ferguson, '98). In other situations in the embryo, the Dpp ligand acts on its own as an on/off signal, without graded effects from Screw. The interaction circuitry of signaling components can be very complex. Gradient formation in vertebrates is not yet well understood.

In vertebrates there are many protein ligands, encoded by at least 28 genes. Among these are the subfamilies of bone morphogenetic proteins (BMPs), Veg-related proteins (Vgrs), TGF- $\beta$ s, Nodals, and growth and differentiation factors (GDFs). Some ligands stick to ECM components such as sulfated proteoglycans, affecting their diffusion and receptor binding. As described in Table 3, the pathway is used widely in vertebrate early development, in the formation of early mesoderm, endoderm, epidermis, and ventral mesoderm. It is later used in limb development, neural tube patterning, gut development, lens formation, bone and

cartilage formation, various neural crest differentiations to neurons and teeth, and many other steps.

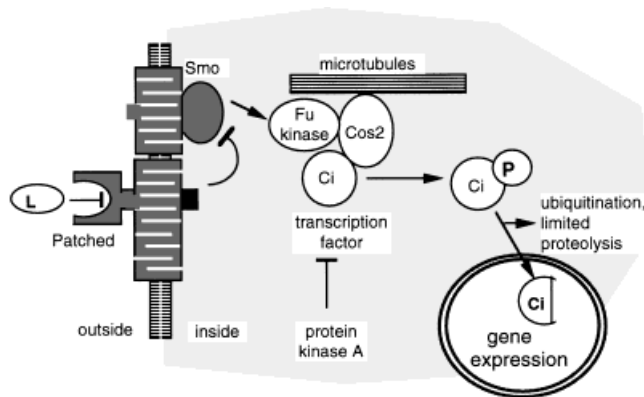
### The Hedgehog pathway

The name derives from a *Drosophila* ligand-deficient mutant that forms excess spikes ("denticles") on the larva and looks like a hedgehog. This remarkable pathway involves two transmembrane proteins: one (called Patched) activates intracellular intermediates of the pathway, such as the Fused kinase; the other (called Smoothened) inhibits this activator (Fig. 5). When Smoothened binds the Hedgehog ligand, it ceases to inhibit Patched, which then can activate the Fused kinase. Thus, the ligand's inhibition of inhibition is used to achieve activation. Intermediates of the pathway include a transcription factor (cubitus interruptus/Gli) which is phosphorylated by the Fused kinase and a kinesin-like protein that may attach the kinase and transcription factor to microtubules in the cytoplasm. When the factor is phosphorylated, it is cleaved proteolytically and a fragment of it translocates to the nucleus and activates specific gene expression (Johnson and Scott, '98). Protein kinase A exerts a negative effect on the pathway, and this is overcome when the ligand is bound.

The Hedgehog ligand is secreted as a cholesterol-modified protein which diffuses poorly, but which has the capacity to cleave itself by limited proteolysis (an "intein") to yield a smaller and more diffusible ligand. The pathway is used in *Drosophila* in segment formation and maintenance, being produced by cells of the posterior compartment of each of the 14 segments. It is also used in appendage, eye, and wing development. In the developing eye, a wave of Hedgehog secretion sweeps across the eye in a posterior to anterior direction, signaling the end of cell proliferation and the orientation and the start of ommatidium differentiation.

In vertebrates, there are several ligands, such as Sonic, Indian, and Desert Hedgehog. The pathway is widely used (see Table 3). In early development the notochord, floorplate of the neural tube, and prechordal mesoderm secrete the Sonic Hedgehog ligand. Nearby cells of the neural tube respond to the signal to establish dorsoventral differences within the neural tube and brain. As a toxicological example of the pathway's disruption, when pregnant cows eat *Veratrum*, a plant containing the steroidal alkaloid cyclopamine, their calves are sometimes born with holoprosencephaly and cyclopia. This is now understood as the effect of cyclopamine in blocking reception of the Hedgehog ligand, and hence disrupting the prechordal mesoderm's signal to the diencephalon rudiment to inhibit eye formation in the center of the rudiment. Hence the diencephalon forms one large central eye (Chiang et al., '96; Cooper et al., '98). The pathway is also used in the differentiation of a portion of the somite into the sclerotome, as distinct from the myotome and dermatome, when somite cells adjacent to the notochord respond to the notochord's





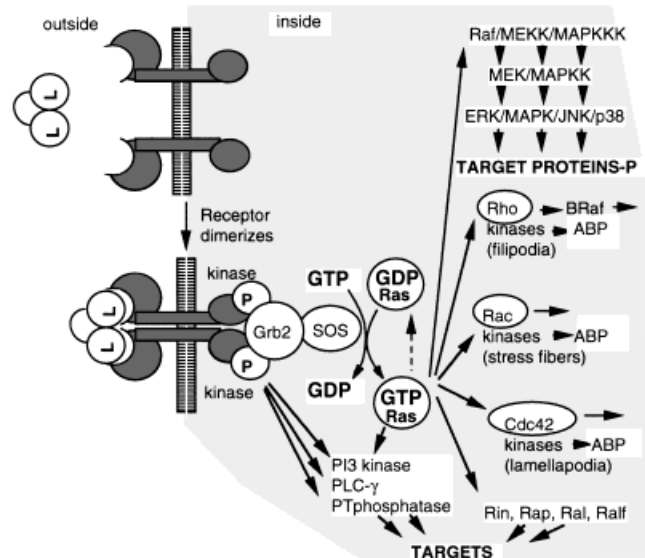
**Fig. 5.** The Hedgehog pathway. When the signal is present, the Fused kinase (Fu) phosphorylates a specific transcription factor (cubitus interruptus [Ci]/Gli) which is then proteolytically cleaved. Thereafter it can enter the nucleus, and affects gene expression. The pathway contains a double inhibition. The Smoothened (Smo) protein activates the Fu kinase but is itself shut down by the Patched (Ptc) receptor. The Hedgehog signal binds to the Ptc receptor and inhibits its inhibition of Smo, so that Smo can then activate the Fu kinase. L designates the ligand. A circled P designates a phosphate group covalently added to a protein. See text for details.

Sonic Hedgehog signal. This too is blocked by cyclopamine (Incardona et al., '98). The pathway is also used in regionalization of the gut by the visceral mesoderm, in limiting the domain of pancreas development, and in the zone of polarizing activity (ZPA) of the limb bud to establish the anteroposterior dimension of the limb. In some humans, the Smoothened receptor is partially inactive, and the pathway operates continuously at a low level, even without ligand. The defect is associated with a high level of basal-cell carcinoma (Ming et al., '98).

### The receptor tyrosine kinase (RTK) pathway

As the name implies, the cytoplasmic tail of the transmembrane receptor is a kinase which specifically phosphorylates tyrosine residues on proteins. When the receptor binds its ligand, it dimerizes and each member cross-phosphorylates the cytoplasmic tail of its partner (Fig. 6). This renders the tails accessible to interaction with the initial components of any or all of at least four major transduction series, a truly bewildering diversity of effects (Hunter and Van Der Geer, '94). One of these components is the ras protein, a small G-protein. Another is a PI3 kinase, and the third is a phospholipase (PLC $\gamma$ ) and the fourth is a phosphotyrosine phosphate. The ras-mediated series leads to the activation of one or more of three pathways of protein kinases (MAPKs; each series three deep). Some of these kinases are also used in stress responses.

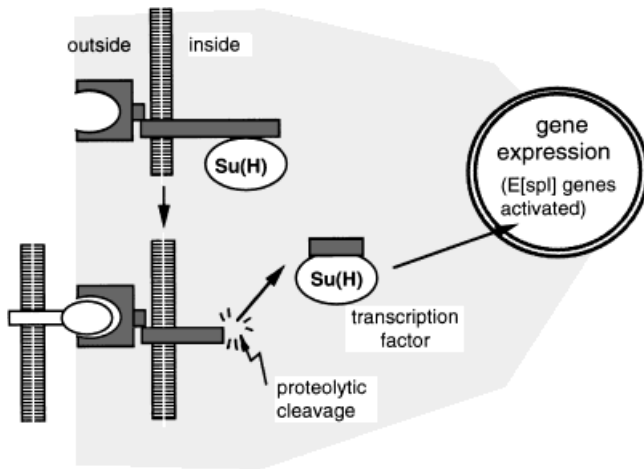
An immense variety of ligands and receptors feed into this complex pathway. All ligands are proteins secreted into the intercellular space or retained on the surface of nearby cells. Among the ligands are subfamilies such as epidermal growth factors (EGFs), fibroblast growth factors (FGFs), and platelet derived growth



**Fig. 6.** The receptor tyrosine kinase (RTK) pathway. The receptor, which is a tyrosine kinase, dimerizes when it binds a signal ligand. The two cytoplasmic tails cross-phosphorylate each other. The phosphorylated tails serve as sites where at least three intermediates bind and gain activity. These three are the ras protein, a PI3 kinase, and phospholipase C $\gamma$ . Active ras then activates a series of intermediates, including many protein kinases (MAP kinases). A wide variety of responses are triggered, including specific transcription and cell motility. L designates the ligand. A circled P designates a phosphate group covalently added to a protein. See text for details.

factors (PDGFs). The particular names are mostly of historical interest. The factors are now known to be produced from many sources and to have many effects in addition to growth effects. In the early development of *Drosophila*, an EGF-like ligand and the RTK pathway have a central role in oogenesis in the interactions of follicle cells with the oocyte, as the oocyte develops anteroposterior and dorsoventral polarity. In the egg, the development of the termini and endoderm depends on an RTK of the egg transducing a protein signal released from opposite poles of the extracellular chorion/vitelline complex. In the eye, the development of the R7 light receptor cell in the ommatidium depends on an RTK (called Sevenless) which binds a ligand (called Boss) specific to this single usage. An FGF-like ligand is used in tracheogenesis, in the formation of the branched airways of the larva and fly.

Vertebrates have an enormous variety of ligands and receptors, with many developmental roles (see Table 3). The FGF family, for example, includes at least 18 members encoded by 18 genes, each protein differing slightly in sequence (two of them even lack secretion signal sequences), receptor specificity, and time and place of production. FGF ligands are used widely in development, in early mesoderm maintenance, in notochord formation, limb development, the eye, craniofacial development, the nasal and otic placodes, muscle differentiation, vertebral bone growth, vasculogenesis, and hair follicles. EGF-like ligands are also widely used.



**Fig. 7.** The Notch/Delta pathway. When the signal is bound, the receptor is cleaved in its cytoplasmic domain, releasing a fragment which binds to the Su(H) protein. The complex enters the nucleus and affects specific gene expression. See text for details.

### The Notch/Delta pathway

This pathway is named after the *Drosophila* mutants in which the components were discovered. It operates between adjacent cells because the ligand (the Delta or Serrate protein) is a transmembrane protein not released from the signaling cell. The receptor, when liganded, is proteolytically cleaved within its cytoplasmic tail. A protein fragment of the tail associates with the Su(H) protein and enters the nucleus to activate specific gene expression (Fig. 7). Among the genes expressed in fruit flies (and also in mice) in response to the Delta signal are those of the E(spl) gene complex, whose several encoded transcription factors include Groucho, a widely used negative regulator (Kimble, '97).

In *Drosophila* this pathway is important in the development of the central and peripheral nervous systems, muscle, the eye, and the gut. Its circuitry generates lateral inhibition and differentiation within groups of equivalent cells. Initially these indifferent cells have the potential to develop to either of two cell types, and each cell releases and receives low levels of the Delta signal. However, no cell can remain both a releaser and receiver for long. When cells receive a little more Delta than they produce, they soon lose the ability to produce it. When cells produce more Delta than they receive, they soon lose the capacity to receive it. Hence, cells become either producers or receivers. The signaling difference is used to choose one cell type or the other. The population is metastable when all cells are equivalent, and stable as a pattern of spaced differences. In vertebrates the pathway is used in the development of somites, the nervous system, and the immune system (see Table 3). Some cancer cells produce a shortened Notch protein that is active even without ligand binding (Capobianco et al., '97).

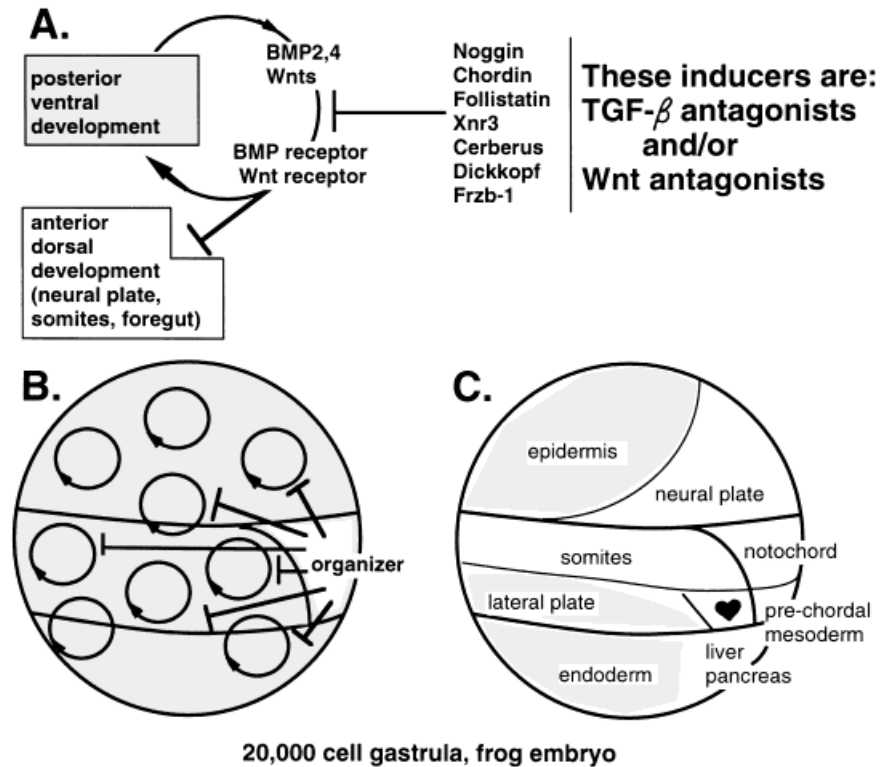
### CURRENT UNDERSTANDING OF THE ORGANIZER'S INDUCTIVE SIGNALS

As noted above, Spemann's discovery of the organizer in 1924 introduced the scientific world to the importance of cell-cell interactions in the early development of vertebrates. By 1932, Holtfreter had shown that the organizer's signals could survive the heating, freezing, and alcohol extraction of organizer tissue, implying that the signals are rather hardy molecules. This set off an international race to isolate inducers. The next 50 years yielded no purified inducer and much frustration. Part of the problem was that the responsive test tissue (the ectoderm from the gastrula-stage embryo) had a capacity to embark on neural development even without the inducer, when exposed to an osmotic or pH shock. This inherent differentiation capacity implied that the inducer is not indispensably instructive. Thereafter many researchers lost interest, and the inducer problem was largely ignored.

Work on the organizer's signals was resumed about 8 years ago by researchers aware of the new techniques and insights of molecular biology. In the past 3 years the action of these inducers has been greatly clarified, especially through analysis of development of the anuran amphibian, *Xenopus*. In several laboratories, the mRNA species transcribed by cells of the amphibian organizer were extracted and reverse transcribed into cDNAs that could be cloned and reexpressed as individual RNAs to inject into frog eggs, to test for the capacity of the translated proteins to trigger the formation of secondary axes (Cho et al., '91; Smith and Harland, '92). The positive outcome resembles that of the organizer graft of Spemann and Mangold. By this axis-duplication assay, at least 13 sequences have now been isolated and identified, namely, those encoding the proteins Noggin, Chordin, Xnr1, Xnr2, Xnr3, Follistatin, eFGF, Cerberus, Dickkopf, Frzb, Goosecoid, Siamois, and Twin. All the corresponding genes are expressed exclusively in the organizer at the early gastrula stage. All but the last three, which are transcription factors, are secreted protein signals or antagonists of signals, as discussed below.

As background to understanding the organizer's effect, it should be said that three large territories of cells surround the organizer at the time of its inductive signaling, namely, the ectoderm, mesoderm, and endoderm. Unique to each territory, cells have a wide range of developmental options, but the cells inhibit themselves from taking certain of these. They accomplish this inhibition by an interesting intercellular circuit. They secrete and receive BMP and Wnt ligands, which do not spread very far, affecting mostly the cell of origin and its few closest neighbors. This is the context in which the organizer works. The organizer releases antagonists that spread several cell diameters and interrupt the autoinhibition in the three territories (Hemmati-Brivanlou and Melton, '94). Noggin, Chordin, Follistatin, Cerberus, Dickkopf, Xnr3, and Frzb are secreted proteins that antagonize Wnt and BMP signal-

**Fig. 8.** Signaling and the organizer. **A:** At left, boxes and circle indicate the state of cells near but outside the organizer. They produce and receive their own TGF- $\beta$  and Wnt signals, keeping themselves in a path of ventral posterior development, and suppressing dorsal anterior development. The organizer releases proteins which bind to the TGF- $\beta$  and Wnt signals and block their ability to bind to receptors. The cells in which the Wnt and TGF- $\beta$  signaling is disrupted then undertake dorsal anterior development. At right are listed the antagonists. The blunt-headed line indicates inhibition. **B:** Schematic drawing of the early gastrula frog embryo. The organizer (dorsal blastopore lip) is at right. The blunt-headed lines extending from it indicate the antagonistic action of its signals, antagonists of Wnt and TGF- $\beta$  signaling. Circular arrows within the grey region indicate the Wnt, TGF- $\beta$  circuitry of the ectoderm, mesoderm, and endoderm, by which these cells perpetuate their ventral posterior development. This circuitry is blocked by the antagonists. **C:** Fate map of the gastrula, showing the development expected of various regions around the organizer. Those close to the organizer take on dorsal anterior development. These are the regions receiving the organizer's antagonists. The regions farther away from the organizer (gray shading) do not receive the antagonists and maintain their ventral posterior development.



ing (reviewed in Harland and Gerhart, '97). Cerberus, as a single protein, is able to inhibit three different signals, Nodal, Wnt, and BMP (Piccolo et al., '99). When these antagonists block the autoinhibition of the cells surrounding the organizer, those cells embark on several new developmental paths of which they were inherently capable but previously repressed (Fig. 8).

The same antagonists from the organizer act on ectoderm, mesoderm, and endoderm, but each territory's cells have different responses. Ectoderm develops to the neural plate instead of the epidermis. Mesoderm develops to somites, heart, and kidney instead of the coelomic and lateral plate mesoderm. Endoderm develops to the anterior gut instead of the posterior gut. These released paths of development can be seen as default options taken when BMP and Wnt signaling fails to prevent them. The antagonists do not bind to receptors on the surrounding cells but stick directly to the ligands, preventing their binding to their receptors. Thus, the organizer's inductions are not instructive but permissive. They only release an inherent capacity of nearby cells to develop into the nervous system, somites, and anterior gut. They are instructive only with regard to their establishing a time, place, orientation, and size of the induced tissue. In retrospect, when researchers of the 1930s–1960s exposed a piece of gastrula ectoderm to a pH or osmotic shock, they probably interfered with the BMP-Wnt signaling of the piece; hence it was released to develop into neural tissue even without an inducer.

In *Drosophila* there is no such organizer. However, there is a surprisingly similar signaling circuitry, and knowledge of these circuits assisted researchers in the analysis of the vertebrate organizer. As mentioned above (see The TGF- $\beta$  Pathway), the dorsalmost territories of cells (prospective dorsal ectoderm and amnioserosa) of the fly embryo release the Dpp and Screw proteins, which are similar to the BMP2,4 and BMP7 proteins of vertebrates, respectively. The adjacent neurogenic ectoderm releases the Sog protein, which is an antagonist of the Screw protein. Sog is similar to the Chordin protein of vertebrates, which is one of the organizer's antagonists of BMPs. The neurogenic ectoderm of *Drosophila* has the inherent capacity to develop as neural or epidermal tissue. Dpp in conjunction with Screw normally represses neural development, but when these are mutationally impaired, the dorsalmost territory develops as neurogenic ectoderm. In normal embryos, the Sog protein antagonizes Screw so that neurogenic ectoderm itself is unrepressed by Dpp and Screw to develop by the neural pathway. Furthermore, the Sog protein, when complexed to Screw, is degraded by a secreted metalloproteinase, the Tollid protein. This proteinase is similar to the Xolloid/BMP-1 proteinase of vertebrates, which degrades the Chordin protein of vertebrates. The complex signaling networks are similar in insects and vertebrates, but are used in different spatial contexts and times, with different spatial outcomes.



## MOUSE KNOCKOUTS OF SIGNALING PATHWAYS

In light of the pervasive use and fundamental role of signaling pathways in development, one might think that the mutational loss of a pathway component would be lethal at an early stage of development. By the same thinking, one might also expect that a strong inhibition of a component by a candidate pharmaceutical or environmental toxicant might be lethal. In *Drosophila* a null mutant for a signaling component (one entirely lacking the encoded functional protein) is indeed usually lethal early in development.

However, the situation is different in mice and other vertebrates. It is now thought that there was a quadruplication of the genome in the earliest days of vertebrate evolution, perhaps 450 million years ago (Holland et al., '94). Vertebrate genomes are comparatively large. In general, genes occur in four copies instead of the single copy found in the genomes of nonvertebrate chordates and most invertebrates, such as *Drosophila*. Also, some genes in vertebrates have locally duplicated into additional copies. All these copies (called paralogs) have diversified in two ways. Some have diversified their cis-regulatory sequences so that they are now expressed at different times and places in the animal. Others have diversified in their coding sequences so that the encoded proteins now differ slightly or greatly in function. For signaling proteins, this functional difference concerns their specificity for receptors or the receptors' specificity of interaction with pathway intermediates.

Methods have become highly efficient for generating a desired null mutant by knockout of the gene in mouse embryonic stem (ES) cells in culture, followed by the controlled introduction of the cells into a blastocyst where some develop into cells of the germ line of the offspring. When two such heterozygous mice are bred, a homozygous null mutant can be obtained for study. It was surprising at first that many kinds of homozygous knockout animals, lacking a particular component of a signaling pathway, succeeded in extensive development. As shown in Table 4, homozygous knockout animals differ greatly in the severity of their defects. A few kinds do show drastic effects accompanied by pre- or periimplantation death, but other kinds show no phenotype at all, some even growing to fertile adults. Many kinds of homozygous null mutants show mild defects, many of which could be classified as birth defects. Does this mean that signaling pathways are not very important in mammalian development? No, the answer seems to be that signaling is indeed crucial, but that the genes for signaling components duplicated and diverged long ago, and the duplicated components are still partially redundant. They still overlap partially in the time and place of expression, and they still overlap partially in the function of their protein products. The defect in a mildly affected phenotype is often limited to those small regions or times where two or more related genes do not overlap in their expression.

Some null mutants for ligands and receptors only show strong defects of development when two or more related genes are knocked out in combination.

The genetic background is also important for the phenotype of the homozygous knockout animal. For example, an EGF receptor can be eliminated in one mouse strain and result in periimplantation death, whereas in another strain the null animal lives 3 weeks beyond birth (Threadgill et al., '95). Also, when the APC (adenomatous polyposis coli) protein intermediate of the Wnt pathway is eliminated in some strains, the adult forms numerous intestinal adenomas. But in another mouse strain, the APC gene can be eliminated with almost no effect (Cormier et al., '97). A suppressor gene of the unaffected strain has been identified as encoding a secreted phospholipase, although it is not yet understood how this activity offsets APC loss.

The phenotypes of these mouse knockout mutants of signaling components may hold lessons for developmental toxicologists. Signaling components may be frequent targets of toxicants, and null mutants for such components may have the phenotypes expected for "ideal" toxicants, i.e., a toxicant so specific as to inactivate just one kind of signaling component in the embryo. The database of knockout phenotypes could be useful in the analysis of toxicant-caused phenotypes. This database is also expected to hold information relevant to genetically-based human birth defects. If one looks for a general relatedness of toxicant effects, heritable birth defects, and experimental null mice, one can say that many of the examples of each are likely to be "loss-of-function" phenotypes. On the other hand, many oncogenes seem to represent "gain-of-function" effects on signaling. Due to mutational alteration, such genes encode signaling components with autonomous and unregulated activity rather than no activity, or components produced at abnormal times and places rather than not produced at all. However, the distinction of gain or loss of function blurs in signaling pathways for those components which act negatively. For example, when the Smoothened protein in the Hedgehog pathway is lost, the pathway is autonomously active. Also, when the axin or APC proteins are lost in the Wnt pathway,  $\beta$ -catenin is more stable and the pathway is autonomously active.

## RELEVANCE TO DEVELOPMENTAL TOXICOLOGY

Although the importance of cell-cell interactions in development has been generally appreciated for 75 years, the evidence is now overwhelming that signaling pervades all aspects of development at all stages, from oogenesis to organogenesis, and further to the ongoing proliferation and differentiation of renewing tissues in the adult. Only a few kinds of signaling pathways, perhaps 10, are used over and over at different times and places in development. These are widely conserved among metazoa, e.g., among nematodes, insects, and vertebrates. In vertebrates, the genes for ligands, recep-

TABLE 4. Mouse null mutants lacking signaling components\*

Signaling component	Viability of null mutant	Phenotype of null mutant	References
Wnt-1	Adulthood	No midbrain, cerebellum, and rhombomere 1	McMahon et al. ('93)
Axin	Early lethal (day 8–10)	Twining	Vasicek et al. ('97); Zeng et al. ('97)
TGF $\beta$ -1 TGF $\beta$ -2	Adulthood Perinatal death	Immune defects, inflammation Defects of heart, lung, spine, limb, craniofacial, and spinal regions	McCartney-Francis, et al. ('97) Sanford et al. ('97)
GDF5	Adulthood	Fused skeletal elements in limbs, one third of joints missing. Like <i>brachypodism</i> mutant.	Storm and Kingsley ('96)
BMP5	Adulthood	Thin axial bones. Abnormal lungs, liver, ureter, and bladder. Like <i>short ear</i> mutant.	Mikic et al. ('96)
BMP7	Adulthood	Defects in eye and kidney. Skeletal abnormalities. Polydactyly of hindlimbs.	Dudley and Robertson ('97)
Noggin	Juvenile	Bone hyperplasia, joints not formed; neural tube and somite defects	Brunet et al. ('98)
FGF4	Early lethal (implantation)	Poor ICM proliferation	Feldman et al. ('95)
FGF5	Adulthood	Long hair. Like <i>Angora</i> mutant.	Hebert et al. ('94)
FGFR1	Early lethal (implantation)	Severe growth retardation, mesoderm not formed	Deng et al. ('97)
FGFR3	Adulthood	Prolonged endochondral bone growth (e.g., long neck)	Deng et al. ('96)
EGFR	Death at implantation or at 3 weeks postnatal, depending on background	Disorganized hair follicles Defective wound healing of the skin	Hansen et al. ('97) Threadgill et al. ('95)
Sonic Hedgehog (Shh)	Perinatal death	Abnormalities of kidney, brain, liver, GI tract Cyclopia, defects of spinal cord, axial skeleton, and limbs	Chiang et al. ('96)
Patched receptor	Homozygotes, early lethality Heterozygotes, adulthood	Open neural tube Rhabdomyosarcomas, large size, radiation-induced teratogenesis, hindlimb defects. Like Gorlin syndrome in humans.	Goodrich et al. ('97) Hahn et al. ('98)
Notch 1	Perinatal death	Disordered somites Like Danforth short-tall mutation?	Swiatek et al. ('94); Conlon et al. ('95)
Delta (Dll1)	Perinatal death	Disordered somites	Hrabe De Angelis et al. ('97)

\*See text for details. Null mutants are shown for components of the five signaling pathways of early development. Note the differing viability. References are minimally indicated. The information is sufficient to find the citation in a widely available database such as Medline, using the author and signaling component.

tors, and some transduction components have duplicated and diversified, so that there are many subtypes of components expressed at different times and places. However, they still belong to the same basic kinds of pathways distinguished by their signal transduction intermediates. These are shared widely among metazoa, reflecting their great antiquity.

What does this deep conservation of signaling components and genetic regulatory circuitry imply for developmental toxicology? It certainly attests to the continued usefulness of studying model organisms such as nematodes, *Drosophila*, zebra fish, frogs, and chicks to gain insights about the development of mammals, including humans. This usefulness is expected to continue in the next decade of study of many kinds of organogenesis. The carry-over of information from model organisms to mammals will be great. It is self-evident that the more

one knows about development, the more one can learn about the action of developmental toxicants, and ultimately the better the tests one can devise for the detection of toxicants.

The analysis of the effects of toxicants on development may be accelerated by the use of genetically modified model organisms to identify targeted developmental processes. With genetically tractable model organisms (nematodes, *Drosophila*, mice), a wealth of null mutants and hypomorphs is available, and more can be made on demand. Their phenotypes, which can be explained in terms of specific failed mechanisms of development, may be comparable to phenotypes generated by toxicants. Also, there are genetically modified model organisms in which to assess toxicant effects favorably, models sensitized for specific signaling pathways and developmental processes and/or transgenic

for various reporter genes (artificially introduced biologic markers). Finally, the possibility is at hand for massive analysis of toxicant effects by DNA array technology (Iyer et al., '99). For those toxicants with very specific effects, model organisms may be especially useful in the elucidation of their action. Such toxicants may not be so abundant at present, but with the increased use of rational drug design and of the function-based selection of rare potent compounds from immense chemical libraries, specific toxicants may become ever more prevalent. For broad specificity toxicants (e.g., heavy metals, arsenate), on the other hand, the toxicant's activation of stress pathways, checkpoint pathways, and apoptosis may continue to be the best indicators of their effect, rather than looking at a specific developmental process.

As developmental toxicologists have demonstrated incontrovertibly, a model organism may give misleading or incomplete information about human toxicant responses for any of several well-known reasons: the model may detoxify or potentiate a toxicant in a way different from that in humans, or the model may have a developmental process basically dissimilar to that of humans. But so far, model organisms have shown that even dissimilar developmental processes make use of the same conserved signaling components and genetic regulatory circuitry. Those developmental processes truly unique to humans will probably be few in number compared to those shared with other mammals, vertebrates, and even invertebrates such as *Drosophila*. Toxicants, taken as a whole, presumably hit targets over the entire range of developmental processes and molecular components of an embryo, most of which are shared. This is not to minimize the need to study the uniquely human modifications of toxicants by metabolizing enzymes or the uniquely mammalian and human aspects of development, but there is no reason to think that potentiated toxicants show a preference at the molecular level for those few uniquely human developmental processes.

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