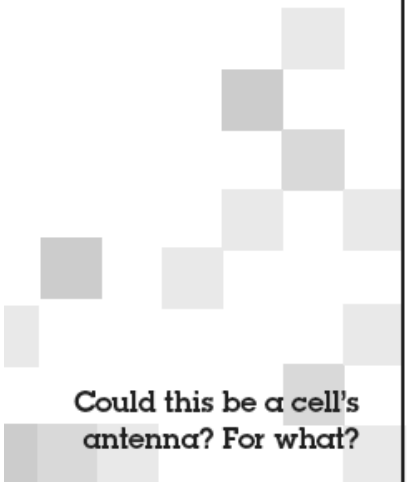
Cell-to-Cell Communication

Mechanisms of Morphogenesis



Could this be a cell's antenna? For what?

DEVELOPMENT IS MORE THAN JUST DIFFERENTIATION. The different cell types of an organism do not exist as random arrangements. Rather, they form organized structures such as limbs and hearts. Moreover, the types of cells that constitute our fingers—bone, cartilage, neurons, blood cells, and others—are the same cell types that make up our pelvis and legs. Somehow, the cells must be ordered to create different shapes and make different connections. This construction of organized form is called **morphogenesis**, and it has been one of the great sources of wonder for humankind.

The twelfth-century rabbi and physician Maimonides framed the question of morphogenesis beautifully when he noted that the pious men of his day (around 1190 CE) believed that an angel of God had to enter the womb to form the organs of the embryo. That act, the man said, was a miracle. How much more powerful a miracle would life be, Maimonides asked, if the Deity had made matter such that it could generate this remarkable order without a matter-molding angel having to intervene in every pregnancy? The problem addressed today is the secular version of Maimonides' question: *How can matter alone construct itself into the organized tissues of the embryo?*

In the mid-twentieth century, E. E. Just (1939) and Johannes Holtfreter (Townes and Holtfreter 1955) predicted that embryonic cells could have differences in their cell

membrane components that would enable the formation of organs. In the late twentieth century, these membrane components—the molecules by which embryonic cells are able to adhere to, migrate over, and induce gene expression in neighboring cells—began to be discovered and described. Today these pathways and networks are being modeled, and we are beginning to understand how the cell integrates the information from its nucleus and from its surroundings to take its place in the community of cells in a way that fosters unique morphogenetic events.

As we discussed in Chapter 1, the cells of an embryo are either epithelial or mesenchymal (see Table 1.1). Epithelial cells adhere to one another and can form sheets and tubes, whereas mesenchymal cells often migrate individually and form extensive extracellular matrices that can keep individual cells separate. An organ is formed from an epithelium and an underlying mesenchyme. There appear to be only a few processes through which cells create structured organs (Newman and Bhat 2008), and all these processes involve the cell surface. This chapter will concentrate on three behaviors requiring cell-to-cell communication via the cell surface: cell adhesion, cell shape, and cell signaling.

A Primer on Cell-to-Cell Communication

An embryo at any stage is held together, organized, and formed by the interactions that occur between cells. The interactions exhibited by cells define their methods of *communication*. For communication to occur successfully between humans, there needs to be some initial "voice" or signal from one person that is "heard" or received by the other person, which results in a specific response (a change in mood, a hug, or perhaps a sarcastic remark back), much like friends conversing. Molecular communication between cells is largely carried out through highly diverse and specific protein-protein interactions, which have evolved to elicit an array of cellular responses, from changes in gene transcription and glucose metabolism to cell migration and cell death. Interactions (or *communication*) between cells and between cells and their environment begin at the plasma membrane, with proteins that are housed within, anchored to, or secreted through the membrane.

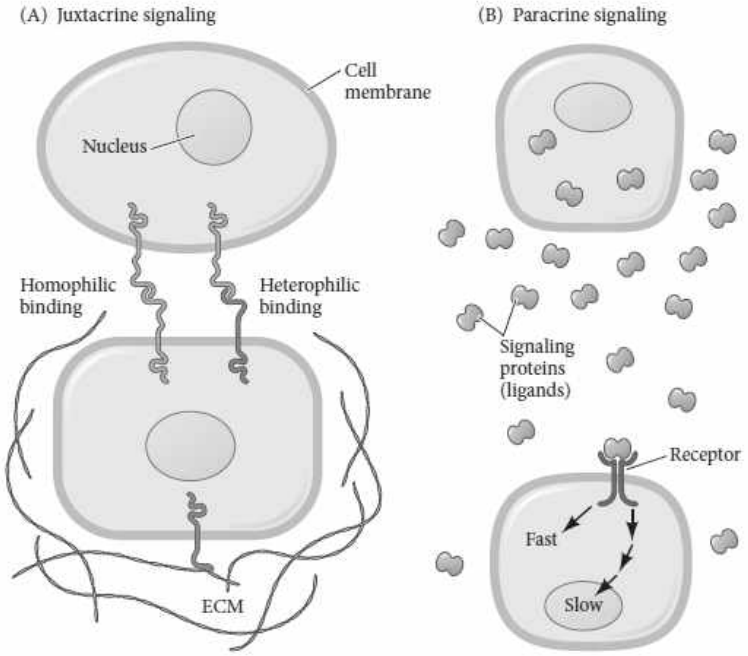


FIGURE 4.1 Local and long-range modes of cell-to-cell communication. (A) Local cell signaling is carried out via membrane receptors that bind to proteins in the extracellular matrix (ECM) or directly to receptors from a neighboring cell in a process called juxtacrine signaling. (B) One mechanism for long-range signaling is through paracrine signaling, such that one cell secretes a signaling protein (ligand) into the environment and across the distance of many cells. Only those cells expressing this ligand's corresponding receptor can respond, either rapidly through chemical reactions in the cytosol, or more slowly through the process of gene and protein expression.

In an embryo, communication between cells can occur across short distances, such as between two neighboring cells in direct contact, called **juxtacrine signaling**, or across long distances through the secretion of proteins into the extracellular matrix, called **paracrine signaling** (**FIGURE 4.1**). Proteins that are secreted from a cell and designed to communicate a response in another cell are generally referred to as signaling proteins (generally called **ligands**), while the proteins within a membrane that function to bind either other membrane-associated proteins or signaling proteins are called **receptors**. A receptor in the membrane of one cell that binds the same type of receptor in another cell represents a **homophilic binding**. In contrast, **heterophilic binding** occurs between different receptor types (see Figure 4.1A).

Binding to a receptor of any kind generally alters the shape, or *conformation*, of the receptor. This conformational change on

the outside of the cell affects the shape of the receptor inside the cell, and this latter change can give the intracellular portion of the receptor a new property. It now has the ability to activate the enzymatic reactions that constitute a signal transduction pathway. Often the "signal" is relayed or "transduced" through successive conformational changes in the molecules of the pathway, changes orchestrated through the binding of phosphate groups or other small molecules (cAMP, Ca^{2+}) that eventually lead to cellular responses. Signal transduction pathways that culminate in activating gene expression in the nucleus are typically slower than those that enzymatically activate biochemical pathways or regulate cytoskeletal proteins, thereby affecting physiological functions or movement, respectively. These signal transduction pathways are fundamental to animal development.

Adhesion and Sorting: Juxtacrine Signaling and the Physics of Morphogenesis

How are separate tissues formed from populations of cells and organs constructed from tissues? How do organs form in particular locations and migrating cells reach their destinations? For example, how do bone cells stick to other bone cells to create a bone rather than merging with adjacent capillary cells or muscle cells? What keeps the mesoderm separate from the ectoderm such that the skin has both a dermis and an epidermis? Why do eyes only form in the head? How do some cells—such as the precursors of our pigment cells and germ cells—travel long distances to reach their final destinations?

Could there be a simple common answer to all these questions? After all, an embryo, from its molecular strands of RNA to its systemic vasculature, develops within the same physical constraints that define our universe. Consider a snowman made out of sand (**FIGURE 4.2**). The thermodynamic properties governing the surface tension between water molecules and the grains of sand serve to hold the parts of Olaf together. Moreover, the sunlight on this sand sculpture establishes differential temperatures and associated water evaporation on the surface compared to the inner composition; consequently, the adhesion between sand grains on the surface rapidly becomes reduced, whereas more centrally located grains hold tight (that is, until the tide changes). Could these same thermodynamic principles govern the connections between cells that support morphogenesis of the embryo?

FIGURE 4.2 Adhesion between sand grains holds this sand sculpture of the Disney character Olaf together.

Differential cell affinity

The experimental analysis of morphogenesis began with the experiments of Townes and Holtfreter in 1955. Taking advantage of the discovery that amphibian tissues become dissociated into single cells when placed in alkaline solutions, they prepared single-cell suspensions from each of the three germ layers of amphibian embryos soon after the neural tube had formed. Two or more of these single-cell suspensions could be combined in various ways. When the pH of the solution was normalized, the cells adhered to one another, forming aggregates on agar-coated petri dishes. By using embryos from species having cells of different sizes and colors, Townes and Holtfreter were able to follow the behavior of the recombined cells.

The results of their experiments were striking. Townes and Holtfreter found that reaggregated cells become spatially segregated. That is, instead of two cell types remaining mixed, each type sorts

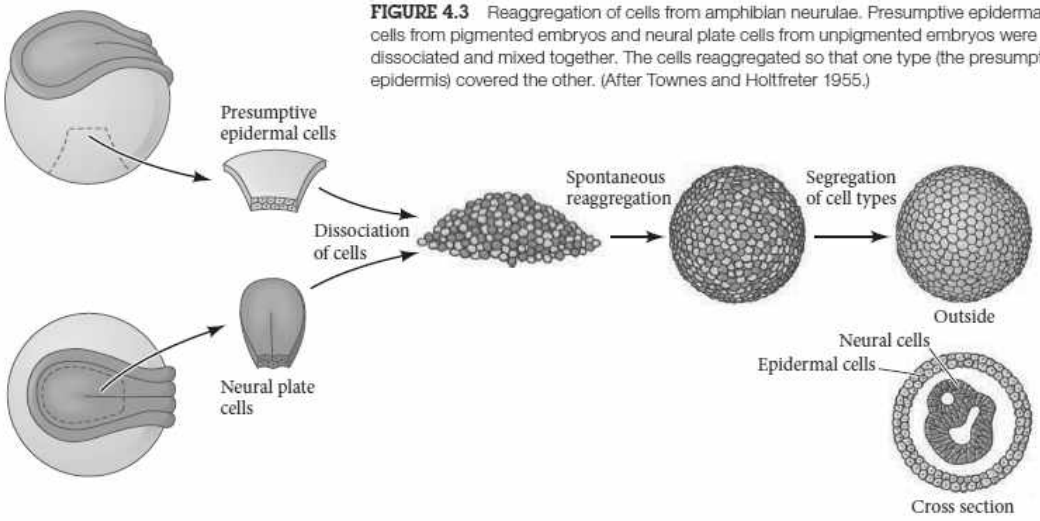


FIGURE 4.3 Reaggregation of cells from amphibian neurulae. Presumptive epidermal cells from pigmented embryos and neural plate cells from unpigmented embryos were dissociated and mixed together. The cells reaggregated so that one type (the presumptive epidermis) covered the other. (After Townes and Holtfreter 1955.)

out into its own region. Thus, when epidermal (ectodermal) and mesodermal cells are brought together in a mixed aggregate, the epidermal cells move to the periphery of the aggregate, and the mesodermal cells move to the inside (**FIGURE 4.3**). Importantly, the researchers found that the final positions of the reaggregated cells reflect their respective positions in the embryo. The reaggregated mesoderm migrates centrally with respect to the epidermis, adhering to the inner epidermal surface (**FIGURE 4.4A**). The mesoderm also migrates centrally with respect to the gut or endoderm (**FIGURE 4.4B**). When the three germ layers are mixed together, however, the endoderm separates from the ectoderm and mesoderm and is then enveloped by them (**FIGURE 4.4C**). In the final configuration, the ectoderm is on the periphery, the endoderm is internal, and the mesoderm lies in the region between them.

Holtfreter interpreted this finding in terms of **selective affinity**. The inner surface of the ectoderm has a positive affinity for mesodermal cells and a negative affinity for the endoderm, whereas the mesoderm has positive affinities for both ectodermal and endodermal cells. Mimicry of normal embryonic structure by cell aggregates is also seen in the recombination of epidermis and neural plate cells (**FIGURE 4.4D**). The presumptive epidermal cells migrate to the periphery as before; the neural plate cells migrate inward, forming a structure reminiscent of the neural tube. When axial mesoderm (notochord) cells are added to a suspension of presumptive epidermal and presumptive neural cells, cell segregation results in an external epidermal layer, a centrally located neural tissue, and a layer of mesodermal tissue between them (**FIGURE 4.4E**). *Somehow, the cells are able to sort out into their proper embryonic positions.* Holtfreter and colleagues concluded that selective affinities change during development. For development to occur, cells must interact differently with other cell populations at specific times. Such changes in cell affinity are extremely important in the processes of morphogenesis.

The thermodynamic model of cell interactions

Cells, then, do not sort randomly, but they can actively move to create tissue organization. What forces direct cell movement during morphogenesis? In 1964, Malcolm Steinberg proposed the **differential adhesion hypothesis**, a model that sought to explain patterns of cell sorting based on thermodynamic principles. Using cells derived from trypsinized embryonic tissues, Steinberg showed that certain cell types

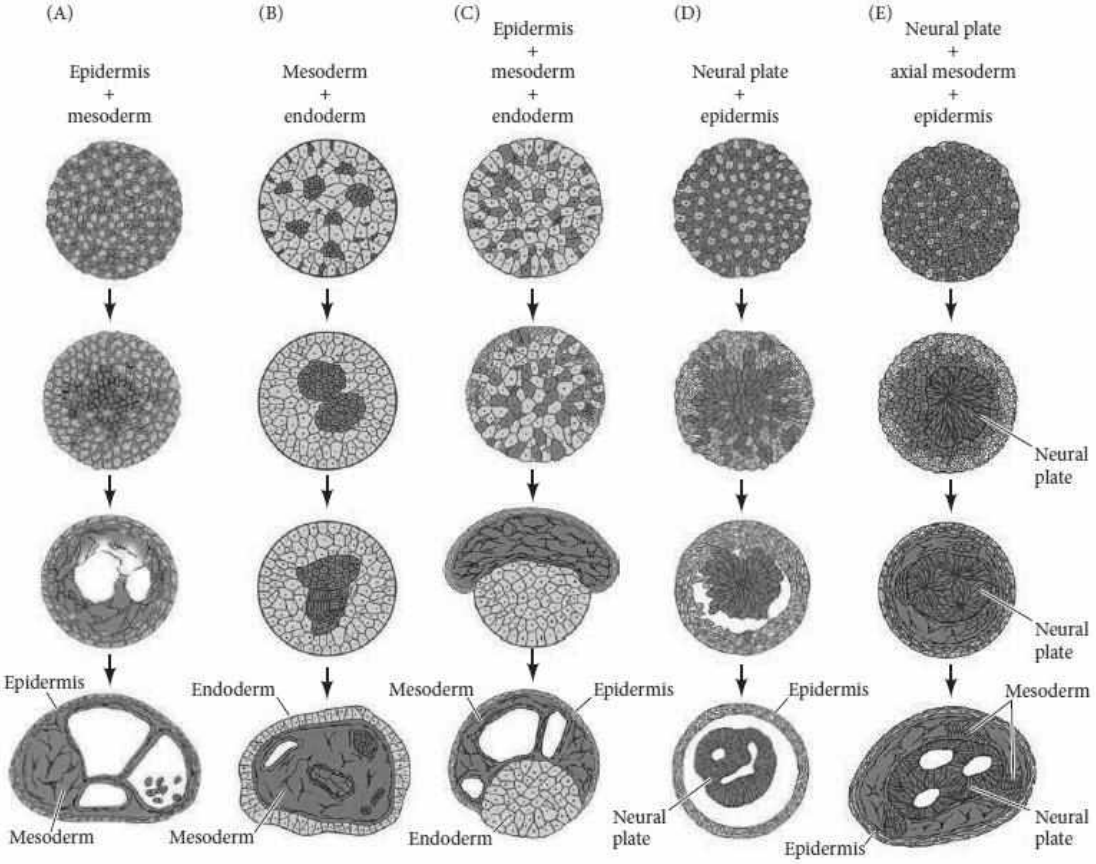


FIGURE 4.4 Sorting out and reconstruction of spatial relationships in aggregates of embryonic amphibian cells. (After Townes and Holtfreter 1955.)

migrate centrally when combined with some cell types, but migrate peripherally when combined with others. Such interactions form a hierarchy (Steinberg 1970). If the final position of cell type A is internal to a second cell type B and if the final position of B is internal to a third cell type C, the final position of A will always be internal to C (**FIGURE 4.5A**; Foty and Steinberg 2013). For example, pigmented retina cells migrate internally to neural retina cells, and heart cells migrate internally to pigmented retina cells. Therefore, heart cells migrate internally to neural retina cells. This observation led Steinberg to propose that cells interact so as to form an aggregate with the smallest interfacial free energy. In other words, the cells rearrange themselves into the most thermodynamically stable pattern. If cell types A and B have different strengths of adhesion and if the strength of A-A connections is greater than the strength of A-B or B-B connections, sorting will occur, with the A cells becoming central. However, if the strength of A-A connections is less than or equal to the strength of A-B connections, the aggregate will remain as a random mix of cells. Finally, if the strength of A-A connections is far greater than the strength of A-B connections or, in other words, if A and B cells show essentially no adhesivity toward one another, A cells and B cells will form separate aggregates. According to this hypothesis, the early embryo can be viewed as existing in an equilibrium state until some change in the adhesive properties of the cell's plasma membrane changes. The movements that result seek to restore the cells

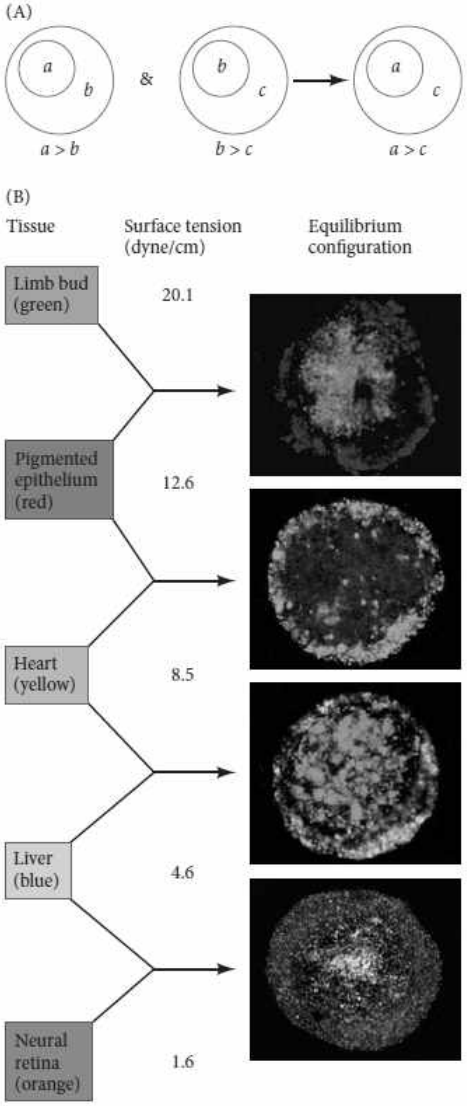


FIGURE 4.5 Hierarchy of cell sorting of decreasing surface tensions. (A) Simple schematic demonstrating a logic statement for the properties of differential cell adhesion. (B) The equilibrium configuration reflects the strength of cell cohesion, with the cell types having the greater cell cohesion segregating inside the cells with less cohesion. These images were obtained by sectioning the aggregates and assigning colors to the cell types by computer. Black areas represent cells whose signal was edited out in the program of image optimization. (From Foty et al. 1996, courtesy of M. S. Steinberg and R. A. Foty.)

to a new equilibrium configuration. All that is required for sorting to occur is that cell types differ in the strengths of their adhesion; differential adhesion is caused by changes in the amount or repertoire of cell surface molecules.

In several meticulous experiments using numerous tissue types, researchers showed that those cell types that had greater surface cohesion migrated centrally compared to cells that had less surface tension (**FIGURE 4.5B**; Foty et al. 1996; Krens and Heisenberg 2011). In the simplest form of this model, all cells could have the same type of "glue" on the cell surface. The amount of this "glue," or the cellular architecture that allows such a substance to be differentially distributed across the surface, could create a difference in the number of stable contacts made between cell types. In a more specific version of this model, the thermodynamic differences could be caused by different types of adhesion molecules (see Moscona 1974). When Holtfreter's studies were revisited using modern techniques, Davis and colleagues (1997) found that the tissue surface tensions of the individual germ layers were precisely those required for the sorting patterns observed both in vitro and in vivo.

Cadherins and cell adhesion

Evidence shows that boundaries between tissues can indeed be created by different cell types having both different types and different amounts of cell adhesion molecules. Several classes of molecules can mediate cell adhesion, but the major cell adhesion molecules appear to be the cadherins.

As their name suggests, **cadherins** are *ca*lcium-*d*ependent ad*he*sion molecules. They are critical for establishing and maintaining intercellular connections, and they appear to be crucial to the spatial segregation of cell types and to the organization of animal form (Takeichi 1987). Cadherins are transmembrane proteins that interact with other cadherins on adjacent cells. The cadherins are anchored inside the cell by a complex of proteins called **catenins** (**FIGURE 4.6**), and the cadherin-catenin complex forms the classic adherens junctions that help hold epithelial cells together. Moreover, because the cadherins and the catenins bind to the actin (microfilament) cytoskeleton of the cell, they integrate the epithelial cells into a mechanical unit. Blocking cadherin *function* (by antibodies that bind and inactivate cadherin) or blocking cadherin *synthesis* (with antisense RNA that binds cadherin messages and prevents their translation) can prevent the formation of epithelial tissues and cause the cells to disaggregate (Takeichi et al. 1979).

Cadherins perform several related functions. First, their external domains serve to adhere cells together. Second, cadherins link to and help assemble the actin cytoskeleton, thereby providing the mechanical forces for forming sheets and tubes. Third, cadherins can serve to initiate and transduce signals that can lead to changes in a cell's gene expression.

VADE MECUM

Movies depict how pioneering experiments by Townes and Holtfreter and by Malcolm Steinberg demonstrated how cell surface adhesion molecules can direct cell sorting behaviors.

In vertebrate embryos, several major cadherin types have been identified. For example, **E-cadherin** is expressed on all early mammalian embryonic cells, even at the zygote stage. In the zebrafish embryo, E-cadherin is needed for the formation and migration of the epiblast as a sheet of cells during gastrulation. Loss of E-cadherin in the *"half-baked"* zebrafish mutant results in a failure of deep epiblast cells to move radially into the more superficial epiblast layer, an in vivo cell sorting process known as **radial intercalation** that helps power epiboly during gastrulation (**FIGURE 4.7**; see also Chapter 11 and Kane et al. 2005). Later in development, this E-cadherin is restricted to epithelial tissues of embryos and adults.

In mammals, **P-cadherin** is found predominantly on the placenta, where it helps the placenta stick to the uterus (Nose and Takeichi 1986; Kadokawa et al. 1989). **N-cadherin** becomes highly expressed on the cells of the developing central nervous system (Hatta and Takeichi 1986), and it may play a role in mediating neural signals. **R-cadherin** is critical in retina formation (Babb et al. 2005). A class of cadherins called **protocadherins** (Sano et al. 1993) lacks the attachment to the actin cytoskeleton through catenins. Expressing similar protocadherins is an important means of keeping migrating epithelial cells together, and expressing dissimilar protocadherins is an important way of separating tissues (as when

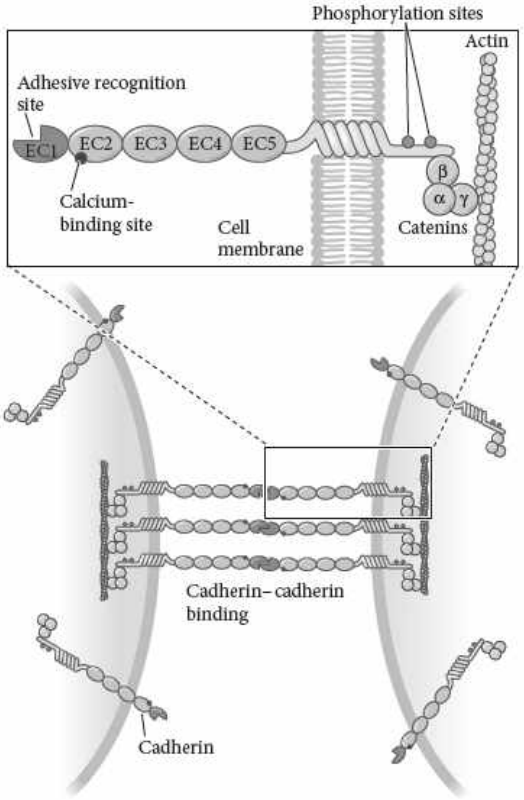


FIGURE 4.6 Simplified scheme of cadherin linkage to the cytoskeleton via catenins. (After Takeichi 1991.)

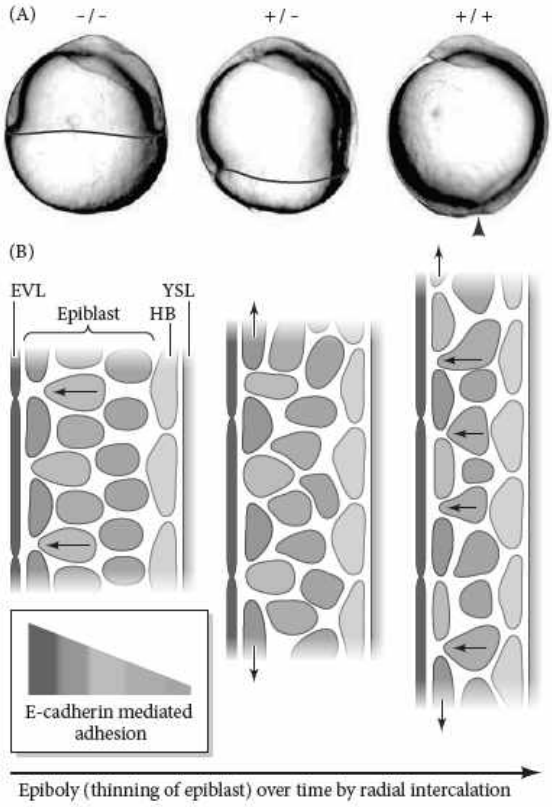


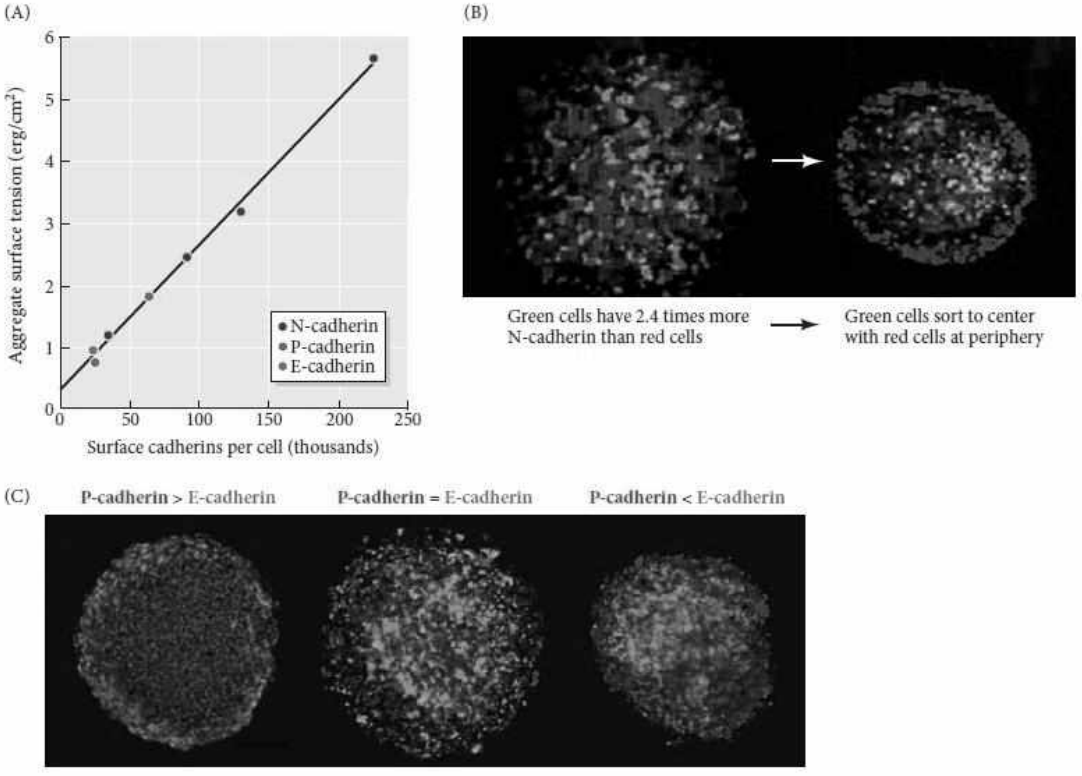
FIGURE 4.7 E-cadherin is required for epiboly in zebrafish. (A) Wild-type embryos (right), and embryos heterozygous (center) and homozygous (left) for the E-cadherin mutation called *half-baked*. During normal gastrulation, cells merge into a thinner but more expansive epiblast layer that envelops the entire yolk (the red arrowhead points to the location of final yolk enclosure in the wild-type). E-cadherin mutants fail to complete epiboly, which is most severely impaired in the homozygous mutant (red lines denote the leading edge of epiblast). (B) Schematic of radial intercalating cell movements in the zebrafish epiblast over time during gastrulation. Cells move toward the superficial enveloping layer in relationship to increasing expression of E-cadherin. E-cadherin is expressed at higher levels in the more superficial layers of the epiblast, including the enveloping layer, and it is this differential expression (and consequently differential adhesion) that powers the radial movement of deep cells to the periphery. EVL, enveloping layer; HB, hypoblast; YSL, yolk syncytial layer. (Data and images based on Kane et al. 2005, courtesy of R. Warga.)

the mesoderm forming the notochord separates from the surrounding mesoderm that will form somites).

Differences in cell surface tension and the tendency of cells to bind together depend on the strength of cadherin interactions (Duguay et al. 2003). This strength can be achieved quantitatively (the more cadherins on the apposing cell surfaces, the tighter the adhesion) or qualitatively (some cadherins will bind to different cadherin types, whereas other cadherins will not bind to different types).

QUANTITY AND COHESION The ability of cells to sort themselves based on the *amount* of cadherin expression was first shown when Steinberg and Takeichi (1994) collaborated on an experiment using two cell lines that were identical except that they synthesized different amounts of P-cadherin. When these two groups of cells, each expressing a different amount of cadherin, were mixed, the cells that expressed more P-cadherin had a higher surface cohesion and migrated internally to the lower-expressing group of cells. Foty and Steinberg (2005) demonstrated that this quantitative cadherin-dependent sorting directly correlated with surface tension (**FIGURE 4.8A,B**). The surface tensions of these homotypic aggregates (all cells have same type of cadherin) are linearly related to the amount of cadherin they express on the cell surface. The cell sorting hierarchy is strictly dependent on the amount of cadherin interactions between the cells. This thermodynamic principle also applies to heterotypic aggregates, in which the relative amounts of different cadherin types still predict cell-sorting behavior in vitro (Foty and Steinberg 2013) (**FIGURE 4.8C**).

FIGURE 4.8 Importance of the amount of cadherin for correct morphogenesis. (A) Aggregate surface tension correlates with the number of cadherin molecules on the cell membranes. (B) Sorting out of two subclones having different amounts of cadherin on their cell surfaces. The green-stained cells had 2.4 times as many N-cadherin molecules in their membrane as did the other cells. (These cells had no normal cadherin genes being expressed.) At 4 hours of incubation (left), the cells are randomly distributed, but after 24 hours of incubation (right), the red cells (with a surface tension of about 2.4 erg/cm^2) have formed an envelope around the more tightly cohering (5.6 erg/cm^2) green cells. (C) Sorting can occur based on cadherin number even if the two cells express different cadherin proteins (i.e., are heterotypic). Red indicates P-cadherin, green E-cadherin. (A,B from Foty and Steinberg 2005; C from Foty and Steinberg 2013.)



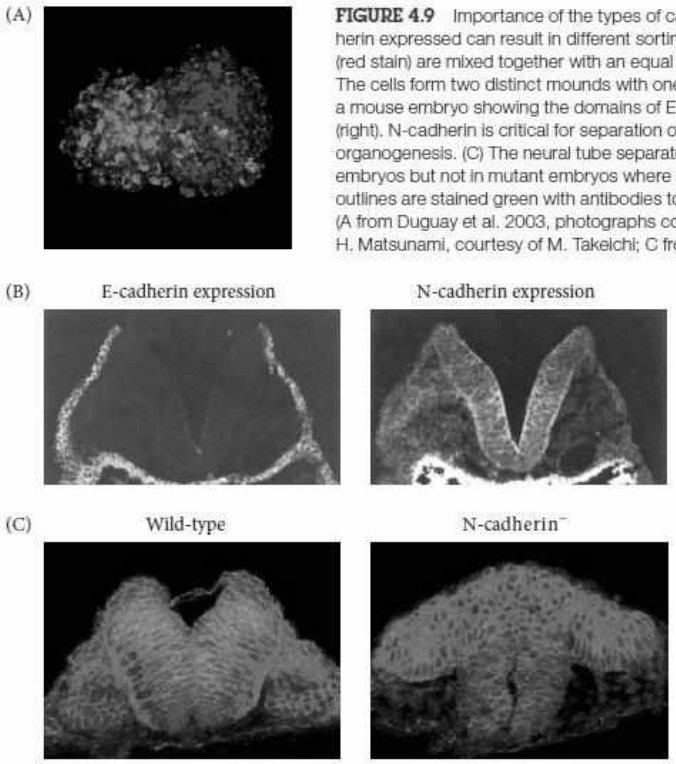


FIGURE 4.9 Importance of the types of cadherin for correct morphogenesis. (A) The type of cadherin expressed can result in different sorting behaviors, as seen when cells expressing R-cadherin (red stain) are mixed together with an equal number of cells expressing B-cadherin (green stain). The cells form two distinct mounds with one common boundary of contact. (B) Cross section of a mouse embryo showing the domains of E-cadherin expression (left) and N-cadherin expression (right). N-cadherin is critical for separation of presumptive epidermal and neural tissues during organogenesis. (C) The neural tube separates cleanly from surface epidermis in wild-type zebrafish embryos but not in mutant embryos where N-cadherin fails to be made. In these images, the cell outlines are stained green with antibodies to β-catenin, while the cell interiors are stained blue. (A from Duguay et al. 2003, photographs courtesy of R. Foty; B photographs by K. Shimamura and H. Matsunami, courtesy of M. Takeichi; C from Hong and Brewster 2006, courtesy of R. Brewster.)

TYPE, TIMING, AND BORDER FORMATION The quantitative effects of cadherins are crucial, but *qualitative* interactions—that is, the *type* and *timing* of cadherin expression—also can be important. The timing of particular developmental events can depend on cadherin expression. For instance, N-cadherin appears in the mesenchymal cells of the developing chick leg just before these cells condense and form nodules of cartilage (which are the precursors of the limb skeleton). N-cadherin is not seen prior to condensation, nor is it seen afterward. If the limbs are injected just prior to condensation with antibodies that block N-cadherin, the mesenchyme cells fail to condense and cartilage fails to form (Oberlender and Tuan 1994). It therefore appears that the signal to begin cartilage formation in the chick limb is the appearance of N-cadherin.

The type of cadherin can matter as well. Duguay and colleagues (2003) showed, for instance, that R-cadherin and B-cadherin do *not* bind well to each other. When two populations of cells expressing either R-cadherin or B-cadherin at equal levels are mixed together, they sort out into two opposing mounds of cells with a distinct border between them (**FIGURE 4.9A**). The formation of boundaries is a critical physical achievement necessary for many morphogenetic events. For instance in the developing ectoderm, the expression of N-cadherin is important in separating the precursors of the neural cells from the precursors of the epidermal cells (**FIGURE 4.9B**). Initially, all early embryonic cells contain E-cadherin, but those cells destined to become the neural tube lose E-cadherin and gain N-cadherin. If epidermal cells are experimentally made to express N-cadherin or if N-cadherin synthesis is blocked in prospective neural cells, the border between the skin and the nervous system fails to form properly (**FIGURE 4.9C**; Kintner et al. 1992). Thus, through the differential expression of two different cadherin types,

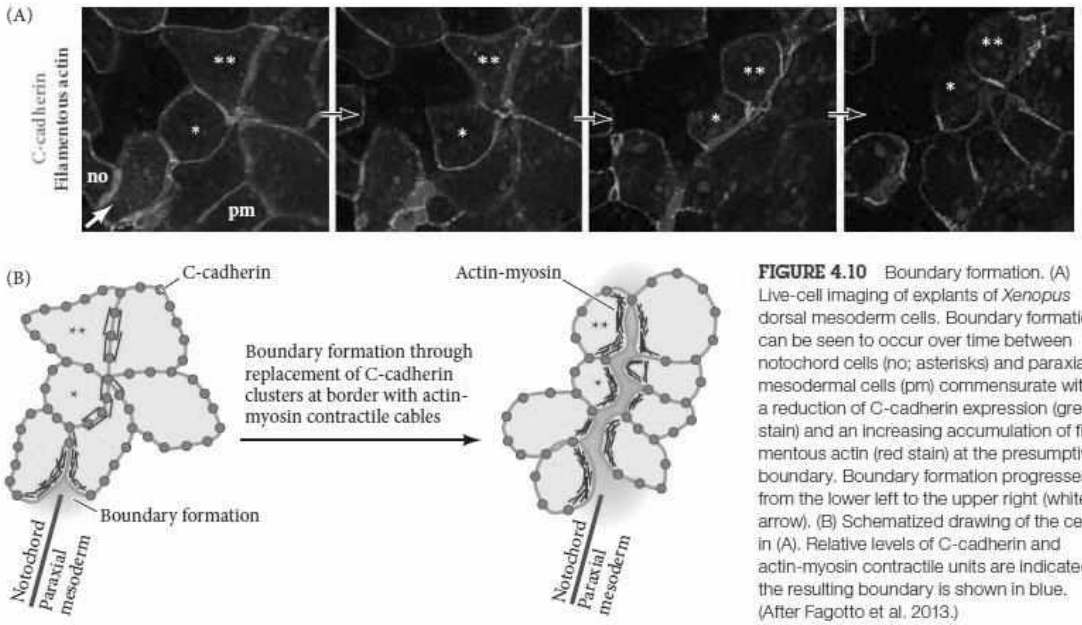


FIGURE 4.10 Boundary formation. (A) Live-cell imaging of explants of *Xenopus* dorsal mesoderm cells. Boundary formation can be seen to occur over time between notochord cells (no; asterisks) and paraxial mesodermal cells (pm) commensurate with a reduction of C-cadherin expression (green stain) and an increasing accumulation of filamentous actin (red stain) at the presumptive boundary. Boundary formation progresses from the lower left to the upper right (white arrow). (B) Schematized drawing of the cells in (A). Relative levels of C-cadherin and actin-myosin contractile units are indicated; the resulting boundary is shown in blue. (After Fagotto et al. 2013.)

Developing Questions

The underlying actin cytoskeleton appears to be crucial in organizing cadherins for forming stable linkages between cells. Although the energetic value of cadherin-cadherin binding is remarkably strong—about 3400 kcal/mole, or some 200 times stronger than most metabolic protein-protein interactions—actin-myosin contractile forces are also important for establishing the tensile forces of a cell. Recently, a "differential interfacial tension hypothesis" proposed that cell cortex contractility governs cell sorting more than cell-to-cell adhesion. As better in vivo tools are developed to quantitatively measure forces on the cellular and molecular levels, it will be exciting to learn how differential adhesion and differential interfacial tension cooperatively regulate morphogenesis. In the coming years, keep an eye out for a building understanding of the role biophysical properties play in mechanisms of morphogenesis.

different tissues can become separated by the formation of a border at the cell membrane occupying the weaker heterophilic interaction (Fagotto 2014).

Another example of boundary formation in the embryo occurs within the mesoderm to separate the axial (notochordal) mesoderm from the paraxial (somitic) mesoderm. The primary mechanism for forming this boundary rests in the reduction of C-cadherins in the apposing membranes of the border cells (Fagotto et al. 2013). Fagotto and colleagues examined this mechanism in live *Xenopus laevis* embryos and found that actin-myosin contractile cables line up parallel to the border interface and are required for both C-cadherin reduction and boundary formation (**FIGURE 4.10**).

WEB TOPIC 4.1 SHAPE CHANGE AND EPITHELIAL MORPHOGENESIS: "THE FORCE IS STRONG IN YOU" The ability of epithelial cells to form sheets and tubes depends on cell shape changes that usually involve cadherins and the actin cytoskeleton.

The Extracellular Matrix as a Source of Developmental Signals

Cell-to-cell interactions do not happen in the absence of an environment; rather, they occur in coordination with and often due to the environmental conditions surrounding the cells. This environment is called the **extracellular matrix**, which is an insoluble network consisting of macromolecules secreted by cells. These macromolecules form a region of noncellular material in the interstices between the cells. Cell adhesion, cell migration, and the formation of epithelial sheets and tubes all depend on the ability of cells to form attachments to extracellular matrices. In some cases, as in the formation of epithelia, these attachments have to be extremely strong. In other instances, as when

cells migrate, attachments have to be made, broken, and made again. In some cases, the extracellular matrix merely serves as a permissive substrate to which cells can adhere or on which they can migrate. In other cases, it provides the directions for cell movement or the signal for a developmental event. Extracellular matrices are made up of the matrix protein collagen, proteoglycans, and a variety of specialized glycoprotein molecules such as fibronectin and laminin.

Proteoglycans play critically important roles in the delivery of the paracrine factors. These large molecules consist of core proteins (such as syndecan) with covalently attached glycosaminoglycan polysaccharide side chains. Two of the most widespread proteoglycans are heparan sulfate and chondroitin sulfate. Heparan sulfate can bind many members of different paracrine families, and it appears to be essential for presenting the paracrine factor in high concentrations to its receptors. In *Drosophila*, *C. elegans*, and mice, mutations that prevent proteoglycan protein or carbohydrate synthesis block normal cell migration, morphogenesis, and differentiation (García-García and Anderson 2003; Hwang et al. 2003; Kirn-Safran et al. 2004).

The large glycoproteins are responsible for organizing the matrix and the cells into an ordered structure. **Fibronectin** is a very large (460-kDa) glycoprotein dimer synthesized by numerous cell types. One function of fibronectin is to serve as a general adhesive molecule, linking cells to one another and to other substrates such as collagen and proteoglycans. Fibronectin has several distinct binding sites, and their interaction with the appropriate molecules results in the proper alignment of cells with their extracellular matrix (**FIGURE 4.11A**). Fibronectin also has an important role in cell migration because the "roads" over which certain migrating cells travel are paved with this protein. Fibronectin paths lead germ cells to the gonads and heart cells to the midline of the embryo. If chick embryos are injected with antibodies to fibronectin, the heart-forming cells fail to reach the midline, and two separate hearts develop (Heasman et al. 1981; Linask and Lash 1988).

SCIENTISTS SPEAK 4.1 A question-and-answer session with Dr. Doug DeSimone and Dr. Tania Rozario about the role of fibronectin during *Xenopus* gastrulation.

Laminin (another large glycoprotein) and **type IV collagen** are major components of a type of extracellular matrix called the **basal lamina**. The basal lamina is characterized by closely knit sheets that underlie epithelial tissue (**FIGURE 4.11B**). The adhesion of epithelial cells to laminin (on which they sit) is much greater than the affinity of mesenchymal cells for fibronectin (to which they must bind and release if they are to migrate). Like fibronectin, laminin plays a role in assembling the extracellular matrix, promoting cell adhesion and growth, changing cell shape, and permitting cell migration (Hakamori et al. 1984; Morris et al. 2003).

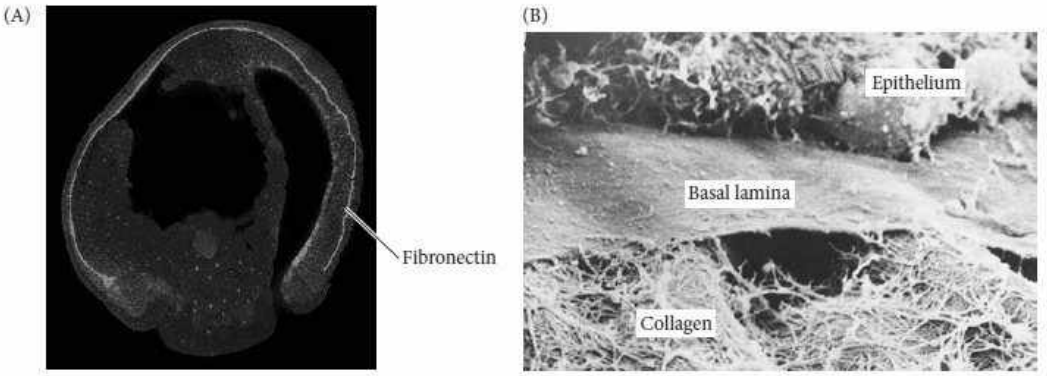


FIGURE 4.11 Extracellular matrices in the developing embryo. (A) Fluorescent antibodies to fibronectin show fibronectin deposition as a green band in the *Xenopus* embryo during gastrulation. The fibronectin will orient the movements of the mesoderm cells. (B) Fibronectin links together migrating cells, collagen, heparan sulfate, and other extracellular matrix proteins. This scanning electron micrograph shows the extracellular matrix at the junction of the epithelial cells (above) and mesenchymal cells (below). The epithelial cells synthesize a tight, laminin-based basal lamina, whereas the mesenchymal cells secrete a loose reticular lamina made primarily of collagen. (A courtesy of M. Marsden and D. W. DeSimone; B courtesy of R. L. Trelsted.)

VADE MECUM

Movies review the molecular components of the extracellular matrix and the experiments of Elizabeth Hay, whose work showed the importance of the ECM to tissue differentiation.

Integrins: Receptors for extracellular matrix molecules

The ability of a cell to bind to adhesive glycoproteins such as laminin or fibronectin depends on its expressing membrane receptors for the cell-binding sites of these large molecules. The fibronectin receptors were identified by using antibodies that block the attachment of cells to fibronectin (Chen et al. 1985; Knudsen et al. 1985). The main fibronectin receptor was found to be an extremely large protein that could bind fibronectin on the outside of the cell, span the membrane, and bind cytoskeletal proteins on the inside of the cell (**FIGURE 4.12**).

This family of receptor proteins are called integrins because they *integrate* the extracellular and intracellular scaffolds, allowing them to work together (Horwitz et al. 1986; Tamkun et al. 1986). On the extracellular side, integrins bind to the amino acid sequence arginine-glycine-aspartate (RGD), found in several extracellular matrix adhesive proteins, including fibronectin, vitronectin (found in the basal lamina of the eye), and laminin (Ruoslahti and Pierschbacher 1987). On the cytoplasmic side, integrins bind to talin and α-actinin, two proteins that connect to actin microfilaments. This dual binding enables the cell to move by contracting the actin microfilaments against the fixed extracellular matrix.

Integrins can also signal from the outside of the cell to the inside of the cell, altering gene expression (Walker et al. 2002). Bissell and colleagues (Bissell et al. 1982; Martins-Green and Bissell 1995) have shown that integrin is critical for inducing specific gene expression in developing tissues, especially those of the liver, testis, and mammary gland. In the mammary gland, extracellular laminin is able to signal the expression of estrogen receptor and casein protein genes through the integrin proteins (Streuli et al. 1991; Notenboom et al. 1996; Muschler et al. 1999; Novaro et al. 2003).

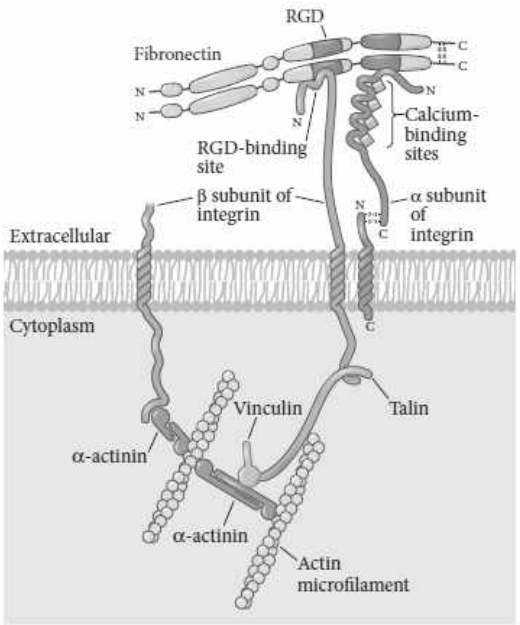


FIGURE 4.12 Simplified diagram of the fibronectin receptor complex. The integrins of the complex are membrane-spanning receptor proteins that bind fibronectin on the outside of the cell while binding cytoskeletal proteins on the inside of the cell. (After Luna and Hitt 1992.)

The presence of bound integrin prevents the activation of genes that promote apoptosis, or programmed cell death (Montgomery et al. 1994; Frisch and Ruoslahti 1997). For instance, the chondrocytes that produce the cartilage of our vertebrae and limbs can survive and differentiate only if they are surrounded by an extracellular matrix and are joined to that matrix through their integrins (Hirsch et al. 1997). If chondrocytes from the developing chick sternum are incubated with antibodies that block the binding of integrins to the extracellular matrix, they shrivel up and die. Indeed, when focal adhesions linking an epithelial cell to its extracellular matrix are broken, the caspase-dependent apoptosis pathway is activated, and the cell dies. Such "death-on-detachment" is a special type of apoptosis called **anoikis**, and it appears to be a major weapon against cancer (Frisch and Francis 1994; Chiarugi and Giannoni 2008).

Although the mechanisms by which bound integrins inhibit apoptosis remain controversial, the extracellular matrix is obviously an important source of signals that can be transduced into the nucleus to produce specific gene expression. Some of the genes induced by matrix attachment are being identified. When plated onto tissue culture plastic, mouse mammary gland cells will divide (**FIGURE 4.13**). Indeed, genes for cell division (*c-myc*, *cyclinD1*) are expressed, whereas genes for differentiated products of the mammary gland (casein, lactoferrin, whey acidic protein) are not expressed. If the same cells are plated onto plastic coated with a basal lamina, the cells stop dividing, and the genes of differentiated mammary gland cells are expressed. That happens only after the integrins of the mammary gland cells bind to the laminin of the basal lamina. Then the gene for lactoferrin is expressed, as is the gene for p21, a cell division inhibitor. The *c-myc* and *cyclinD1*

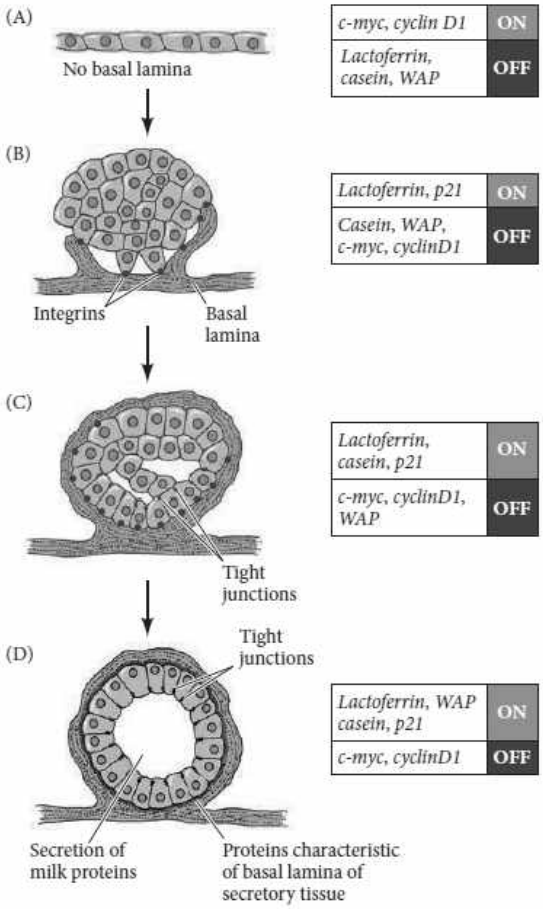


FIGURE 4.13 Basal lamina-directed gene expression in mammary gland tissue. (A) Mouse mammary gland tissue divides when placed on tissue culture plastic (no basal lamina). The genes encoding cell division proteins are on, and the genes capable of synthesizing the differentiated products of the mammary gland—lactoferrin, casein, and whey acidic protein (WAP)—are off. (B) When these cells are placed on a basal lamina, the genes for cell division proteins are turned off, while the genes encoding inhibitors of cell division (such as p21) and the gene for lactoferrin are turned on. (C,D) The mammary gland cells wrap the basal lamina around them, forming a secretory epithelium. The genes for casein and WAP are sequentially activated. (After Bissell et al. 2003.)

genes become silent. Eventually, all the genes for the developmental products of the mammary gland are expressed, and the cell division genes remain turned off. By this time, the mammary gland cells have enveloped themselves in a basal lamina, forming a secretory epithelium reminiscent of the mammary gland tissue. The binding of integrins to laminin is essential for transcription of the casein gene, and the integrins act in concert with prolactin (see Figure 4.27) to activate that gene's expression (Roskelley et al. 1994; Muschler et al. 1999).

The Epithelial-Mesenchymal Transition

One important developmental phenomenon, the **epithelial-mesenchymal transition**, or **EMT**, integrates all the processes we have discussed so far in this chapter. EMT is an orderly series of events whereby epithelial cells are transformed into mesenchymal cells. In this transition, a polarized stationary epithelial cell, which normally interacts with basal lamina through its basal surface, becomes a migratory mesenchymal cell that can invade tissues and help form organs in new places (**FIGURE 4.14A**; see Sleepman and Thiery 2011). EMT is usually initiated when paracrine factors from neighboring cells activate gene expression in the target cells, thereby instructing the target cells to downregulate their cadherins, release their attachment to laminin and other basal lamina components, rearrange their actin cytoskeleton, and secrete new extracellular matrix molecules characteristic of mesenchymal cells.

The epithelial-mesenchymal transition is critical during development (**FIGURE 4.14B,C**). Examples of developmental processes in which this transition is active include (1) the formation of neural crest cells from the dorsalmost region of the neural tube; (2) the formation of mesoderm in chick embryos, wherein cells that had been part of an epithelial layer become mesodermal and migrate into the embryo; and (3) the formation of vertebrae precursor cells from the somites, wherein these cells detach from the somite and migrate around the developing spinal cord. EMT is also important in adults, in whom it is needed for wound healing. The most critical adult form of EMT, however, is seen in cancer metastasis, wherein cells that have been part of a solid tumor mass leave that tumor to invade other tissues and form secondary tumors elsewhere in the body. It appears that in metastasis, the processes that generated the cellular transition in the embryo are reactivated, allowing cancer cells to migrate and become invasive. Cadherins are downregulated, the actin cytoskeleton is reorganized, and the cells secrete enzymes such as metalloproteinases to degrade the basal lamina and mesenchymal extracellular matrix while also undergoing cell division (Acloque et al. 2009; Kalluri and Weinberg 2009).

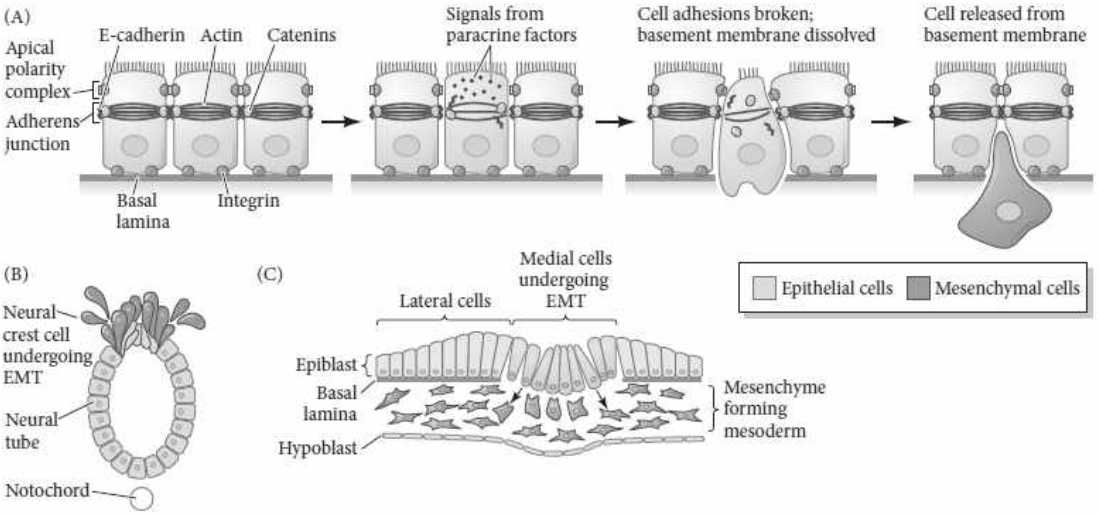


FIGURE 4.14 Epithelial-mesenchymal transition, or EMT. (A) Normal epithelial cells are attached to one another through adherens junctions containing cadherin, catenins, and actin rings. They are attached to the basal lamina through integrins. Paracrine factors can repress the expression of genes that encode these cellular components, causing the cell to lose polarity, lose attachment to the basal lamina, and lose cohesion with other epithelial cells. Cytoskeletal remodeling occurs, as well as the secretion of proteases that degrade the basal lamina and extracellular matrix molecules, enabling the migration of the newly formed mesenchymal cell. (B,C) EMT is seen in vertebrate embryos during the normal formation of neural crest from the dorsal region of the neural tube (B) and during the formation of the mesoderm by mesenchymal cells delaminating from the epiblast (C).

Cell Signaling

We have just learned how cell-to-cell adhesion (a juxtacrine interaction) can influence how cells position themselves within an embryo, and in previous chapters, we discussed the importance that a cell's position in the embryo can have on regulating its fate. What is so special about a given position in the embryo that it can determine a cell's fate? As you know, the experiences one has in early life greatly influence the type of person one becomes as an adult in terms of personality, career choice, or food preferences. Similarly, the experiences a cell has in its embryonic position influence the gene regulatory network under which it develops. Therefore, the real question is, in a given location, what defines the cell's experience?

Induction and competence

From the earliest stages of development through the adult, cell behaviors such as adhesion, migration, differentiation, and division are regulated by signals from one cell being received by another cell. Indeed, these interactions (which are often reciprocal, as we will describe later) are what allow organs to be constructed. The development of the vertebrate eye is a classic example used to describe the modus operandi of tissue organization via intercellular interactions.

In the vertebrate eye, light is transmitted through the transparent corneal tissue and focused by the lens tissue (the diameter of which is controlled by muscle tissue), eventually impinging on the tissue of the neural retina. The precise arrangement of tissues in the eye cannot be disturbed without impairing its function. Such coordination in the construction of organs is accomplished by one group of cells changing the behavior of an adjacent set of cells, thereby causing them to change their shape, mitotic rate, or cell fate. This kind of interaction at close range between two or more cells or tissues of different histories and properties is called **induction**.

DEFINING INDUCTION AND COMPETENCE There are at least two components to every inductive interaction. The first component is the **inducer**, the tissue that produces a signal (or signals) that changes the cellular behavior of the other tissue. Often this signal is a secreted protein called a paracrine factor. **Paracrine factors** are proteins made by a cell or a group of cells that alter the behavior or differentiation of adjacent cells. In contrast to endocrine factors (hormones), which travel through

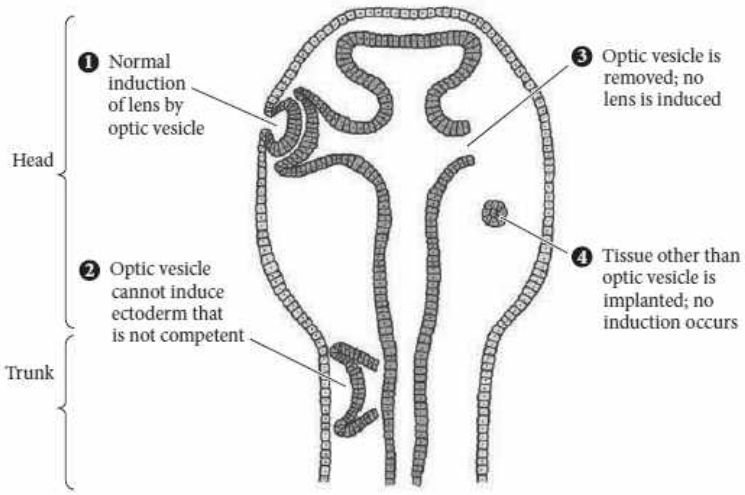


FIGURE 4.15 Ectodermal competence and the ability to respond to the optic vesicle inducer in *Xenopus*. The optic vesicle is able to induce lens formation in the anterior portion of the ectoderm (1) but not in the presumptive trunk and abdomen (2). If the optic vesicle is removed (3), the surface ectoderm forms either an abnormal lens or no lens at all. Most other tissues are not able to substitute for the optic vesicle (4).

the blood and exert their effects on cells and tissues far away, paracrine factors are secreted into the extracellular space and influence their close neighbors. The second component, the **responder**, is the cell or tissue being induced. Cells of the responding tissue must have both a receptor protein for the inducing factor and the *ability* to respond to the signal. The ability to respond to a specific inductive signal is called **competence** (Waddington 1940).

BUILDING THE VERTEBRATE EYE In the initiation of the vertebrate eyes, paired regions of the brain bulge out and approach the surface ectoderm of the head. The head ectoderm is competent to respond to the paracrine factors made by these brain bulges (the **optic vesicles**), and the head ectoderm receiving these paracrine factors is induced to form the lens of the eye. The genes for lens proteins become induced in the head ectoderm cells and are expressed in these cells. The Rho-family GTPases are activated to control the elongation and curvature of the lens fibers (see Chapter 16; Maddala et al. 2008). Moreover, the prospective lens cells secrete paracrine factors that instruct the optic vesicle to form the retina. Thus, the two major parts of the eye co-construct each other, and the eye forms from reciprocal paracrine interactions. The head ectoderm is the only region capable of responding to the optic vesicle. If an optic vesicle from a *Xenopus laevis* embryo is placed underneath head ectoderm in a different part of the head from where the frog's optic vesicle normally occurs, the vesicle will induce that ectoderm to form lens tissue; trunk ectoderm, however, will not respond to the optic vesicle (**FIGURE 4.15**; Saha et al. 1989; Grainger 1992). Only head ectoderm is *competent* to respond to the signals from the optic vesicle by producing a lens.

Often, one induction will give a tissue the competence to respond to another inducer. Studies on amphibians suggest that the first inducers of the lens may be the foregut endoderm and heart-forming mesoderm that underlie the lens-forming ectoderm during the early and mid gastrula stages (Jacobson 1963, 1966). The anterior neural plate may produce the next signals, including a signal that promotes the synthesis of the Paired box 6 (Pax6) transcription factor in the anterior ectoderm which is required for the competence to respond to the optic vesicle's signals (**FIGURE 4.16**; Zygar et al. 1998). Thus, although the optic vesicle appears to be *the* lens inducer, the anterior ectoderm has already been induced by at least two other tissues. The optic vesicle's situation is like that of the player who kicks the "winning" goal in a soccer match, yet many others helped to position that ball for the final kick!

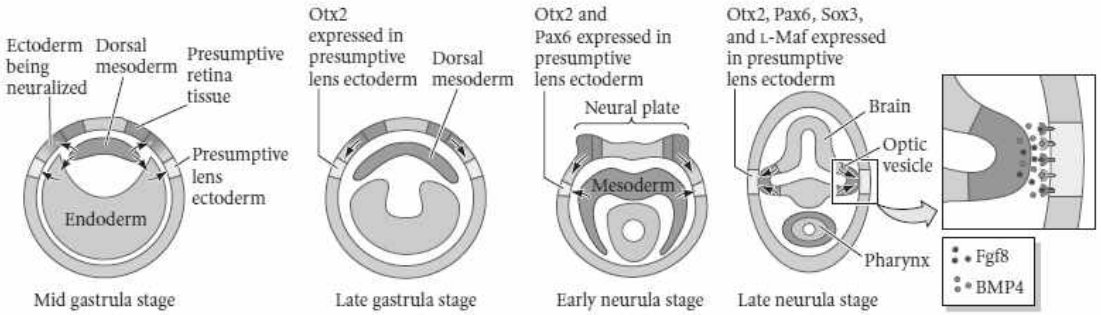


FIGURE 4.16 Sequence of amphibian lens induction postulated by experiments on embryos of the frog *Xenopus laevis*. Unidentified inducers (possibly from the foregut endoderm and cardiac mesoderm) cause the synthesis of the Otx2 transcription factor in the head ectoderm during the late gastrula stage. As the neural folds rise, inducers from the anterior neural plate (including the region that will form the retina) induce Pax6 expression in the anterior ectoderm that can form lens tissue. Expression of Pax6 protein may constitute the competence of the surface ectoderm to respond to the optic vesicle during the late neurula stage. The optic vesicle secretes BMP and FGF family paracrine factors (see signals in higher magnification of boxed area) that induce the synthesis of the Sox transcription factors and initiate observable lens formation. (After Grainger 1992.)

The optic vesicle appears to secrete two paracrine factors, one of which is BMP4 (Furuta and Hogan 1998), a protein that is received by the lens cells and induces the production of the Sox transcription factors (see Figure 4.16, right-most panels). The other is Fgf8, a secreted signal that induces the appearance of the L-Maf transcription factor (Ogino and Yasuda 1998; Vogel-Höpker et al. 2000). As we saw in Chapter 3, the combination of Pax6, Sox2, and L-Maf in the ectoderm is needed for the production of the lens and the activation of lens-specific genes such as δ-*crystallin*. Pax6 is important in providing the competence for the ectoderm to respond to the inducers from the optic cup (Fujiwara et al. 1994). If *Pax6* is lost, whether it is in fruit flies, frogs, rats, or humans, it results in a complete loss or reduction of the eyes (Quiring et al. 1994). Experiments recombining surface ectoderm with the optic vesicle from wild-type and *Pax6* mutant rat embryos demonstrated that Pax6 must be functional in the surface ectoderm for it to form a lens (**FIGURE 4.17A,B**). In humans, a spectrum of eye malformations have been associated with a variety of *Pax6* mutations. These malformations include aniridia, in which the iris is reduced or lacking (**FIGURE 4.17C**); *Pax6* mutations in *Xenopus* have revealed remarkably similar aniridia-like symptoms, enabling researchers to model and further investigate the developmental role of Pax6 in this human disease (Nakayama et al. 2015).

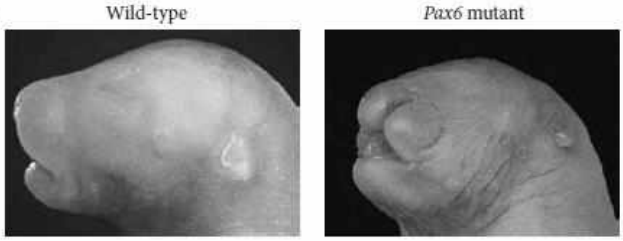
Reciprocal induction

Another feature of induction is the reciprocal nature of many inductive interactions. To continue the above example, once the lens has formed, it induces other tissues. One of these responding tissues is the optic vesicle itself; thus, the inducer becomes the induced. Under the influence of factors secreted by the lens, the optic vesicle becomes the optic cup, and the wall of the optic cup differentiates into two layers: the pigmented retina and the neural retina (see Figure 16.8; Cvekl and Piatigorsky 1996; Strickler et al. 2007). Such interactions are called **reciprocal inductions**.

Another principle can be seen in such reciprocal inductions: a structure does not need to be fully differentiated to have a function. As we will detail in Chapter 16, the optic vesicle induces the surface ectoderm to become a lens before the optic vesicle has become the retina. Similarly, the developing lens reciprocates by inducing the optic vesicle before the lens forms its characteristic fibers. Thus, before a tissue has its "adult" functions, it has critically important transient functions in building the organs of the embryo.

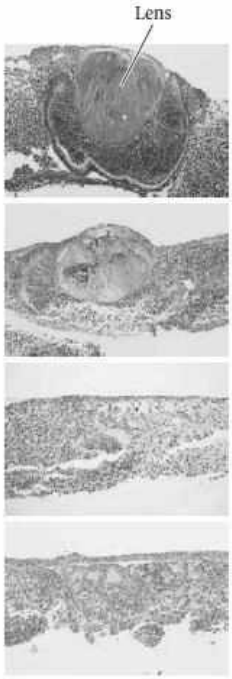
INSTRUCTIVE AND PERMISSIVE INTERACTIONS Howard Holtzer (1968) distinguished two major modes of inductive interaction. In **instructive interaction**, a signal from the inducing cell is *necessary* for initiating new gene expression in the responding cell. Without the inducing cell, the responding cell is not capable of differentiating in that particular way. For example, one instructive interaction is when a *Xenopus* optic vesicle

(A)



(B)

Optic vesicles	Surface ectoderm	Lens induction
Wild-type	Wild-type	Yes
Pax6^{-}/*Pax6*$^{-}$	Wild-type	Yes
Wild-type	*Pax6*$^{-}$/*Pax6*$^{-}$	No
Pax6^{-}/*Pax6*$^{-}$	*Pax6*$^{-}$/*Pax6*$^{-}$	No



(C)

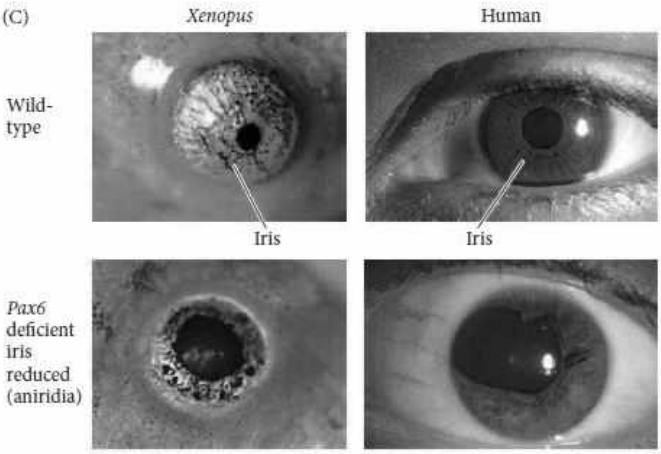


FIGURE 4.17 The *Pax6* gene is similarly required for eye development in frogs, rats, and humans. (A) Loss of *Pax6* in rats results in the failure to form eyes as well as significant reductions in nasal structures. (B) An analysis of lens induction following recombination experiments of the optic vesicle and surface ectoderm between wild-type and *Pax6* null rat embryos. Pax6 is required only in the surface ectoderm for proper lens induction. (C) Mutations in the *Pax6* gene in *Xenopus* and humans result in similar reductions in the iris of the eye as compared to wild-type individuals. This phenotype is characteristic of aniridia. (A from Fujiwara et al. 1994; B photographs courtesy of M. Fujiwara; C from Yaoita et al. 2015, courtesy of R. M. Grainger.)

Developing Questions

Although rebuilding a decellularized heart is clearly an example of permissive interactions, could there be instructive interactions too? Recently, iPSC-derived cardiovascular progenitor cells successfully seeded a decellularized mouse heart and differentiated into cardiomyoctyes, smooth muscle and endothelial cells (Lu et al. 2013). What could the ECM be providing to directly influence the differentiation of progenitor cells into these varied cell types?

experimentally placed under a new region of head ectoderm causes that region of the ectoderm to form a lens.

The second type of inductive interaction is **permissive interaction**. Here, the responding tissue has already been specified and needs only an environment that allows the expression of these traits. For instance, many tissues need an extracellular matrix to develop. The extracellular matrix does not alter the type of cell that is produced, but it enables what has already been determined to be expressed.[1] A dramatic example of permissive interactions at work comes from the regenerative medicine field, in which an extracellular matrix scaffold can promote the differentiation and rebuilding of a beating heart. Doris Taylor's research group used detergents to remove all the cells from a cadaveric rat heart, which leaves behind the natural extracellular matrix (**FIGURE 4.18A**; Ott et al. 2008). Proteins like fibronectin, collagen, and laminin held together the rest of the ECM and maintained the intricate shape of the heart. The researchers then infused this ECM scaffold with cardiomyocytes. Surprisingly, these cells differentiated and organized into a functionally contracting "recellularized" heart (**FIGURE 4.18B**). Therefore, the environmental conditions of the decellularized ECM were permissive in allowing the cardiomyocytes to recreate contracting heart muscle. You will be reading more about regenerative medicine in Chapter 5.

SCIENTISTS SPEAK 4.2 Dr. Doris Taylor discusses the use of decellularized organs for regeneration.

Epithelial-mesenchymal interactions

Some of the best-studied cases of induction involve the interactions of sheets of epithelial cells with adjacent mesenchymal cells. All organs consist of an epithelium and an associated mesenchyme, so these interactions are among the most important phenomena in nature. Some examples are listed in **TABLE 4.1**.

[1] It is easy to distinguish permissive and instructive interactions using an analogy. This textbook is made possible by both permissive and instructive interactions. A reviewer can convince us to change the material in the chapters, which is an instructive interaction because the information expressed in the book is changed from what it would have been. However, the information in the book could not be expressed at all without permissive interactions with the publisher and printer.

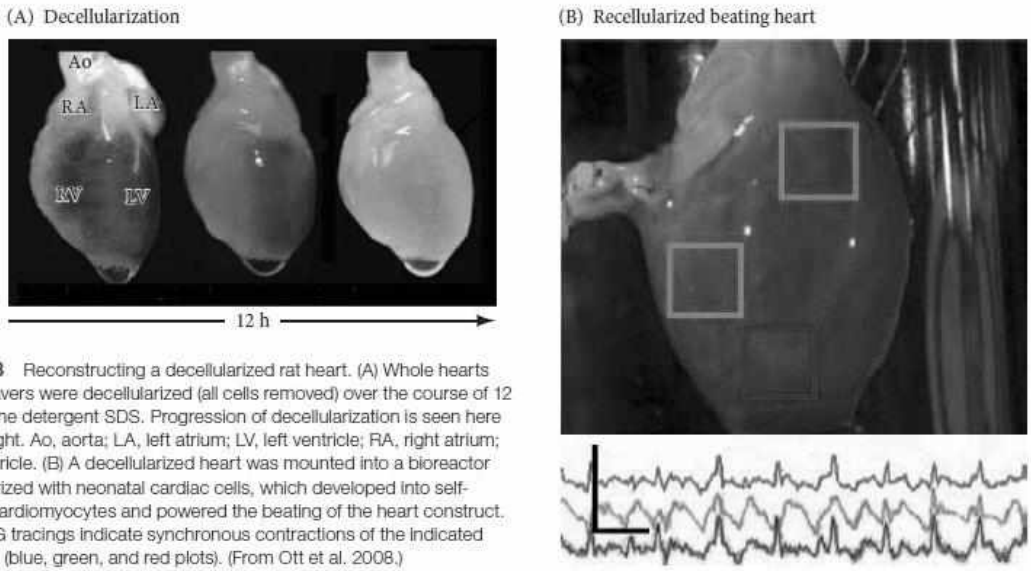


FIGURE 4.18 Reconstructing a decellularized rat heart. (A) Whole hearts from rat cadavers were decellularized (all cells removed) over the course of 12 hours using the detergent SDS. Progression of decellularization is seen here from left to right. Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. (B) A decellularized heart was mounted into a bioreactor and recellularized with neonatal cardiac cells, which developed into self-contracting cardiomyocytes and powered the beating of the heart construct. Regional ECG tracings indicate synchronous contractions of the indicated heart regions (blue, green, and red plots). (From Ott et al. 2008.)

TABLE 4.1 Some epithelial-mesenchymal interactions

Organ	Mesenchymal component	Epithelial component
Cutaneous structures (hair, feathers, sweat glands, mammary glands)	Epidermis (ectoderm)	Dermis (mesoderm)
Limb	Epidermis (ectoderm)	Mesenchyme (mesoderm)
Gut organs (liver, pancreas, salivary glands)	Epithelium (endoderm)	Mesenchyme (mesoderm)
Foregut and respiratory-associated organs (lungs, thymus, thyroid)	Epithelium (endoderm)	Mesenchyme (mesoderm
Kidney	Ureteric bud (mesoderm)	Mesenchyme epithelium (mesoderm)
Tooth	Jaw epithelium (ectoderm)	Mesenchyme (neural crest)

REGIONAL SPECIFICITY OF INDUCTION Using the induction of cutaneous (skin) structures as our examples, we will look at the properties of epithelial-mesenchymal interactions. The first of these properties is the regional specificity of induction. Skin is composed of two main tissues: an outer epidermis (an epithelial tissue derived from ectoderm) and a dermis (a mesenchymal tissue derived from mesoderm). The chick epidermis secretes proteins that signal the underlying dermal cells to form condensations, and the condensed dermal mesenchyme responds by secreting factors that cause the epidermis to form regionally specific cutaneous structures (Nohno et al. 1995; Ting-Berreth and Chuong 1996). These structures can be the broad feathers of the wing, the narrow feathers of the thigh, or the scales and claws of the feet (**FIGURE 4.19**). The dermal mesenchyme is responsible for the regional specificity of induction in the competent epidermal epithelium. Researchers can separate the embryonic epithelium and mesenchyme from each other and recombine them in different ways (Saunders et al. 1957). The same epithelium develops cutaneous structures according to the region from which the mesenchyme was taken. Here, the mesenchyme plays an instructive role, calling into play different sets of genes in the responding epithelial cells.

GENETIC SPECIFICITY OF INDUCTION The second property of epithelial-mesenchymal interactions is the genetic specificity of induction. Whereas the mesenchyme may instruct the epithelium as to what sets of genes to activate, the responding epithelium can comply with these instructions only so far as its genome permits. This property was

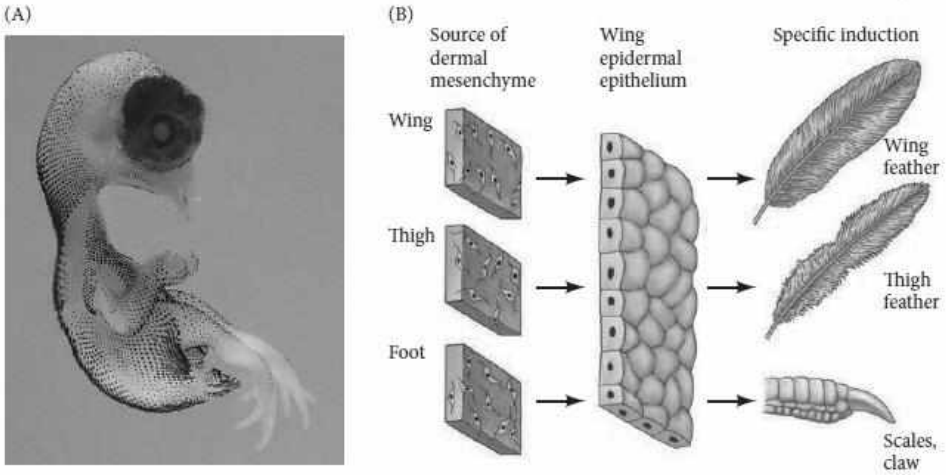


FIGURE 4.19 Feather induction in the chick. (A) In situ hybridization of a 10-day chick embryo shows Sonic hedgehog expression (dark spots) in the ectoderm of the developing feathers and scales. (B) When cells from different regions of the chick dermis (mesenchyme) are recombined with wing epidermis (epithelium), the type of cutaneous structure made by the epidermal epithelium is determined by the source of the mesenchyme. (A courtesy of W.-S. Kim and J. F. Fallon; after Saunders 1980.)

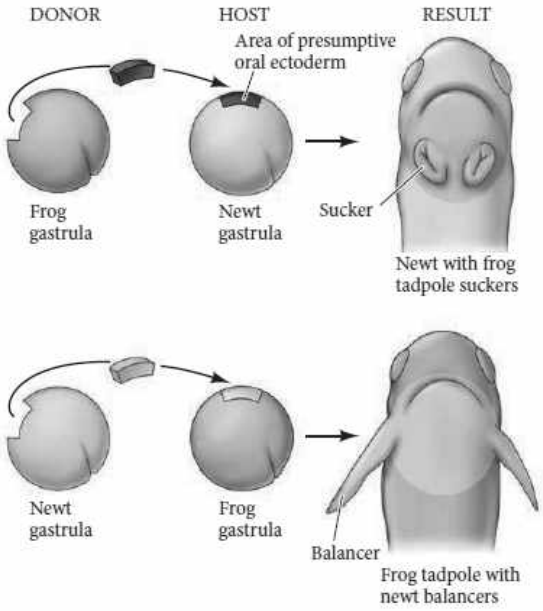


FIGURE 4.20 Genetic specificity of induction in amphibians. Reciprocal transplantation between the presumptive oral ectoderm regions of salamander and frog gastrulae leads to newts with tadpole suckers and tadpoles with newt balancers. (After Hamburgh 1970.)

discovered through experiments involving the transplantation of tissues from one species to another.

In one of the most dramatic examples of interspecific induction, Hans Spemann and Oscar Schotté (1932) transplanted flank ectoderm from an early frog gastrula to the region of a newt gastrula destined to become parts of the mouth. Similarly, they placed presumptive flank ectodermal tissue from a newt gastrula into the presumptive oral regions of frog embryos. The structures of the mouth region differ greatly between salamander and frog larvae. The salamander larva has club-shaped balancers beneath its mouth, whereas the frog tadpole produces mucus-secreting glands and suckers. The frog tadpole also has a horny jaw without teeth, whereas the salamander has a set of calcareous teeth in its jaw. The larvae resulting from the transplants were chimeras. The salamander larvae had frog-like mouths, and the frog tadpoles had salamander teeth and balancers (**FIGURE 4.20**). In other words, the mesenchymal cells instructed the ectoderm to make a mouth, but the ectoderm responded by making the only kind of mouth it "knew" how to make, no matter how inappropriate.[2]

Thus, the instructions sent by the mesenchymal tissue can cross species barriers. Salamanders respond to frog inducers, and chick tissue responds to mammalian inducers. The response of the epithelium, however, is species-specific. So, whereas organ-type specificity (e.g., feather or claw) is usually controlled by the mesenchyme, species specificity is usually controlled by the responding epithelium. As we will see in Chapter 26, major evolutionary changes in the phenotype can be brought about by changing the response to a particular inducer.

The insect trachea: Combining inductive signals with cadherin regulation

Earlier in this chapter we talked about the shared role of cadherins and actinomyosin cortical contraction in mediating cell-to-cell adhesions involved in tissue morphogenesis. Instructions from outside the cell can influence cell shape change through modulation of the cadherin-actinomyosin mechanism. For instance, the tracheal (respiratory) system in *Drosophila* embryos develops from epithelial sacs. The approximately 80 cells in each of these sacs become reorganized into primary, secondary, and tertiary branches without any cell division or cell death (Ghabrial and Krasnow 2006). This reorganization is initiated when nearby cells secrete a protein called Branchless, which acts as a **chemoattractant** (usually a diffusible molecule that attracts a cell to migrate along an increasing concentration gradient toward the source secreting the factor).[3] Branchless binds to a receptor on the cell membranes of the epithelial cells. The cells receiving the most Branchless protein lead the rest, whereas the followers (connected to one another by cadherins) receive a signal from the leading cells to form the tracheal tube (**FIGURE 4.21**). It is the lead cell that will change its shape (by rearranging its actin-myosin cytoskeleton via a Rho GTPase-mediated process) to migrate and form the secondary branches. During this migration, cadherin proteins are regulated such that the epithelial

[2]Spemann is reported to have put it this way: "The ectoderm says to the inducer, 'you tell me to make a mouth; all right, I'll do so, but I can't make your kind of mouth; I can make my own and I'll do that.' " (Quoted in Harrison 1933.)

[3]There are also *chemorepulsive* factors that send the migrating cells in an opposite direction. Generally speaking, chemotactic factors—soluble factors that cause cells to move in a particular direction—are assumed to be chemoattractive unless otherwise described.

cells can migrate over one another to form a tube while keeping their integrity as an epithelium (Cela and Llimagas 2006).

Another external force is also at work, however. The dorsalmost secondary branches of the sacs move along a groove that forms between the developing muscles. These tertiary cell migrations cause the trachea to become segmented around the musculature (Franch-Marro and Casanova 2000). In this way, the respiratory tubes are placed close to the larval musculature.

Paracrine Factors: Inducer Molecules

How are the signals between inducer and responder transmitted? While studying the mechanisms of induction that produce the kidney tubules and teeth, Grobstein (1956) and others (Saxén et al. 1976; Slavkin and Bringas 1976) found that some inductive events could occur despite a filter separating the epithelial and mesenchymal cells. Other inductions, however, were blocked by the filter. The researchers therefore concluded that some of the inducers were soluble molecules that could pass through the small pores of the filter and that other inductive events required physical contact between the epithelial and mesenchymal cells.

When membrane proteins on one cell surface interact with receptor proteins on adjacent cell surfaces (as seen with cadherins), the event is called a **juxtacrine interaction** (since the cell membranes are *juxtaposed*). When proteins synthesized by one cell can diffuse over small distances to induce changes in neighboring cells, the event is called a **paracrine interaction**. Paracrine factors are diffusible molecules that work in a range of about 15 cell diameters, or about 40–200 μm (Bollenbach et al. 2008; Harvey and Smith 2009).

A specific type of paracrine interaction is the **autocrine interaction**. Autocrine interactions occur when the same cells that secrete the paracrine factors also respond to them. In other words, the cell synthesizes a molecule for which it has its own receptor. Although autocrine regulation is not common, it is seen in placental cytotrophoblast cells; these cells synthesize and secrete platelet-derived growth factor, whose receptor is on the cytotrophoblast cell membrane (Goustin et al. 1985). The result is the explosive proliferation of that tissue.

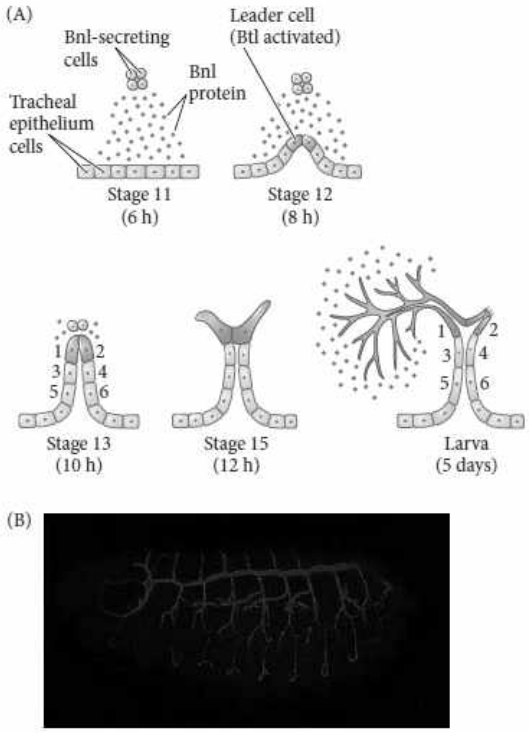


FIGURE 4.21 Tracheal development in *Drosophila*. (A) Diagram of dorsal tracheal branch budding from tracheal epithelium. Nearby cells secrete Branchless protein (Bnl; blue dots), which activates Breathless protein (Btl) on tracheal cells. The activated Btl induces migration of the leader cells and tube formation; the dorsal branch cells are numbered 1 through 6. Branchless also induces unicellular secondary branches (stage 15). (B) Larval *Drosophila* tracheal system visualized with a fluorescent red antibody. Note the intercalated branching pattern. (A after Ghabrial and Krasnow 2006; B from Casanova 2007.)

Morphogen gradients

One of the most important mechanisms governing cell fate specification involves gradients of paracrine factors that regulate gene expression; such signaling molecules are called morphogens. A **morphogen** (Greek, "form-giver") is a diffusable biochemical molecule that can determine the fate of a cell by its concentration.[4] That is, cells exposed to high levels of a morphogen activate different genes than those cells exposed to lower levels. Morphogens can be transcription factors produced within a syncytium of nuclei as in the *Drosophila* blastoderm (see Chapter 2). They can also be paracrine factors that are produced in one group of cells and then travel to another population of cells, specifying the target cells to have similar or different fates according to the concentration of the morphogen. Uncommitted cells exposed to high concentrations of the morphogen

[4]Although there is overlap in the terminology, a *morphogen* specifies cells in a quantitative ("more or less") manner, whereas a *morphogenetic determinant* specifies cells in a qualitative ("present or absent") way. Morphogens are analog; morphogenetic determinants are digital.

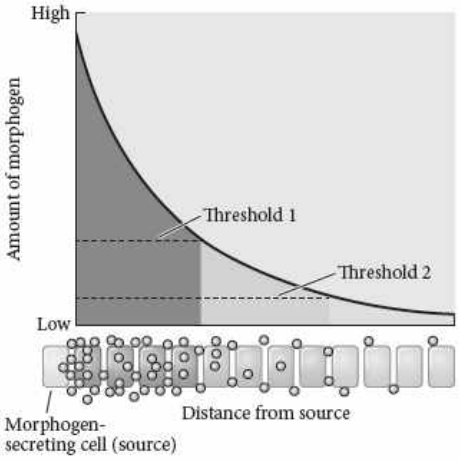


FIGURE 4.22 Specification of uniform cells into three cell types by a morphogen gradient. A morphogenetic paracrine factor (yellow dots) is secreted from source cells (yellow) and forms a concentration gradient within the responsive tissue. Cells exposed to morphogen concentrations above threshold 1 activate certain genes (red). Cells exposed to intermediate concentrations (between thresholds 1 and 2) activate a different set of genes (pink) and also inhibit the genes induced at the higher concentrations. Those cells encountering low concentrations of morphogen (below threshold 2) activate a third set of genes (blue). (After Rogers and Schier 2011.)

(nearest its source of production) are specified as one cell type. When the morphogen's concentration drops below a certain threshold, a different cell fate is specified. When the concentration falls even lower, a cell that initially was of the same uncommitted type is specified in yet a third distinct manner (**FIGURE 4.22**).

DEV TUTORIAL ***Morphogen Signaling*** A lecture and demonstration by Dr. Michael Barresi of some ways in which morphogen signaling operates.

Regulation by gradients of paracrine factor concentration was elegantly demonstrated by the specification of different mesodermal cell types in the frog *Xenopus laevis* by activin, a paracrine factor of the TGF-β family (**FIGURE 4.23**; Green and Smith 1990; Gurdon et al. 1994). Activin-secreting beads were placed on unspecified cells from an early *Xenopus* embryo. The activin then diffused from the beads. At high concentrations (about 300 molecules/cell), activin induced expression of the *goosecoid* gene, whose product is a transcription factor that specifies the frog's dorsal-most structures. At slightly lower concentrations of activin (about 100 molecules per cell), the same tissue activated the *Xbra* gene and was specified to become muscle. At still lower concentrations, these genes were not activated, and the "default" gene expression instructed the cells to become blood vessels and heart (Dyson and Gurdon 1998).

The range of a paracrine factor (and thus the shape of its morphogen gradient) depends on several aspects of that factor's synthesis, transport, and degradation. In some cases, cell surface molecules stabilize the paracrine factor and aid in its diffusion, while in other cases, cell surface moieties retard diffusion and enhance degradation. Such diffusion-regulating interactions between morphogens and extracellular matrix factors are very important in coordinating organ growth and shape (Ben Zvi et al. 2010, 2011).

The induction of numerous organs is effected by a relatively small set of paracrine factors that often function as morphogens. The embryo inherits a rather compact genetic "tool kit" and uses many of the same proteins to construct the heart, kidneys, teeth, eyes, and other organs. Moreover, the same proteins are used throughout the animal kingdom; for instance, the factors active in creating the *Drosophila* eye or heart are very similar to those used in generating mammalian organs. Many paracrine factors can be grouped into one of four major families on the basis of their structure:

1. The fibroblast growth factor (FGF) family
2. The Hedgehog family
3. The Wnt family
4. The TGF-β superfamily, encompassing the TGF-β family, the activin family, the bone morphogenetic proteins (BMPs), the Nodal proteins, the Vg1 family, and several other related proteins

Signal transduction cascades: The response to inducers

For a ligand to induce a cellular response in a cell, it must bind to a receptor, which starts a cascade of events within the cell that ultimately regulate a response. Paracrine

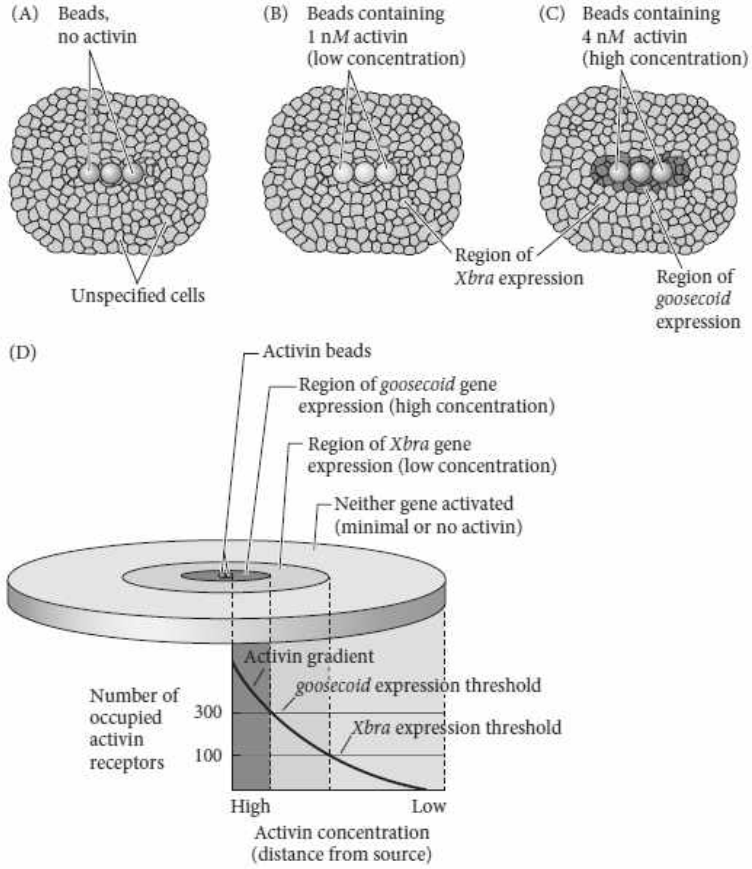


FIGURE 4.23 A gradient of the paracrine factor activin, a morphogen, causes concentration-dependent expression differences of two genes in unspecified amphibian cells. (A) Beads containing no activin did not elicit expression (i.e., mRNA transcription) of either the *Xbra* or *goosecoid* gene. (B) Beads containing 1 n*M* activin elicited *Xbra* expression in nearby cells. (C) Beads containing 4 n*M* activin elicited *Xbra* expression, but only at a distance of several cell diameters from the beads. A region of *goosecoid* expression is seen near the source bead, however. Thus, it appears that *Xbra* is induced at particular concentrations of activin and that *goosecoid* is induced at higher concentrations. (D) Interpretation of the *Xenopus* activin gradient. High concentrations of activin activate *goosecoid*, whereas lower concentrations activate *Xbra*. A threshold value appears to exist that determines whether a cell will express *goosecoid*, *Xbra*, or neither gene. In addition, Brachyury (the *Xbra* protein product in *Xenopus*) inhibits the expression of *goosecoid*, thereby creating a distinct boundary. This pattern correlates with the number of activin receptors occupied on individual cells. (After Gurdon et al. 1994; Dyson and Gurdon 1998.)

factors function by binding to a receptor that initiates a series of enzymatic reactions within the cell. These enzymatic reactions have as their end point either the regulation of transcription factors (such that different genes are expressed in the cells reacting to these paracrine factors) and/or the regulation of the cytoskeleton (such that the cells responding to the paracrine factors alter their shape or are permitted to migrate). These pathways of responses to the paracrine factor often have several end points and are called **signal transduction cascades**.

The major signal transduction pathways all appear to be variations on a common and rather elegant theme, exemplified in **FIGURE 4.24**. Each receptor spans the cell membrane and has an extracellular region, a transmembrane region, and a cytoplasmic region. When a paracrine factor binds to its receptor's extracellular domain, the paracrine factor induces a conformational change in the receptor's structure. This shape change is transmitted through the membrane and alters the shape of the receptor's cytoplasmic domain, giving that domain the ability to activate cytoplasmic proteins. Often such a conformational change confers enzymatic activity on the domain, usually a kinase activity that can use ATP to phosphorylate specific tyrosine residues of particular proteins. Thus, this type of receptor is often called a **receptor tyrosine kinase** (**RTK**). The active receptor can now catalyze reactions that phosphorylate other proteins, and this phosphorylation in turn activates their latent activities. Eventually, the *cascade* of phosphorylation activates a dormant transcription factor or a set of cytoskeletal proteins.

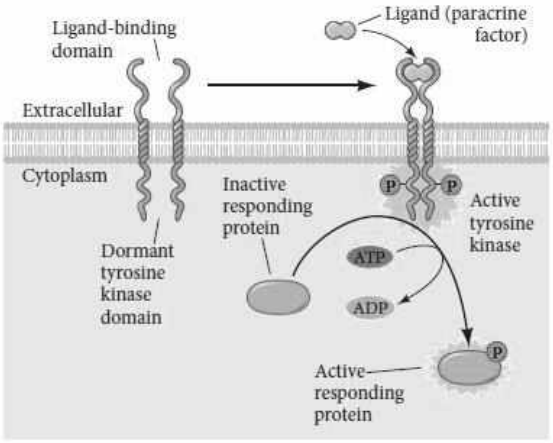
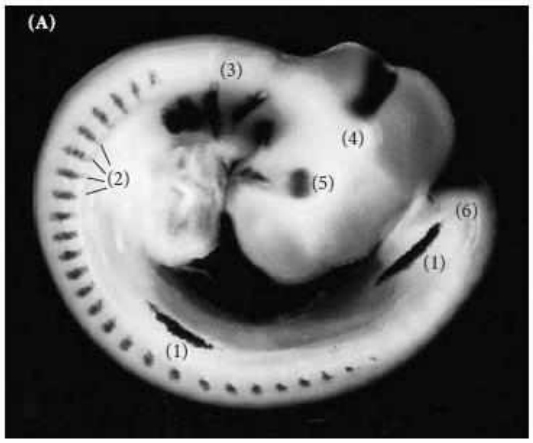
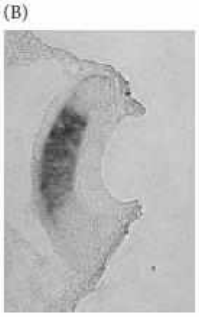
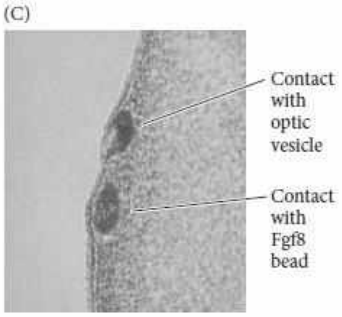


FIGURE 4.24 Structure and function of a receptor tyrosine kinase. The binding of a paracrine factor (such as Fgf8) by the extracellular portion of the receptor protein activates the dormant tyrosine kinase, whose enzyme activity phosphorylates its reciprocal receptor partner followed by specific tyrosine residues of certain intracellular proteins.

FIGURE 4.25 Fgf8 in the developing chick. (A) *Fgf8* gene expression pattern in the 3-day chick embryo, shown by in situ hybridization. Fgf8 protein (dark areas) is seen in the distalmost limb bud ectoderm (1), in the somitic mesoderm (the segmented blocks of cells along the anterior-posterior axis (2), in the branchial arches of the neck (3), at the boundary between the midbrain and hindbrain (4), in the optic vesicle of the developing eye (5), and in the tail (6). (B) In situ hybridization of *Fgf8* in the optic vesicle. The *Fgf8* mRNA (purple) is localized to the presumptive neural retina of the optic cup and is in direct contact with the outer ectoderm cells that will become the lens. (C) Ectopic expression of L-Maf in competent ectoderm can be induced by the optic vesicle (above) and by an Fgf8-containing bead (below). (A courtesy of E. Laufer, C.-Y. Yeo, and C. Tabin; B,C courtesy of A. Vogel-Höpker.)







Below we will describe some of the major characteristics of the four families of paracrine factors, their modes of secretion, gradient manipulation, and the mechanisms underlying transduction in the responding cells. The distinctive roles of each paracrine factor in a variety of developmental processes will be discussed throughout the book, however.

Fibroblast growth factors and the RTK pathway

The **fibroblast growth factor** (**FGF**) family of paracrine factors comprises nearly two dozen structurally related members, and the FGF genes can generate hundreds of protein isoforms by varying their RNA splicing or initiation codons in different tissues (Lappi 1995). Fgf1 protein is also known as acidic FGF and appears to be important during regeneration (Yang et al. 2005), Fgf2 is sometimes called basic FGF and is very important in blood vessel formation, and Fgf7 sometimes goes by the name of keratinocyte growth factor and is critical in skin development. Although FGFs can often substitute for one another, the expression patterns of the FGFs and their receptors give them separate functions.

One member of this family, Fgf8, is especially important during segmentation, limb development, and lens induction. Fgf8 is usually made by the optic vesicle that contacts the outer ectoderm of the head (**FIGURE 4.25A**; Vogel-Höpker et al. 2000). After contact with the outer ectoderm occurs, *Fgf8* gene expression becomes concentrated in the region of the presumptive neural retina (the tissue directly apposed to the presumptive lens) (**FIGURE 4.25B**). Moreover, if Fgf8-containing beads[5] are placed adjacent to head ectoderm, this ectopic Fgf8 will induce this ectoderm to produce ectopic lenses and express the lens-associated transcription factor L-Maf (**FIGURE 4.25C**). FGFs often work by activating a set of receptor tyrosine kinases called the **fibroblast growth factor receptors** (**FGFRs**). For instance, the Branchless protein is an FGFR in *Drosophila*.

When an FGFR binds an FGF ligand (and *only* when it binds an FGF ligand), the dormant kinase is activated and phosphorylates certain proteins (including other FGFRs) within the

[5]Synthetic beads can be coated with proteins and placed into the tissue of an embryo. These proteins are released from the bead slowly and then diffuse radially, creating concentration gradients.

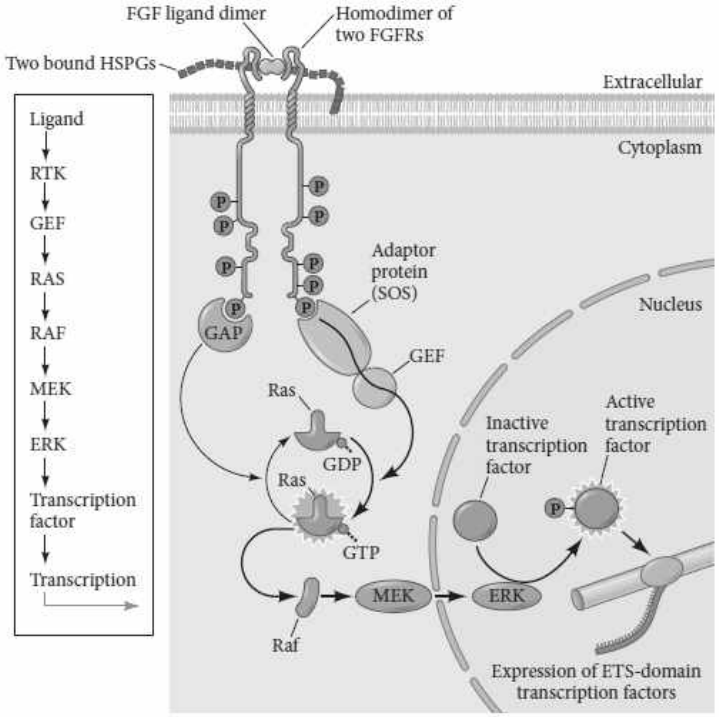


FIGURE 4.26 The widely used RTK signal transduction pathway can be activated by fibroblast growth factor. The receptor tyrosine kinase is dimerized by the ligand (a paracrine factor, such as FGF) along with heparan sulfate proteoglycans (HSPG), which together cause the dimerization and autophosphorylation of the RTKs. The adaptor protein recognizes the phosphorylated tyrosines on the RTK and activates an intermediate protein, GEF, which activates the Ras G-protein by allowing phosphorylation of the GDP-bound Ras. At the same time, the GAP protein stimulates hydrolysis of this phosphate bond, returning Ras to its inactive state. The active Ras activates the Raf protein kinase C, which in turn phosphorylates a series of kinases (such as MEK). Eventually, the activated kinase ERK alters gene expression in the nucleus of the responding cell by phosphorylating certain transcription factors (which can then enter the nucleus to change the types of genes transcribed) and certain translation factors (which alter the level of protein synthesis). In many cases, this pathway is reinforced by the release of calcium ions. A simplified version of the pathway is shown on the left.

responding cell. These proteins, once activated, can perform new functions. The **RTK pathway** was one of the first signal transduction pathways to unite various areas of developmental biology (**FIGURE 4.26**). Researchers studying *Drosophila* eyes, nematode vulvae, and human cancers found that they were all studying the same genes!

Fibroblast growth factors, epidermal growth factors, platelet-derived growth factors, and stem cell factor are all paracrine factors that bind to receptor tyrosine kinase (RTK). Each RTK can bind only one (or one small set) of these ligands, and stable binding requires an additional element, heparan sulfate proteoglycans, or HSPG (Mohammadi et al. 2005; Bökel and Brand 2013). When RTK-ligand binding occurs, RTK undergoes a conformational change that enables it to dimerize with another RTK. This conformational change stimulates the latent kinase activity of each RTK, and these receptors phosphorylate each other on particular tyrosine residues (see Figure 4.26). Thus, the binding of the paracrine factor to its RTK causes a cascade of autophosphorylation of the cytoplasmic domain of the receptor partners. The phosphorylated tyrosine on the receptor is then recognized by an adaptor protein that serves as a bridge linking the phosphorylated RTK to a powerful intracellular signaling system.

While binding to the phosphorylated RTK through one of the RTK's cytoplasmic domains, the adaptor protein also activates a G protein, such as Ras. Normally, the G protein is in an inactive, GDP-bound state. The activated receptor stimulates the adaptor protein to activate the **GTP exchange factor** (**GEF**; also called **guanine nucleotide releasing factor**, or **GNRP**). GEF catalyzes the exchange of GDP with GTP. The GTP-bound G protein is an active form that transmits the signal to the next molecule. After the signal is delivered, the GTP on the G protein is hydrolyzed back into GDP. This catalysis is greatly stimulated by the complexing of the Ras protein with the **GTPase-activating protein** (**GAP**). In this way, the G protein is returned to its inactive state, where it can await further signaling. Without the GAP protein, Ras protein cannot efficiently

catalyze GTP and so remains in its active configuration for a longer time (Cales et al. 1988; McCormick 1989). Mutations in the *RAS* gene account for a large proportion of cancerous human tumors (Shih and Weinberg 1982), and the mutations of *RAS* that make it oncogenic all inhibit the binding of the GAP protein.

The active Ras G protein associates with a kinase called Raf. The G protein recruits the inactive Raf kinase to the cell membrane, where it becomes active (Leevers et al. 1994; Stokoe et al. 1994). Raf kinase activates the MEK protein by phosphorylating it. MEK is itself a kinase, which activates the ERK protein by phosphorylation. In turn, ERK is a kinase that enters the nucleus and phosphorylates certain transcription factors, many of which belong to the Pea3/Etv4-subfamily (Raible and Brand 2001; Firnberg and Neubüser 2002; Brent and Tabin 2004; Willardsen et al. 2014). The end point of the RTK-signaling pathway is the regulation of expression of a variety of different genes, including but not limited to ones involved in the cell cycle.

FGFs and the JAK-STAT pathway

Fibroblast growth factors can also activate the JAK-STAT cascade. This pathway is extremely important in the differentiation of blood cells, the growth of limbs, and the activation of the casein gene during milk production (**FIGURE 4.27**; Briscoe et al. 1994; Groner and Gouilleux 1995). The cascade starts when a paracrine factor is bound by the extracellular domain of a receptor that spans the cell membrane, with the cytoplasmic domain of the receptor being linked to **JAK** (*Ja*nus *k*inase) proteins. The binding of paracrine factor to the receptor activates the JAK kinases and causes them to phosphorylate the **STAT** (signal *t*ransducers and *a*ctivators of *t*ranscription) family of transcription factors (Ihle 1996, 2001). The phosphorylated STAT is a transcription factor that can now enter into the nucleus and bind to its enhancers.

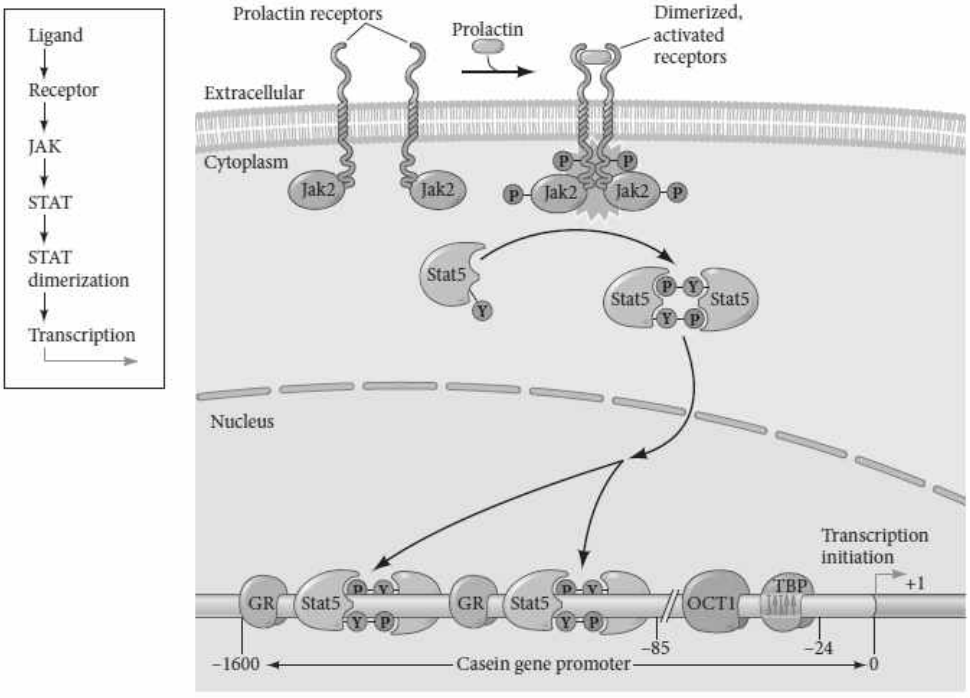


FIGURE 4.27 A JAK-STAT pathway: casein gene activation. The gene for casein is activated during the final (lactogenic) phase of mammary gland development, and its activating signal is the secretion of the hormone prolactin from the anterior pituitary gland. Prolactin causes the dimerization of prolactin receptors in the mammary duct epithelial cells. A particular JAK protein (Jak2) is "hitched" to the cytoplasmic domain of these receptors. When the receptors bind prolactin and dimerize, the JAK proteins phosphorylate each other and the dimerized receptors, activating the dormant kinase activity of the receptors. The activated receptors add a phosphate group to a tyrosine residue (Y) of a particular STAT protein, which in this case is Stat5. This addition allows Stat5 to dimerize, be translocated into the nucleus, and bind to particular regions of DNA. In combination with other transcription factors (which presumably have been waiting for its arrival), the Stat5 protein activates transcription of the casein gene. GR is the glucocorticoid receptor, OCT1 is a general transcription factor, and TBP is the major promoter-binding protein that anchors RNA polymerase II (see Chapter 2) and is responsible for binding RNA polymerase II. A simplified diagram is shown on the left. (For details, see Groner and Gouilleux 1995.)

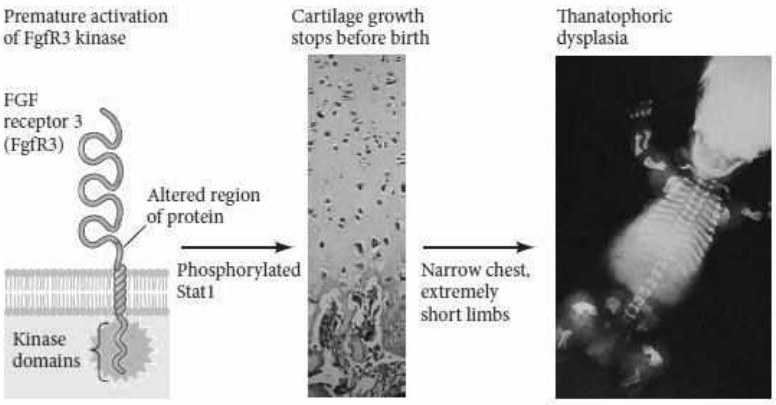


FIGURE 4.28 A mutation in the gene for FgfR3 causes the premature constitutive activation of the STAT pathway and the production of phosphorylated Stat1 protein. This transcription factor activates genes that cause the premature termination of chondrocyte cell division. The result is thanatophoric dysplasia, a condition of failed bone growth that results in the death of the newborn infant because the thoracic cage cannot expand to allow breathing. (After Gilbert-Barness and Opitz 1996.)

The JAK-STAT pathway is critically important in regulating human fetal bone growth. Mutations that prematurely activate the STAT pathway have been implicated in some severe forms of dwarfism, such as the lethal condition thanatophoric dysplasia, in which the growth plates of the rib and limb bones fail to proliferate. The short-limbed newborn dies because its ribs cannot support breathing. The genetic lesion responsible is in *FGFR3*, the gene encoding fibroblast growth factor receptor 3 (**FIGURE 4.28**; Rousseau et al. 1994; Shiang et al. 1994). *FGFR3* is expressed in the cartilage precursor cells (chondrocytes) in the growth plates of the long bones. Normally, the FgfR3 protein (a receptor tyrosine kinase) is activated by a fibroblast growth factor and signals the chondrocytes to stop dividing and begin differentiating into cartilage. This signal is mediated by the Stat1 protein, which is phosphorylated by activated FgfR3 and then translocated into the nucleus. Inside the nucleus, Stat1 activates the genes encoding a cell cycle inhibitor, the p21 protein (Su et al. 1997). Thus, the mutations causing thanatophoric dwarfism result from a gain-of-function mutation in the *FGFR3* gene. The mutant receptor gene is active constitutively; that is, it is without the need to be activated by an FGF signal (Deng et al. 1996; Webster and Donoghue 1996). Chondrocytes stop proliferating shortly after they are formed and the bones fail to grow. Other mutations that activate *FGFR3* prematurely but to a lesser degree produce achondroplasic (short-limbed) dwarfism (Legeai-Mallet et al. 2004).

SCIENTISTS SPEAK 4.3 Dr. Francesca Mariani talks about the role of FGF signaling during limb bud outgrowth.

WEB TOPIC 4.2 FGF RECEPTOR MUTATIONS Mutations of human FGF receptors have been associated with several skeletal malformation syndromes, including syndromes in which skull, rib, or limb cartilages fail to grow or differentiate.

The Hedgehog family

The proteins of the **Hedgehog family** of paracrine factors are multifunctional signaling proteins that act in the embryo through signal transduction pathways to induce particular cell types and through other means to influence cell guidance. The original *hedgehog* gene was found in *Drosophila*, in which genes are named after their mutant phenotypes: the loss-of-function *hedgehog* mutation causes the fly larva to be covered with pointy denticles on its cuticle (hair-like structures), thus resembling a hedgehog. Vertebrates have at least three homologues of the *Drosophila hedgehog* gene: *sonic hedgehog* (*shh*), *desert hedgehog* (*dhh*), and *indian hedgehog* (*ihh*). The Desert hedgehog protein is found in the Sertoli cells of the testes, and mice homozygous for a null allele of *dhh* exhibit defective spermatogenesis. Indian hedgehog is expressed in the gut and cartilage and is important in postnatal

bone growth (Bitgood and McMahon 1995; Bitgood et al. 1996). Sonic hedgehog[6] has the greatest number of functions of the three vertebrate Hedgehog homologues. Among other important functions, Sonic hedgehog is responsible for assuring that motor neurons come only from the ventral portion of the neural tube (see Chapter 13), that a portion of each somite forms the vertebrae (see Chapter 17), that the feathers of the chick form in their proper places (see Figure 4.19), and that our pinkies are always our most posterior digits (see Chapter 19). Hedgehog signaling is capable of regulating these many developmental events because they function as morphogens; Hedgehog proteins are secreted from a cellular source, displayed in a spatial gradient, and induce differential gene expression at different threshold concentrations that result in distinct cell identities.

HEDGEHOG SECRETION Different modes of processing and assembly of Hedgehog proteins can significantly alter the amount secreted and the gradient that is formed (**FIGURE 4.29**). By cleaving off its carboxyl terminus and associating with both cholesterol and palmitic acid moieties, Hedgehog protein can be processed and secreted as monomers or multimers, packaged as lipoprotein assemblies, or even transported out of the cell within exovesicles.

In the mouse limb bud, it was shown that if Shh lacks the cholesterol modification, it diffuses too quickly and dissipates into the surrounding space (Li et al. 2006). These lipid modifications are also required for stable concentration gradients of Hedgehog and pathway activation. Through these varied protein processing and transport mechanisms, stable gradients of Hedgehog can be established over distances of several hundred microns (about 30 cell diameters in the mouse limb).

[6]Yes, it is named after the Sega Genesis character. The vertebrate *Hedgehog* genes were discovered by searching vertebrate gene libraries (chick, rat, zebrafish) with probes that found sequences similar to that of the fruit fly *hedgehog* gene. Riddle and colleagues (1993) discovered three genes homologous to *Drosophila hedgehog*. Two were named after existing species of hedgehog, and the third was named after the animated character. Two other *hedgehog* genes, found only in fish, were originally named *echidna hedgehog* (possibly after Sonic's cartoon friend) and *tiggywinkle hedgehog* (after Beatrix Potter's fictional hedgehog), but they are now referred to as *ihh-b* and *shh-b*, respectively.

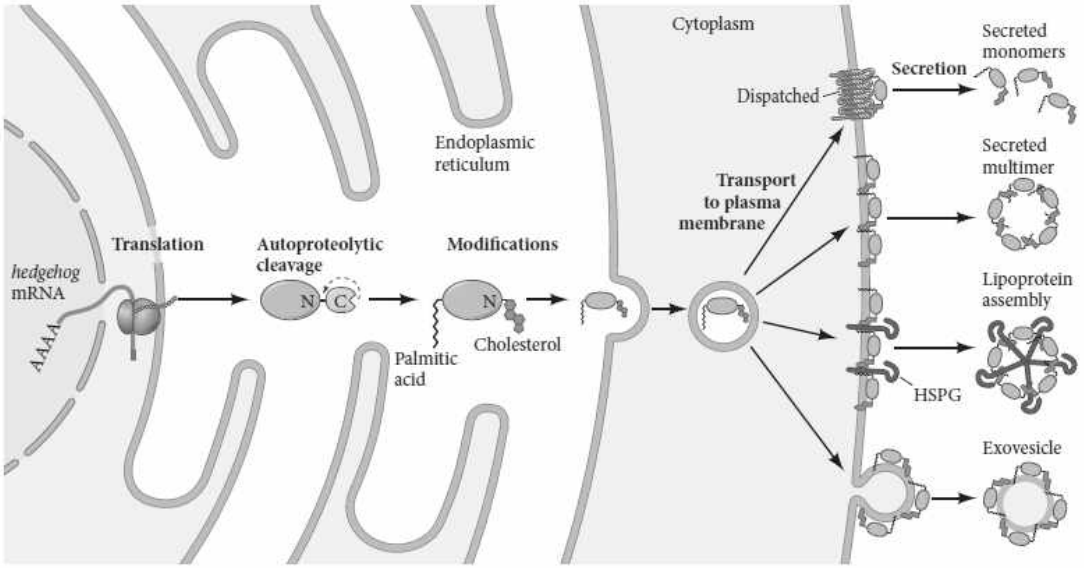


FIGURE 4.29 Hedgehog processing and secretion. Translation of the *hedgehog* gene in the endoplasmic reticulum produces a Hedgehog protein with autoproteolytic activity that cleaves off the carboxyl terminus (C) to reveal a signal sequence that marks the protein for secretion. The freed C-terminal segment is not involved in signaling and is often degraded, whereas the amino-terminal portion (N) of the molecule becomes the active Hedgehog protein intended for secretion. Secretion requires the addition of cholesterol and palmitic acid to the Hedgehog protein (Briscoe and Thérond 2013). Interactions between the cholesterol moiety and a transmembrane protein called Dispatched enables Hedgehog to be secreted and diffuse as monomers; both cholesterol and palmitic acid are required for multimeric assembly. In addition, Hedgehog interactions with a class of membrane-associated heparan sulfate proteoglycans (HSPGs) foster the congregation and secretion of Hedgehog molecules as lipoprotein assemblies (Breitling 2007; Guerrero and Chiang 2007). Similar clustering of Hedgehog can be used to transport Hedgehog out of the cell within exovesicles.

THE HEDGEHOG PATHWAY The cholesterol moiety on Hedgehog is not only important for modulating its extracellular transport; it is also critical for Hedgehog to anchor to its receptor on the receiving cell's plasma membrane (Grover et al. 2011). The Hedgehog binding receptor is called Patched, which is a large, 12-pass transmembrane protein (**FIGURE 4.30**). Patched, however, is not a signal transducer. Rather, the Patched protein represses the function of another transmembrane receptor called Smoothened. *In the absence of Hedgehog* binding to Patched, Smoothened is inactive and degraded, and a

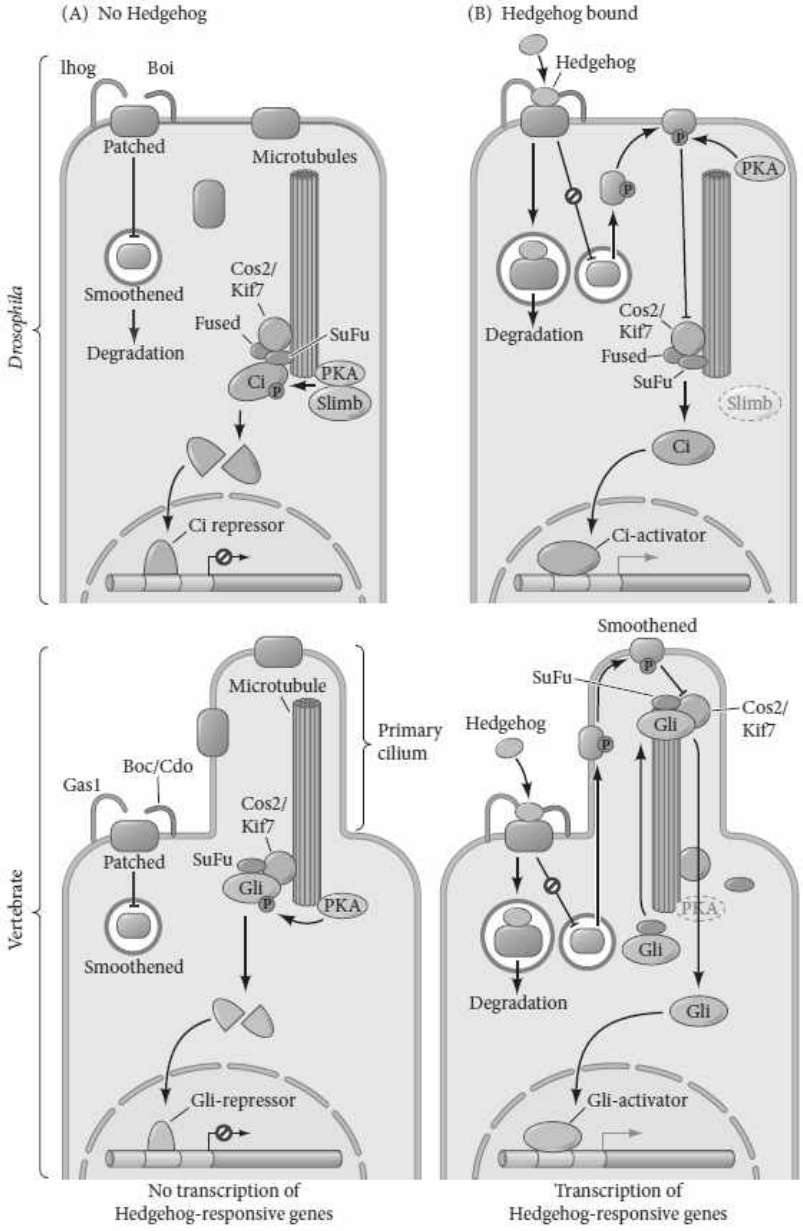


FIGURE 4.30 Hedgehog signal transduction pathway. Patched protein in the cell membrane is an inhibitor of the Smoothened protein. (A) In the absence of Hedgehog binding to Patched, Patched inhibits Smoothened and in *Drosophila melanogaster* the Ci protein remains tethered to the microtubules by the Cos2 and Fused proteins. This tether allows the proteins PKA and Slimb to cleave Ci into a transcriptional repressor that blocks the transcription of particular genes. (B) When Hedgehog binds to Patched, its conformational changes release the inhibition of the Smoothened protein. Smoothened then releases Ci from the microtubules, inactivating the cleavage proteins PKA and Slimb. The Ci protein enters the nucleus, and acts as a transcriptional activator of particular genes. In vertebrates (lower panels), the homologues of Ci are the *Gli* genes, which function similarly as transcriptional activators or repressors when a hedgehog ligand is bound to Patched or absent, respectively. Additionally in vertebrates, for Smoothened to positively regulate Gli processing into an activator form, it needs to gain access into the primary cilium—hedgehog ligand binding to patched enables the transport of Smoothened into the primary cilium. Lastly, several co-receptors such as Gas1 and Boc function to enhance hedgehog signaling. (After Johnson and Scott 1998; Briscoe and Thérond 2013; Yao and Chuang 2015.)

transcription factor—Cubitus interruptus (Ci) in *Drosophila* or one of its vertebrate homologues Gli1, Gli2, and Gli3—is tethered to the microtubules of the responding cell. Although tethered to the microtubules, Ci/Gli is cleaved in such a way that a portion of it enters the nucleus and acts as a transcriptional repressor. This cleavage reaction is catalyzed by several proteins that include Fused, Suppressor of fused (SuFu), and Protein kinase A (PKA). *When Hedgehog is present*, the responding cells express several additional co-receptors (Ihog/Cdo, Boi/Boc, and Gas1) that together foster strong Hedgehog-Patched interactions. Upon binding, the Patched protein's shape is altered such that it no longer inhibits Smoothened, and Patched enters an endocytic pathway for degradation. Smoothened releases Ci/Gli from the microtubules (probably by phosphorylation), and the full-length Ci/Gli protein can now enter the nucleus to act as a transcriptional *activator* of the same genes the cleaved Ci/Gli used to repress (see Figure 4.30; Yao and Chuang 2015; Briscoe and Thérond 2013; Lum and Beachy 2004).

There are other targets for Hedgehog signaling independent of Gli transcription factors, and they involve the fast remodeling of the actin cytoskeleton, resulting in directed migration of the responding cells. For instance, the Charron lab has shown that pathfinding axons in the neural tube can sense the presence of a gradient of Sonic hedgehog emanating from the floorplate, which will serve to attract commissural neurons to turn toward the midline and cross to the other hemisphere of the nervous system (Yam et al. 2009; Sloan et al. 2015). We will discuss the mechanisms of axon guidance in greater detail in Chapter 15.

The Hedgehog pathway is extremely important in vertebrate limb patterning, neural differentiation and pathfinding, retinal and pancreas development, and craniofacial morphogenesis, among many other processes (**FIGURE 4.31A**; McMahon et al. 2003). When mice were made homozygous for a mutant allele of *Sonic hedgehog*, they had major limb and facial abnormalities. The midline of the face was severely reduced, and a single eye formed in the center of the forehead, a condition known as cyclopia after the one-eyed Cyclops of Homer's *Odyssey* (**FIGURE 4.31B**; Chiang et al. 1996). Some human cyclopia syndromes are caused by mutations in genes that encode either Sonic hedgehog or the enzymes that synthesize cholesterol (Kelley et al. 1996; Roessler et al. 1996; Opitz and Furtado 2013). Moreover, certain chemicals that induce cyclopia do so by interfering with the Hedgehog pathway (Beachy et al. 1997; Cooper et al. 1998). Two teratogens[7] known to cause cyclopia in vertebrates are jervine and cyclopamine. Both are alkaloids found in the plant *Veratrum californicum* (corn lily), and both directly bind to and inhibit Smoothened function (see Figure 4.31B; Keeler and Binns 1968).

In later development, Sonic hedgehog is critical for feather formation in the chick embryo, for hair formation in mammals, and, when misregulated, for the formation of

[7]A *teratogen* is an exogenous compound capable of causing malformations in embryonic development; see Chapters 1 and 24.

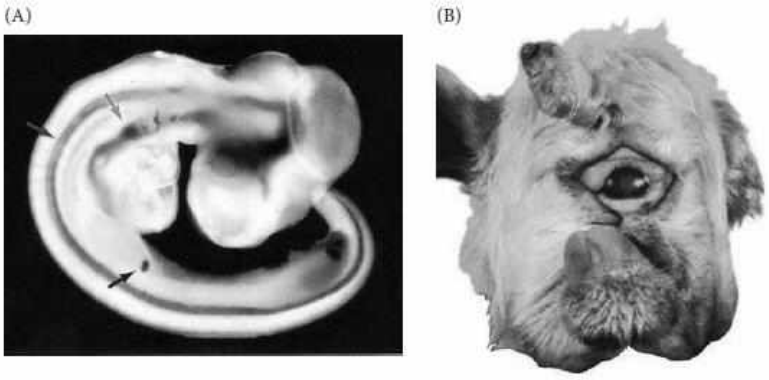


FIGURE 4.31 (A) Sonic hedgehog is shown by in situ hybridization to be expressed in the nervous system (red arrow), gut (blue arrow), and limb bud (black arrow) of a 3-day chick embryo. (B) Head of a cyclopic lamb born of a ewe that ate *Veratrum californicum* early in pregnancy. The cerebral hemispheres fused, resulting in the formation of a single, central eye and no pituitary gland. The jervine alkaloid made by this plant inhibits cholesterol synthesis, which is needed for Hedgehog production and reception. (A courtesy of C. Tabin; B courtesy of L. James and USDA Poisonous Plant Laboratory.)

skin cancer in humans (Harris et al. 2002; Michino et al. 2003). Although mutations that inactivate the Hedgehog pathway can cause malformations, mutations that activate the pathway ectopically can have mitogenic effects and cause cancers. If the Patched protein is mutated in somatic tissues such that it can no longer inhibit Smoothened, it can cause tumors of the basal cell layer of the epidermis (basal cell carcinomas). Heritable mutations of the *patched* gene cause basal cell nevus syndrome, a rare autosomal dominant condition characterized by both developmental anomalies (fused fingers; rib and facial abnormalities) and multiple malignant tumors (Hahn et al. 1996; Johnson et al. 1996). Interestingly, vismodegib, a compound that inhibits Smoothened function similar to cyclopamine, is currently in clinical trials as a therapy to combat basal cell carcinomas (Dreno et al. 2014; Erdem et al. 2015). (What do you think the warnings for pregnancy should be on this drug?)

VADE MECUM

The segment on zebrafish development demonstrates how alcohol can induce cyclopia in these embryos.

SCIENTISTS SPEAK 4.4 Dr. James Briscoe answers questions on the role of Hedgehog signaling during neural tube development.

SCIENTISTS SPEAK 4.5 Dr. Marc Tessier-Lavigne speaks on the role of Hedgehog as a noncanonical axon guidance cue.

The Wnt family

The Wnts are paracrine factors that make up a large family of cysteine-rich glycoproteins with at least 11 conserved Wnt members among vertebrates (Nusse and Varmus 2012); 19 separate *Wnt* genes are found in humans![8] The Wnt family was originally discovered and named *wingless* during a forward genetic screen in *Drosophila melanogaster* in 1980 by Christiane Nüsslein-Volhard and Eric Wieschaus, when mutations in this locus prevented the formation of the wing. The Wnt name is a fusion of the *Drosophila* segment polarity gene *wingless* with the name of one of its vertebrate homologues, *integrated*. The enormous array of different *Wnt* genes across species speaks to their importance in an equally large number of developmental events. For example, Wnt proteins are critical in establishing the polarity of insect and vertebrate limbs, in promoting the proliferation of stem cells, in regulating cell fates along axes of various tissues, in development of the mammalian urogenital system (**FIGURE 4.32**), and in guiding the migration of mesenchymal cells and pathfinding axons. How is it that Wnt signaling is capable of mediating such diverse processes as cell division, cell fate, and cell guidance?

WNT SECRETION Similar to the building of the functional Hedgehog proteins, Wnt proteins are synthesized in the endoplasmic reticulum and modified by the addition of lipids (palmitic and palmitoleic acid). These lipid modifications are catalyzed by the *O*-acetyltransferase Porcupine. (*How do you think this enzyme received this name?*[9]) It is interesting that loss of the *Porcupine* gene results in reduced Wnt secretion paired with its build up in the endoplasmic reticulum (van den Heuvel et al. 1993; Kadowaki et al. 1996), indicating that adding lipids to Wnt is important for transporting it to the plasma membrane. Once at the plasma membrane, Wnt can be secreted by the same mechanisms we saw for Hedgehog

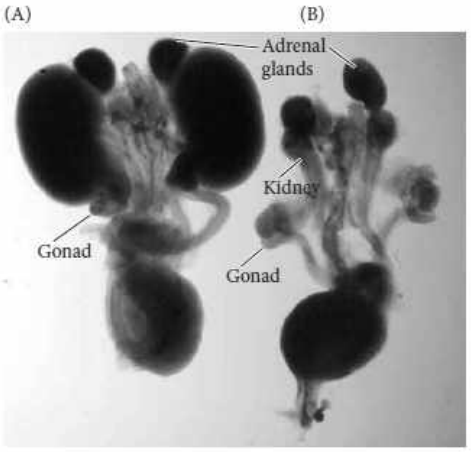


FIGURE 4.32 Wnt4 is necessary for kidney development and for female sex determination. (A) Urogenital rudiment of a wild-type newborn female mouse. (B) Urogenital rudiment of a newborn female mouse with targeted knockout of *Wnt4* shows that the kidney fails to develop. In addition, the ovary starts synthesizing testosterone and becomes surrounded by a modified male duct system. (Courtesy of J. Perasaari and S. Vainio.)

[8] A comprehensive summary of all the Wnt proteins and Wnt signaling components can be found at http://web.stanford.edu/group/nusselab/cgi-bin/wnt/.

[9] In flies, the mutated *Porcupine* gene results in segmentation defects creating denticles resembling porcupine spines in the larva (Perrimon et al. 1989). Do you recall the naming of Hedgehog? Porcupine is specific to Wnt palmitoylation, whereas Hedgehog is palmitoylated by a similar enzyme called Hhat.

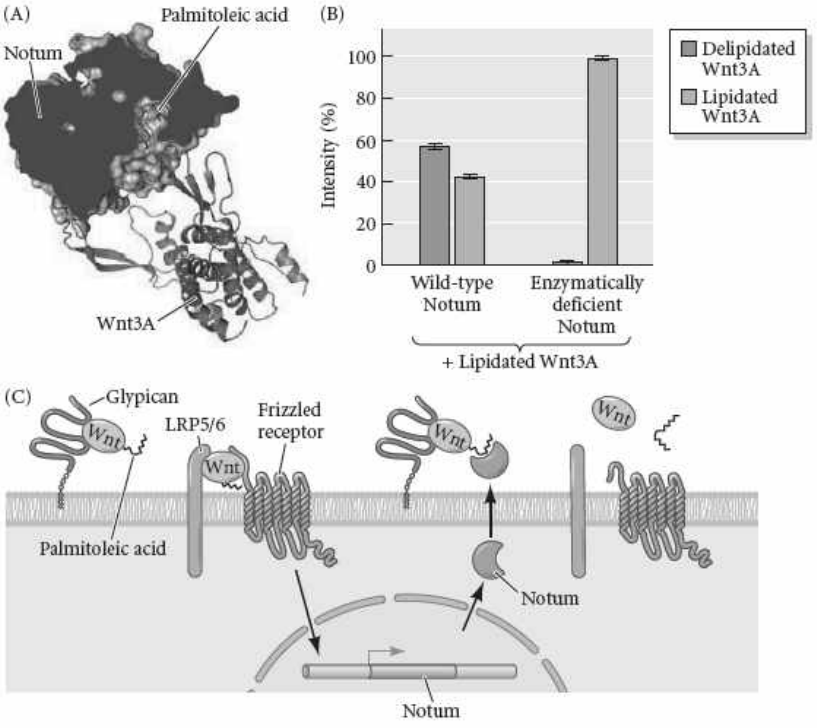
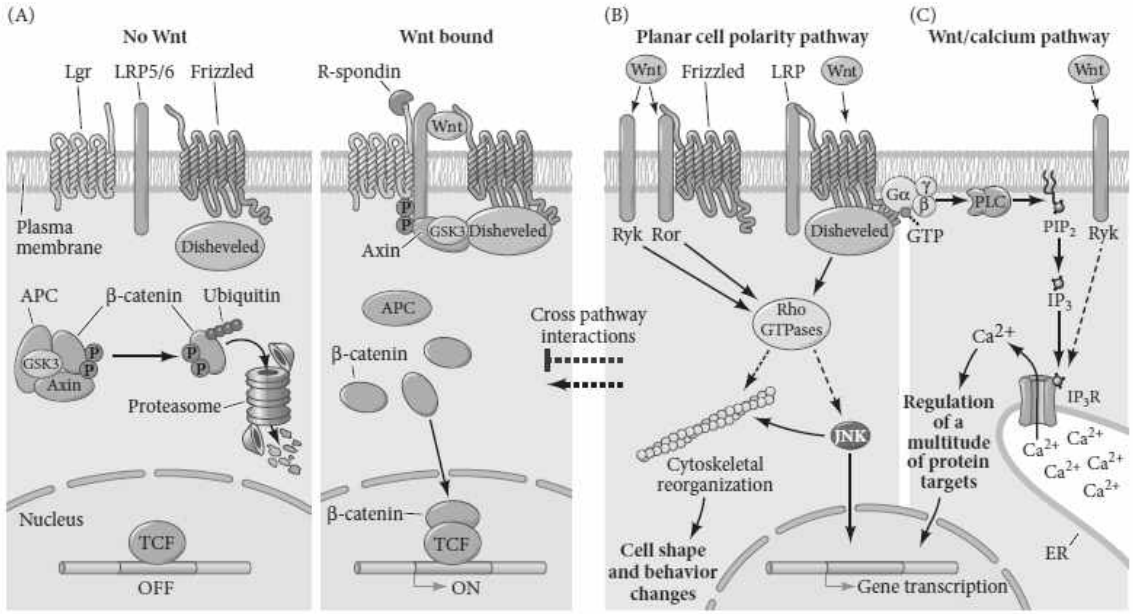


FIGURE 4.33 Notum antagonism of Wnt. (A) Structures of Notum (gray) and Wnt3A (green) bound together. The active site of Notum is visualized in this cutaway view demonstrating the precise binding with the palmitoleic acid moiety of Wnt3A (orange). (B) Once bound, Notum possesses the enzymatic hydrolase activity to cleave this lipid off of Wnt3A, rendering it unable to interact with the Frizzled receptor. The data shown here demonstrate the requirement of this hydrolase function for appropriate delipidation of Wnt3A. Notum lacking its enzymatic activity is unable to remove the lipid group from Wnt3A (Delipidated, purple bars) as compared to wild-type Notum. (C) Model of extracellular regulation of Wnt. Lipidated Wnt can bind both its Frizzled receptor and glypicans (heparan sulfate proteoglycans). Active Wnt signaling leads to the upregulation of Notum, which is secreted and interacts with glypicans, where it will also bind to and cleave off the palmitoleic acid portions of Wnt proteins. In this way, Wnt signaling leads to a Notum-mediated negative feedback mechanism. (A created by Matthias Zebisch; data from Kakugawa et al. 2015; courtesy of Yvonne Jones and Jean-Paul Vincent.)

protein: by free diffusion, by being transported in exosomes, or by being packaged in lipoprotein particles (Tang et al. 2012; Saito-Diaz et al. 2013; Solis et al. 2013).

Upon secretion, Wnt proteins associate with glypicans (a type of heparan sulfate proteoglycan) in the extracellular matrix, which restricts diffusion and leads to a greater accumulation of Wnt closer to the source of production. When Wnt attaches to the Frizzled receptor on a responding cell, the cell secretes Notum, a hydrolase that associates with glypican and then cleaves off Wnt's attached lipids in a process of *deacylation* or *delipidation* (Kakugawa et al. 2015). This process reduces Wnt signaling because the lipids are essential for Wnt to bind to Frizzled, which creates a negative feedback mechanism for preventing excessive Wnt signaling. The Frizzled receptor possesses a unique hydrophobic cleft adapted to interact with lipidated Wnts, a binding conformation mimicked in Notum's structure as well (**FIGURE 4.33A,B**). Overexpression of Notum in the *Drosophila* imaginal wing disc causes a reduction in Wnt/Wg target gene expression; in contrast, clonal loss of *Notum* yields to expanded Wnt target gene expression. Interestingly, *Notum* gene expression is upregulated in Wnt-responsive cells, creating a mechanism of negative feedback (**FIGURE 4.33C**; Kakugawa et al. 2015; Nusse 2015). Notum is not alone in functioning to inhibit binding of Wnt to its receptor; numerous antagonists exist, including the Secreted frizzled-related protein (Sfrp), Wnt inhibitory factor (Wif), and members of the Dickkopf (Dkk) family (Niehrs 2006). Together, the multiple modes of Wnt secretion, glypican-mediated restriction, secreted ligand inhibitors, and negative feedback establish stable gradients of Wnt ligands and pathway response.

THE CANONICAL WNT PATHWAY (β-CATENIN DEPENDENT) The first Wnt pathway to be characterized was the canonical "Wnt/β-catenin pathway," which represents the signaling events that culminate in the activation of the β-catenin transcription factor and modulation of specific gene expression (**FIGURE 4.34A**; Chien et al. 2009; Clevers



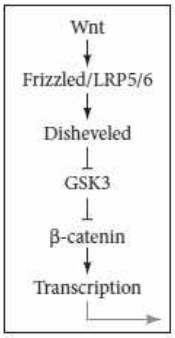


FIGURE 4.34 Wnt signal transduction pathways. (A) The canonical, or β-catenin-dependent, Wnt pathway. The Wnt protein binds to its receptor, a member of the Frizzled family, but it often does so in combination with interactions with LRP5/6 and Lgr receptors. During periods of Wnt absence, β-catenin interacts with a complex of proteins, including GSK3, APC, and Axin, that target Wnt for protein degradation in the proteasome. The downstream transcriptional effector of Wnt signaling is the β-catenin transcription factor. In the presence of certain Wnt proteins, Frizzled then activates Disheveled, allowing Disheveled to become an inhibitor of glycogen synthase kinase 3 (GSK3). GSK3, if it were active, would prevent the dissociation of β-catenin from the APC protein. So, by inhibiting GSK3, the Wnt signal frees β-catenin to associate with it's co-factors (LEF or TCF) and become an active transcription factor. (B,C) Alternatively, noncanonical (β-catenin-independent) Wnt signaling pathways can regulate cell morphology, division, and movement. (B) Certain Wnt proteins can similarly signal through Frizzled to activate Disheveled but in a way that leads to the activation of Rho GTPases, like Rac and RhoA. These GTPases coordinate changes in cytoskeleton organization and also through janus kinase (JNK) regulate gene expression. (C) In a third pathway, certain Wnt proteins activate Frizzled and Ryk receptors in a way that releases calcium ions and can result in Ca^{2+}-dependent gene expression. (After MacDonald et al. 2009.)

and Nusse 2012; Nusse 2012; Saito-Diaz et al. 2013). In Wnt/β-catenin signaling, lipidated Wnt family members interact with a pair of transmembrane receptor proteins: one from the Frizzled family and one large transmembrane protein called LRP5/6 (Logan and Nusse 2004; MacDonald et al. 2009). *In the absence of Wnts,* the transcriptional cofactor β-catenin is constantly being degraded by a protein degradation complex containing several proteins (such as axin and APC) as well as **glycogen synthase kinase 3** (**GSK3**). GSK3 phosphorylates β-catenin so that it will be recognized and degraded by proteosomes. The result is Wnt-responsive genes being repressed by the LEF/TCF transcription factor, which functionally complexes with at least two other proteins, including a histone deacetylase.

When Wnts come into contact with a cell, they bring together the Frizzled and LRP5/6 receptors to form a multimeric complex. This linkage enables LRP5/6 to bind both Axin and GSK3, and enables the Frizzled protein to bind Disheveled—all of which occurs on the intracellular side of the plasma membrane. Disheveled keeps Axin and GSK3 bound to the cell membrane and thereby prevents β-catenin from being phosphorylated by GSK3. This process stabilizes β-catenin, which accumulates and enters the nucleus (see Figure 4.34A). Here it binds to the LEF/TCF transcription factor and converts this former repressor

into a transcriptional activator, thereby activating Wnt-responsive genes (Cadigan and Nusse 1997; Niehrs 2012).

This model is undoubtedly an oversimplification because different cells use the pathway in different ways (see McEwen and Peifer 2001; Clevers and Nusse 2012; Nusse 2012; Saito-Diaz et al. 2013). One overriding principle already evident in both the Wnt and Hedgehog pathways, however, is that *activation is often accomplished by inhibiting an inhibitor.*

THE NONCANONICAL WNT PATHWAYS (β-CATENIN INDEPENDENT) In addition to sending signals to the nucleus, Wnt proteins can also cause changes within the cytoplasm that influence cell function, shape, and behavior. These alternative or *noncanonical* pathways can be divided into two types: the "planar cell polarity" pathway and the "Wnt/calcium" pathway (**FIGURE 4.34B,C**). The planar cell polarity, or PCP, pathway functions to regulate the actin and microtubule cytoskeleton, thus influencing cell shape, and often results in bipolar protrusive behaviors necessary for a cell to migrate. Certain Wnts (such as Wnt5a and Wnt11) can activate Disheveled by binding to a different receptor (Frizzled paired with Ror instead of Lrp5), and this Ror receptor complex phosphorylates Disheveled in a way that allows it to interact with Rho GTPases (Grumolato et al. 2010; Green et al. 2014). Rho GTPases are colloquially viewed as the "master builders" of the cell because they can activate an array of other proteins (kinases and cytoskeletal binding proteins) that remodel cytoskeletal elements to alter cell shape and movement. Wnt signaling through the PCP pathway is most notable for instructing cell behaviors along the same spatial plane within a tissue and hence is called *planar polarity*. Wnt/PCP signaling through cytoskeleton control can direct cells to divide in the same plane (rather than forming upper and lower tissue compartments) and to move within that same plane (Shulman et al. 1998; Winter et al. 2001; Ciruna et al. 2006; Witte et al. 2010; Sepich et al. 2011; Ho et al. 2012; Habib et al. 2013). In vertebrates, this regulation of cell division and migration is important for establishing germ layers and for anterior-posterior axis extension during gastrulation and neurulation.

As its name implies, the Wnt/calcium pathway leads to the release of calcium stored within cells, and this released calcium acts as an important *secondary messenger* to modulate the function of many downstream targets. In this pathway, Wnt binding to the receptor protein (possibly Ryk, alone or in concert with Frizzled) activates a phospholipase (PLC) whose enzyme activities release a compound that in turn releases calcium ions from the smooth endoplasmic reticulum (see Figure 4.34C). The released calcium can activate enzymes, transcription factors, and translation factors. In zebrafish, Ryk deficiency impairs Wnt-directed calcium release from internal stores and as a result impairs directional cell movement (Lin et al. 2010; Green et al. 2014). Ryk has been demonstrated to be cleaved and transported into the nucleus, where it plays roles in mammalian neural development and *C. elegans* vulval development (Lyu et al. 2008; Poh et al. 2014).

Although each of the three Wnt pathways—β-catenin, PCP, and calcium—possess primary functions that are different from one another, mounting evidence suggests that there are significant cross interactions between these pathways (van Amerongen and Nusse 2009; Thrasivoulou et al. 2013). For instance, Wnt5-mediated calcium signaling has been shown to *antagonize* the Wnt/β-catenin pathway during vertebrate gastrulation and limb development (Ishitani et al. 2003; Topol et al. 2003; Westfall et al. 2003).

Developing Questions

How different are the pathways of Wnt/β-catenin, calcium, and PCP? Arguably the most significant challenge to understanding Wnt signaling is figuring out how the different pathways interact. Perhaps we need a more integrated comprehension of signal transduction, one that can predict interactions not only between canonical and noncanonical Wnt signaling pathways, but among those for all the paracrine factors (Wnt, Hedgehog, FGF, BMP, etc.). What do you think? How would you go about trying to examine meaningful pathway interactions?

The TGF-β superfamily

There are more than 30 structurally related members of the **TGF-β superfamily**,[10] and they regulate some of the most important interactions in development (**FIGURE 4.35**). The TGF-β superfamily includes the TGF-β family, the Nodal and activin families, the

[10]TGF stands for Transforming Growth Factor. The designation "superfamily" is often applied when each of the different classes of molecules constitutes a family. The members of a superfamily all have similar structures but are not as similar as the molecules within each family are to one another.

bone morphogenetic proteins (BMPs), the Vg1 family, and other proteins, including glial-derived neurotrophic factor (GDNF; necessary for kidney and enteric neuron differentiation) and anti-Müllerian hormone (AMH), a paracrine factor involved in mammalian sex determination. Below we summarize three of these families most widely used throughout development: TGF-βs, BMPs and Nodal/Activin.

- Among members of the **TGF-β family**, TGF-β1, 2, 3, and 5 are important in regulating the formation of the extracellular matrix between cells and for regulating cell division (both positively and negatively). TGF-β1 increases the amount of extracellular matrix that epithelial cells make (both by stimulating collagen and fibronectin synthesis and by inhibiting matrix degradation). TGF-β proteins may be critical in controlling where and when epithelia branch to form the ducts of kidneys, lungs, and salivary glands (Daniel 1989; Hardman et al. 1994; Ritvos et al. 1995). The effects of the individual TGF-β family members are difficult to sort out because members of the TGF-β family appear to function similarly and can compensate for losses of the others when expressed together.
- The members of the **BMP family** can be distinguished from other members of the TGF-β superfamily by having seven (rather than nine) conserved cysteines in the mature polypeptide. Because they were originally discovered by their ability to induce bone formation, they were given the name **bone morphogenetic proteins**. It turns out, though, that bone formation is only one of their many functions; the BMPs are extremely multifunctional.[11] They have been found to regulate cell division, apoptosis (programmed cell death), cell migration, and differentiation (Hogan 1996). They include proteins such as BMP4 (which in some tissues causes bone formation, in other tissues specifies epidermis, and in other instances causes cell proliferation or cell death) and BMP7 (which is important in neural tube polarity, kidney development, and sperm formation). The BMP4 homologue in *Drosophila* is critically involved in forming appendages, including the limbs, wings, genitalia, and antennae. Indeed, the malformations of 15 such structures have given this homologue the name Decapentaplegic (DPP). As it (rather oddly) turns out, BMP1 is not a member of the BMP family at all; rather, it is a protease. BMPs are thought to work by diffusion from the cells producing them (Ohkawara et al. 2002). Inhibitors such as Noggin and Chordin that bind directly to BMP reduce BMP-receptor interactions. We will cover this morphogenetic mechanism more directly when we discuss dorsoventral axis specification in the gastrula.
- The **Nodal** and **activin** proteins are extremely important in specifying the different regions of the mesoderm and for distinguishing the left and right sides of the vertebrate body axis. The left-right asymmetry of bilateral organisms is strongly influenced by a gradient of Nodal from right to left across the embryo. In vertebrates, this Nodal gradient appears to be created by the beating of motile cilia that promotes the graded flow of Nodal across the midline (Babu and Roy 2013; Molina et al. 2013; Blum et al. 2014; Su 2014).

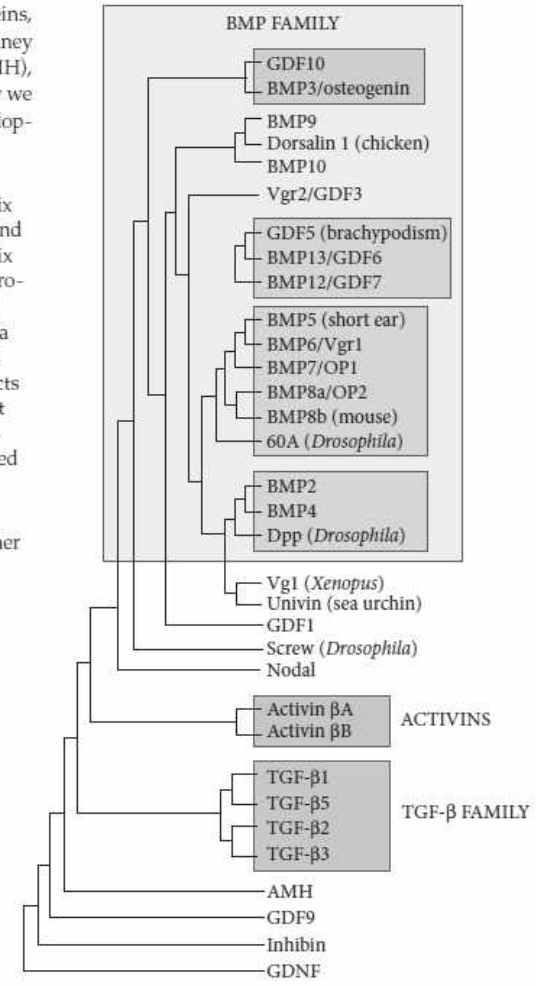


FIGURE 4.35 Relationships among members of the TGF-β superfamily. (After Hogan 1996.)

[11] One of the many reasons humans do not seem to need an enormous genome is that the gene products—proteins—involved in our construction and development often have many functions. Many of the proteins that we are familiar with in adults (such as hemoglobin, keratins, and insulin) do have only one function, which led to the erroneous conclusion that this situation is the norm.

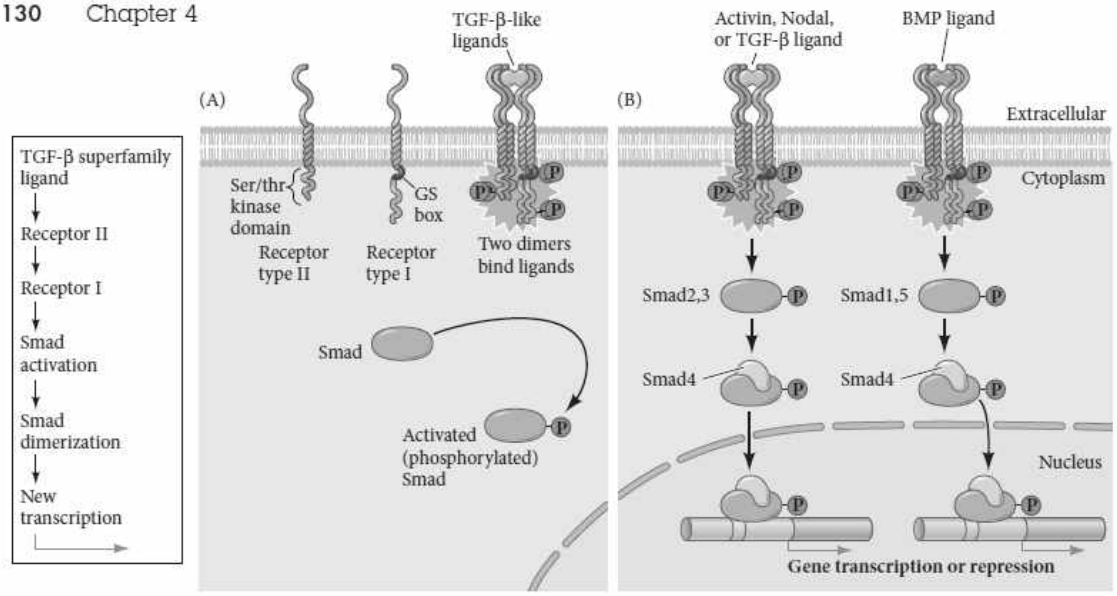


FIGURE 4.36 The Smad pathway is activated by TGF-β superfamily ligands. (A) An activation complex is formed by the binding of the ligand by the type I and type II receptors, which allows the type II receptor to phosphorylate the type I receptor on particular serine or threonine residues. The phosphorylated type I receptor protein can now phosphorylate the Smad proteins. (B) Those receptors that bind TGF-β family proteins or members of the activin family phosphorylate Smads 2 and 3. Those receptors that bind to BMP family proteins phosphorylate Smads 1 and 5. These Smads can complex with Smad4 to form active transcription factors. A simplified version of the pathway is shown on the left.

THE SMAD PATHWAY Members of the TGF-β superfamily activate members of the **Smad family** of transcription factors (Heldin et al. 1997; Shi and Massagué 2003). The TGF-β ligand binds to a type II TGF-β receptor, which allows that receptor to bind to a type I TGF-β receptor. Once the two receptors are in close contact, the type II receptor phosphorylates a serine or threonine on the type I receptor, thereby activating it. The activated type I receptor can now phosphorylate the Smad[12] proteins (**FIGURE 4.36A**). Smads 1 and 5 are activated by the BMP family of TGF-β factors, whereas the receptors binding activin, Nodal, and the TGF-β family phosphorylate Smads 2 and 3. These phosphorylated Smads bind to Smad4 and form the transcription factor complexes that will enter the nucleus (**FIGURE 4.36B**).

Other paracrine factors

Although most paracrine factors are members of one of the four families described above (FGF, Hedgehog, Wnt, or the TGF-β superfamily), some paracrine factors have few or no close relatives. Epidermal growth factor, hepatocyte growth factor, neurotrophins, and stem cell factor are not included among these four groups, but each plays important roles during development. In addition, there are numerous paracrine factors involved almost exclusively with developing blood cells: erythropoietin, the cytokines, and the interleukins. Another class of paracrine factors was first characterized for their role in cell/axon guidance and include members of the Netrin, Semaphorin, and Slit families. These classic guidance molecules (such as netrins) are now being shown to regulate gene expression as well. We will discuss all these paracrine factors in the context of their developmental relevance later in the book.

The Cell Biology of Paracrine Signaling

We have been discussing cell membrane dynamics and cell signaling as if they were two separate entities, but their functioning is closely related. Paracrine factors can rearrange the cell surface, and the cell surface is critical in regulating paracrine factor synthesis, flow, and function. The actions of paracrine signals often change the composition of the cell membrane.

[12]Researchers named the Smad proteins by merging the names of the first identified members of this family: the *C. elegans* SMA protein and the *Drosophila* Mad protein.

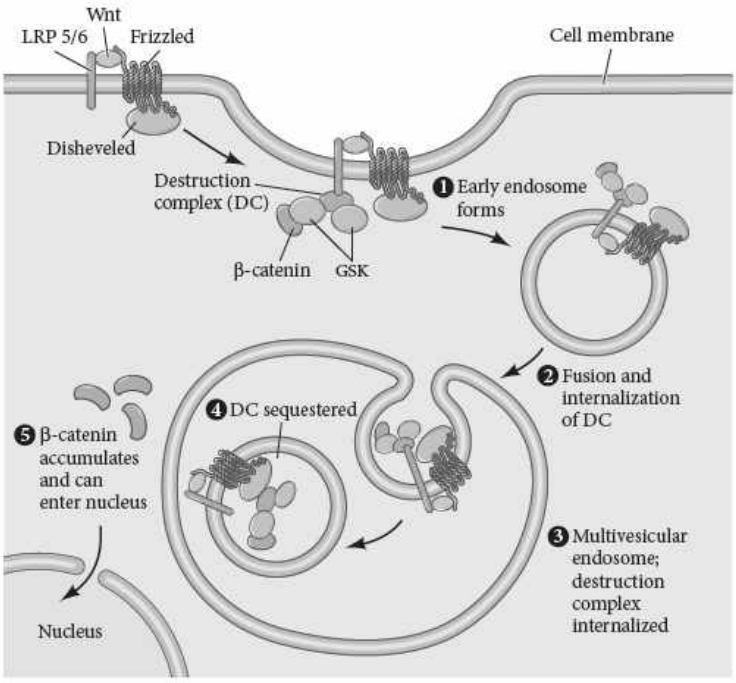


FIGURE 4.37 A Wnt pathway: packaging the β-catenin destruction apparatus into endosomes. A major mechanism for separating β-catenin from enzymes that would otherwise destroy it is to package the complex in membrane-bound vesicles called endosomes. When Wnt binds to Frizzled, Frizzled can bind the destruction complex; the entire complex (including the bound Wnt and its receptor) is internalized, allowing β-catenin to accumulate rather than being degraded. (After Taelman et al. 2010.)

ENDOSOME INTERNALIZATION The type and number of receptors that a cell displays at its cell surface present its potential for response. Endocytosis is one mechanism used to eliminate a receptor at the membrane. Recent studies are revealing that internalization of ligand-receptor complexes into membrane-bound vesicles called **endosomes** is a common mechanism in paracrine signaling. When Wnt binds to its receptors, the β-catenin destruction complex binds to the receptor, and the entire complex (including the receptor and its bound Wnt) is internalized in endosomes (**FIGURE 4.37**; Taelman et al. 2010; Niehrs 2012). This process removes the complex, targets it for degradation, and enables the survival of β-catenin. The internalization of the signaling complex appears to be critical for the accumulation of β-catenin, and proteins that aid in this endocytosis (such as R-spondins; see Figure 4.34) make the Wnt pathway more efficient (Ohkawara et al. 2011). Similarly, Hedgehog-Patched complexes and FGF-FGFR complexes are also internalized in endosomes and targeted for degradation, a process that is required for proper limb development (Briscoe and Thérond 2013; Handschuh et al. 2014; Hsia et al. 2015).

DIFFUSION OF PARACRINE FACTORS Paracrine factors do not flow freely through the extracellular space. Rather, factors can be bound by cell membranes and extracellular matrices of the tissues. In some cases, such binding can impede the spread of a paracrine morphogen and even target the paracrine factor for degradation (Capurro et al. 2008; Schwank et al. 2011). Wnt proteins, for instance, do not diffuse far from the cells secreting them unless helped by other proteins. Thus, the range of Wnt factors is significantly extended when the nearby cells secrete proteins that bind to the paracrine factor and prevent it from binding prematurely to the target tissue (**FIGURE 4.38**; Mulligan et al. 2012). Similarly, as we have mentioned above, **heparan sulfate proteoglycans** (**HSPGs**) in the extracellular matrix often modulate the stability, reception, diffusion rate, and concentration gradient of FGF, BMP, and Wnt proteins (Akiyama et al. 2008; Yan and Lin 2009; Berendsen et al. 2011; Christian 2011; Müller and Schier 2011; Nahmad and Lander 2011).

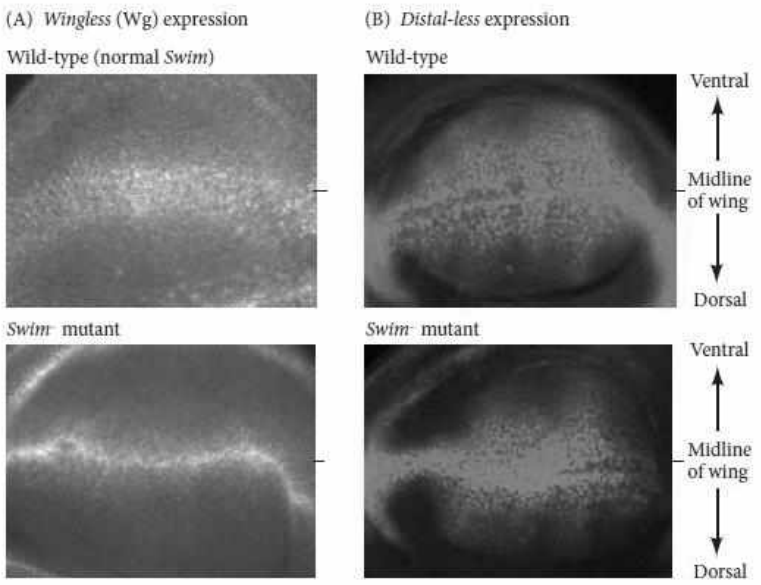


FIGURE 4.38 Wnt diffusion is affected by other proteins. (A) Diffusion of Wingless (Wg, a Wnt paracrine factor) throughout the developing wing of wild-type *Drosophila* (above) is enhanced by Swim, a protein that stabilizes Wg and that is made by some of the wing cells. When Swim is not present, as in the mutant below, Wg does not disperse but is confined to the narrow band of *Wg*-expressing cells. (B) Similarly, Wingless usually activates the *Distal-less* gene (green) in much of the wild-type wing (seen above). However, in *swim*-mutant flies, the range of *Distal-less* expression is confined to those areas near the band of *Wg*-expressing cells. (From Mulligan et al. 2012.)

FGF secretion represents a comprehensive example of the ways that HSPGs can influence paracrine factor diffusion. Cells secrete FGFs into the extracellular matrix, where the FGFs can interact with a diversity of HSPGs that function to both modulate the diffusion of FGF and influence FGF-FGFR binding (Balasubramanian and Zhang 2015). Like all proteoglycans, HSPGs possess chains of sugar molecules that vary in length and type, and different forms of HSPG-FGF interactions can differently shape the FGF gradient. Specifically, the morphogen gradient of Fgf8 is thought to be established through a source-sink model (also known as a "secretion-diffusion-clearance" mechanism; Yu et al. 2009). In this model, cells secreting Fgf8 are the source of the morphogen, and the receiving cells provide the sink through mechanisms of binding, internalization, or protein degradation for clearance of Fgf8 (Balasubramanian and Zhang 2015). Michael Brand's lab tested this model in the zebrafish gastrula by microinjecting a cluster of cells with Fgf8 fused with GFP, quantifying the amount of Fgf8 in the extracellular space at varying distances from the microinjected cells using fluorescence correlation spectroscopy (**FIGURE 4.39A,B**). Remarkably, the researchers were able to visualize an Fgf8-GFP gradient that differed under different circumstances (**FIGURE 4.39C**): free diffusion of the ligand achieved the greatest distance traveled; "directed diffusion" along HSPG fibers fostered rapid movement over several cell distances; "confined clustering" of Fgf8 on dense HSPG matrices significantly restricted diffusion; and endocytosis internalized the Fgf8-FGFR complex for lysosomal degradation in receiving cells (Yu et al. 2009; Bökel and Brand 2013). Thus, the target tissue is not passive. It can promote diffusion, retard diffusion, or degrade the paracrine factor.

CILIA AS SIGNAL RECEPTION CENTERS In many cases, the reception of paracrine factors is not uniform throughout the cell membrane; rather, receptors are often congregated asymmetrically. For instance, the reception of Hedgehog proteins in vertebrates occurs on the primary cilium, a focal extension of the cell membrane made by microtubules (Huangfu et al. 2003; Goetz and Anderson 2010). The primary cilium should not be confused with motile cilia such as those found lining the trachea or in the node of a gastrulating embryo. The primary cilium is much shorter than motile cilia and largely went unnoticed until we realized its direct role in numerous human diseases. In fact,

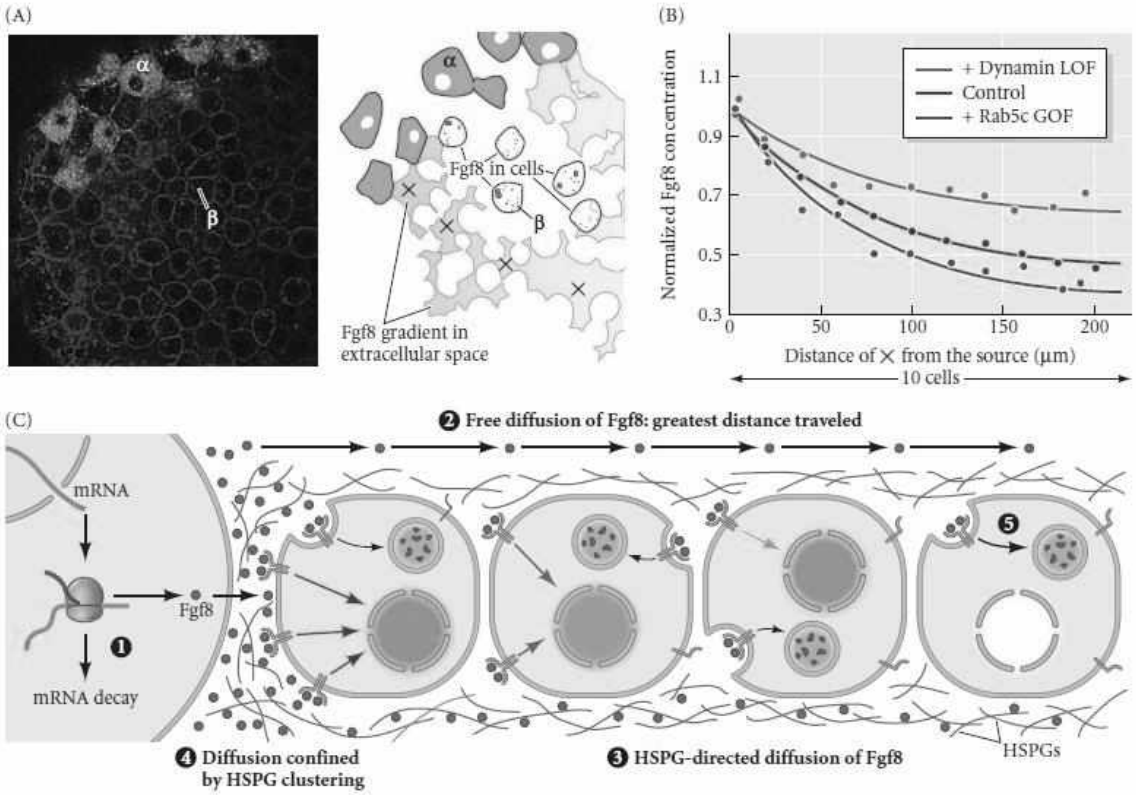


FIGURE 4.39 The Fgf8 gradient. (A) Zebrafish blastulae were injected with DNA encoding Fgf8-GFP (green stain) and mRFP-glycosyl phosphatidylinositol (GPI; red stain) to visualize, respectively, Fgf8 expression and the cell membrane. The confocal image is of a resulting zebrafish gastrula, showing Fgf8 protein being produced by and secreted away from isolated GFP-labeled cells (green). On the right is a schematic representation of select cells and the Fgf8 expression seen in the confocal image (compare α and β identifiers). Fgf8 is seen in a gradient in the extracellular matrix as well as being internalized in receiving cells. (B) Quantification of Fgf8 protein at different locations in (A), indicated by "X" marks in schematic. Manipulation of endocytosis causes predictable changes in the range of Fgf8 secretion. Inhibition of endocytosis with the dominant negative GTPase dynamin causes a shallower Fgf8 gradient over a longer distance (green plot) (LOF, loss of function), whereas increased endocytosis with the overexpression of the endosomal protein Rab5c (GOF, gain of function) yields a steeper and shorter Fgf8 gradient (blue plot). (C) Five primary mechanisms for shaping the Fgf8 gradient. (1) The difference in the rate of *fgf8* transcription and *fgf8* mRNA decay can influence the amount of Fgf8 protein ultimately secreted from a producing cell. Once secreted, Fgf8 can (2) freely diffuse or (3) travel rapidly along HSPG fibers for directed diffusion. (4) In contrast, however, dense areas of HSPGs can also confine and restrict Fgf8 diffusion. (5) The Fgf8-FGFR complex can also be internalized by endocytosis and targeted for lysosomal degradation. Together these different mechanisms result in the displayed gradient of Fgf8, and differential responses in cells that experience different concentrations of Fgf8 signaling (different colored nuclei). (A courtesy of Michael Brand; B after Yu et al. 2009; C after Bökel and Brand 2013; Balasubramanian and Zhang 2015.)

some of these "ciliopathies," such as Bardet-Biedl syndrome, are suspected to be due to an indirect effect on Hedgehog signaling (Nachury 2014). In unstimulated cells, the Patched protein (the Hedgehog receptor; see Figure 4.30) is located in the primary cilium membrane , whereas the Smoothened protein is in the cell membrane close to the cilium or part of an endosome being targeted for degradation. Patched inhibits Smoothened function by preventing it from entering the primary cilium (Milenkovic et al. 2009; Wang et al. 2009). When Hedgehog binds to Patched, however, Smoothened is allowed to join it on the ciliary cell membrane, where it inhibits the PKA and SuFu proteins that make the repressive form of the Gli transcription factor (**FIGURE 4.40**). The microtubules of these

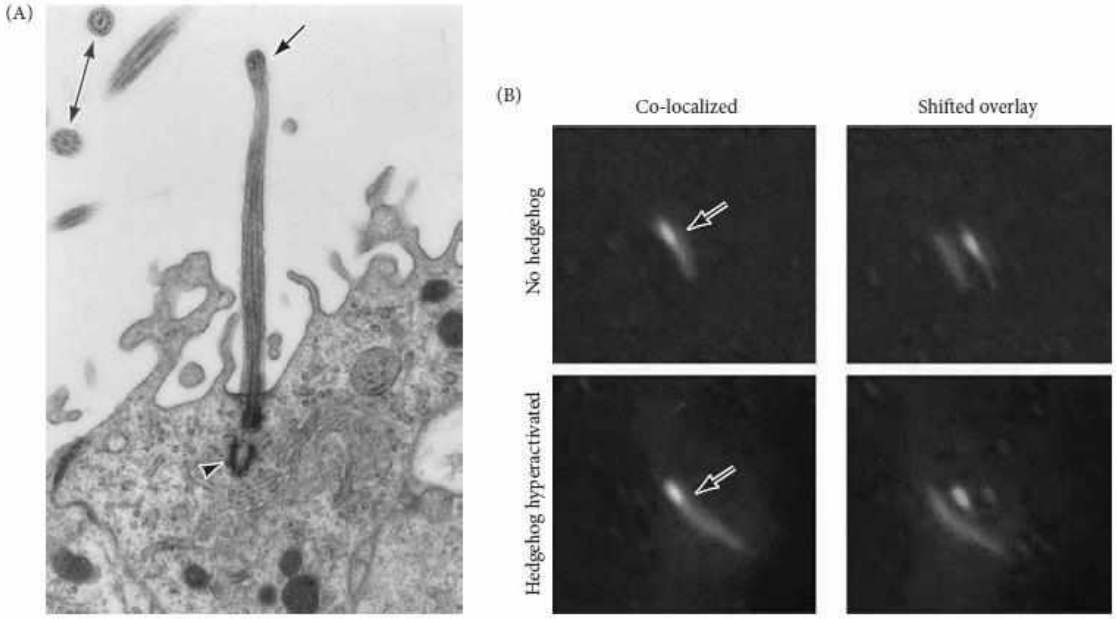


FIGURE 4.40 The primary cilium for Hedgehog reception. (A) Transmission electron micrograph showing a longitudinal section of the primary cilium (black arrow) of a "B-type cell," a neural stem cell in the adult mammalian brain (see Chapter 5). The centriole at the base of this cilium is visible (arrowhead); the microtubules in this primary cilium form an 8+0 structure (other types of cilia, such as motile cilia, typically form a 9+2 arrangement; seen in upper left corner in cross-section [red arrows]). (B) Activation of the Hedgehog pathway requires the transport of Smoothened into the primary cilium. Seen here is the primary cilium (arrow; immunofluorescence stained for acetylated tubulin, blue) on a fibroblast in culture. The ciliary protein Evc (stained green) co-localizes with Smoothened (red) upon hyperactivation of Hedgehog signaling by the drug SAG. Compare the co-localized labeling on the left with the overlays on the right, which have been shifted to show each individual marker. Activation of the Evc-Smo complex in the primary cilium leads to full-length Gli signaling. (A from Alvarez-Bulla et al. 1998; B from Caparrós-Martín et al. 2013.)

cilia provide a scaffold for motor proteins to transport Patched and Smoothened as well as activated Gli proteins, and mutations that knock out cilia formation or their transport mechanism also prevent Hedgehog signaling (Mukhopadhyay and Rohatgi 2014).

Focal membrane protrusions as signaling sources

We have discussed the roles of secreted growth factors for both short- and long-range cell-to-cell communication. But is there a mechanism to present a signal without secreting it? In such a scenario, the producing cell itself *physically reaches out* and presents the signal. Here we highlight emerging ideas of how two types of dynamic membrane extensions can facilitate intercellular communication, and even produce long-range gradients.

LAMELLIPODIA In tunicates, an asymmetric division of a single precardiac founder cell gives rise to the heart progenitors. Although both daughter cells are exposed to the inductive signal Fgf9, only the smaller of the two responds to generate the heart progenitor lineage. During asymmetric division, localized protrusions (**lamellipodia**) form on the ventral-anterior side of the founder cell (Cooley et al. 2011). These protrusions are actin-rich (unlike the microtubule-rich cilia) and result from the polarized localization of a Rho GTPase (Cdc42) in this region. It is possible that the underlying extracellular matrix of the ventral epidermis stimulates this localization. At the same time, FGF receptor activity becomes concentrated in the lamellipodia. When the cell

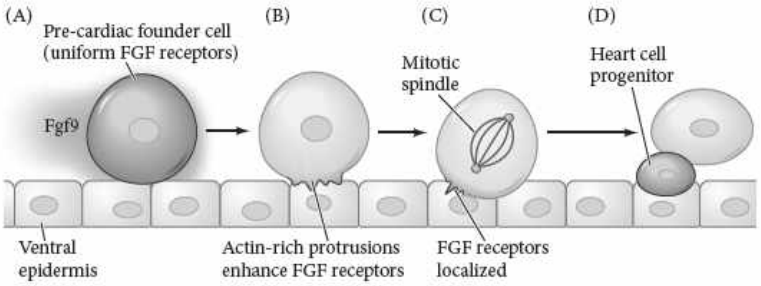


FIGURE 4.41 Model for differential specification of the tunicate heart progenitor lineage. (A) Uniform exposure to Fgf9 leads to uniform FGF receptor occupancy on all parts of the founder cell membrane. (B) Actin-rich protrusions on the ventral-anterior membrane of the cell are associated with high FGF receptor activation. (C) As the progenitor cell enters mitosis, invasive protrusions of the ventral-anterior cell membrane restrict FGF receptors to this region. (D) Following asymmetric cell division, the FGF-activated MAPK pathway is restricted to the ventral daughter cell, leading to differential expression of heart progenitor genes. (After Cooley et al. 2011.)

divides, the smaller daughter inherits these localized, activated FGF receptors, leading to differential activation of the genes that will form heart muscle (**FIGURE 4.41**).

THE FILOPODIAL CYTONEME What if the molecules we thought were diffusible paracrine factors moving through the extracellular matrix were actually transferred from one cell to another at synapse-like connections? There is now significant evidence to support the existence of specialized filopodial projections called **cytonemes**, which stretch out remarkable distances (more than 100 μm) from either the target cells or the signal-producing cells, like long membrane conduits connecting the two types of cells (Roy and Kornberg 2015). Under this model, ligand-receptor binding would initially occur at the tips of cytonemes projecting from the target cells when the tips are positioned in direct apposition to the producing cell's plasma membrane. The ligand-receptor complex would then be transported down the cytoneme to the target cell body.

Cytoneme-mediated morphogen signaling was first described by Thomas Kornberg's laboratory studying development of the air sac and wing disc in *Drosophila* (Roy et al. 2011). A cluster of cells called the air sac primordium (ASP) develops along the basal surface of the wing disc in response to DPP (a BMP homologue) and FGF morphogen gradients in the wing disc (**FIGURE 4.42A,B**). The Kornberg lab discovered that the ASP cells extend cytonemes toward the DPP- and FGF-expressing cells, and that these cytonemes contain receptors for these morphogens—separate receptors in separate cytonemes. Moreover, DPP bound to its receptor on ASP cells has been documented traveling along a cytoneme to the cell body. Anterior-posterior patterning of the wing disc by a gradient of Hedgehog (Hh) signaling also appears to be accomplished through cytonemes (**FIGURE 4.42C**). Hedgehog coming from posterior cells is delivered through cytonemes that extend from the basolateral surface of anterior cells to the Hh-producing posterior cells (**FIGURE 4.42D,E**; Bischoff et al. 2013).

Recent investigations have shown that vertebrates use cytonemes as well. Work in Michael Brand's lab and recent work by Steffen Scholpp's lab have shown that the same gastrulating cells also transport the morphogen Wnt8a along cytoneme-like extensions. In this case, the signal-producing cells are extending the cytonemes, transporting the Wnt8a morphogen to target cells (**FIGURE 4.42F**; Luz et al. 2014; Stanganello et al. 2015). Cytoneme-like interactions are also suspected in one of the classic examples of morphogen signaling, that of anterior-posterior specification in the tetrapod limb bud. Here, a posterior-to-anterior gradient of Sonic hedgehog (Shh) in the limb bud leads to the correct patterning of digits (see Chapter 19). In the chick limb bud, both the Sonic hedgehog-expressing cells and the anterior target cells extend filopodial projections toward each other and make contact at points where Sonic hedgehog (Patch) receptors are localized (**FIGURE 4.42G**; Sanders et al. 2013).

SCIENTISTS SPEAK 4.6 An iBiology Seminar by Dr. Thomas Kornberg of the University of California, San Francisco, discusses cytoneme-directed transport and direct transfer models.

Developing Questions

Are all the molecules that we have considered to be paracrine factors distributed solely by contact through filopodial cytoneme processes, as opposed to diffusion through the extracellular matrix? This question is increasingly coming up in debates among developmental biologists. Where do you stand? Are you a "diffusionist" or a "cytonemist"? Is there room for both mechanisms, or perhaps even a developmental need for both?

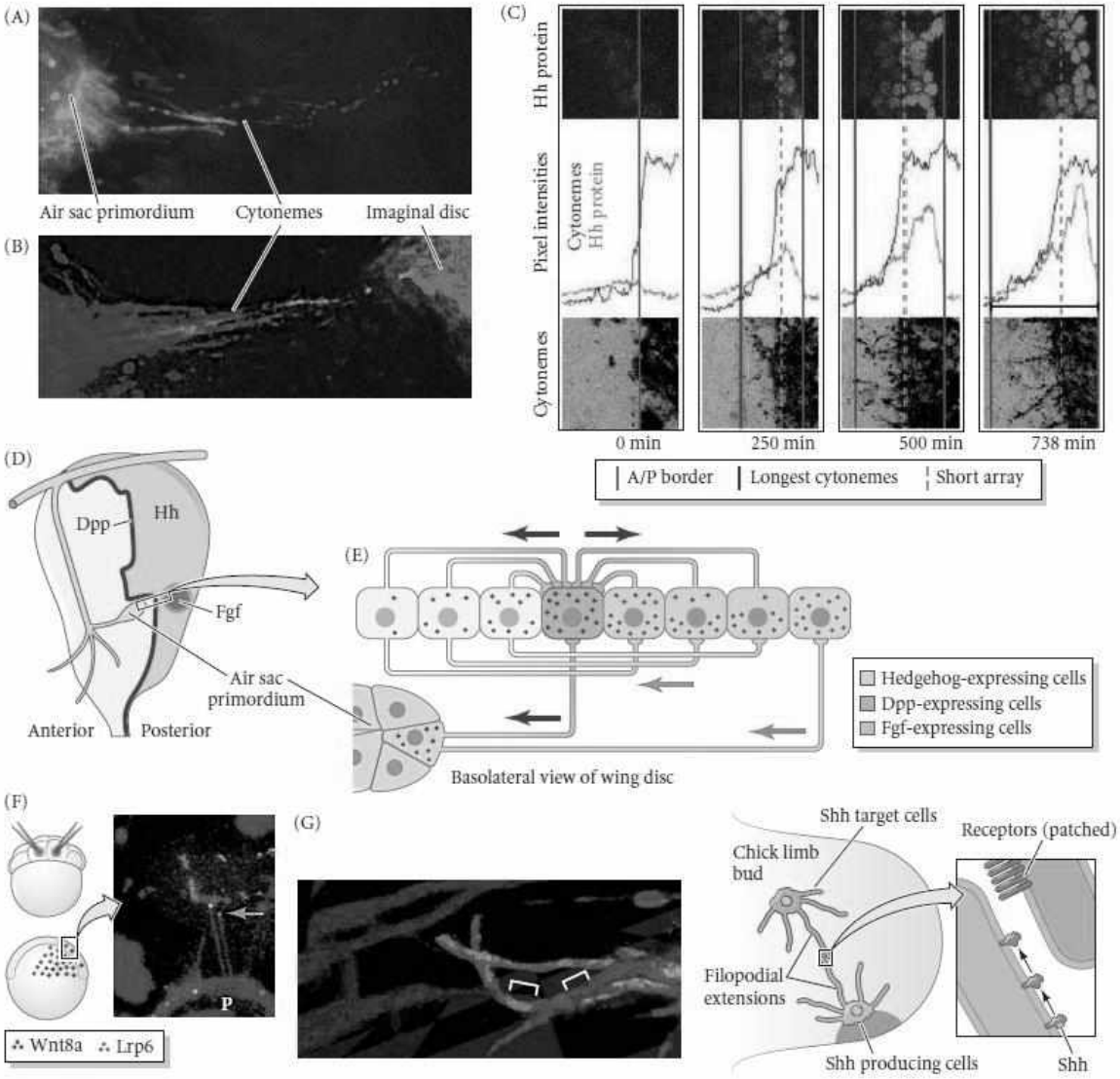


FIGURE 4.42 Filopodia-transported morphogens from fly to fish and mouse. (A) Cytonemes from the air sac primordium (ASP) extend toward the epithelium of the wing imaginal disc in *Drosophila* to shuttle the FGF (green) and DPP (red) morphogens. (B) Transported DPP receptor binds DPP produced by the wing disc cells, which gets transported back down the cytoneme to the ASP. (C) This system of cytonemes in the *Drosophila* wing disc is capable of establishing a gradient of Hedgehog (Hh) protein (green in top panels and in plot) over the course of filopodial extension (black processes in lower panels and red plot line). (D) Illustration of the *Drosophila* wing imaginal disc during its interactions with tracheal cells, namely the air sac primordium. Dpp, Hh, and Fgf expressing cells are represented as blue, red, and green domains. (E) Magnified cross section of the boxed region in (D). Cytoneme extensions from the air sac primordium as well as between cells of the wing disc are illustrated along with the morphogens produced and transported along these cytonemes (arrows). (F) Wnt8a (red) and its receptor Lrp6 (green) were microinjected into two different cells of an early-stage zebrafish blastula. Live cell imaging of these cells at the gastrula stage revealed Wnt8a interactions with the Lrp6 receptor at the tips of filopodial extensions from the producer cells (P, yellow arrow). (G) In the chick limb bud, long, thin filopodial protrusions have been documented extending both from Sonic hedgehog-producing cells in the posterior region (purple cell with green Shh protein in left image) and from the target cells in the anterior limb bud (red cells). These opposing filopodia directly interact (brackets, left image), and at this point of interaction, it is proposed that Shh and its receptor Patch can bind (right illustration). (A,B from Roy and Kornberg 2011; C from Bischoff et al. 2013; F from Stanganello et al. 2015; G from Sanders et al. 2013.)

Juxtacrine Signaling for Cell Identity

In juxtacrine interactions, proteins from the inducing cell interact with receptor proteins of adjacent responding cells without diffusing from the cell producing it. Three of the most widely used families of juxtacrine factors are the **Notch proteins** (which bind to a family of ligands exemplified by the Delta protein); **cell adhesion molecules** such as cadherins; and the **eph receptors** and their **ephrin ligands**. When an ephrin on one cell binds with the eph receptor on an adjacent cell, signals are sent to each of the two cells (Davy et al. 2004; Davy and Soriano 2005). These signals are often those of either attraction or repulsion, and ephrins are often seen where cells are being told where to migrate or where boundaries are forming. We will see the ephrins and the eph receptors functioning in the formation of blood vessels, neurons, and somites. We will now look more closely at the Notch proteins and their ligands, as well as discussing cell adhesion molecules as part of an important developmental pathway called Hippo signaling.

The Notch pathway: Juxtaposed ligands and receptors for pattern formation

Although most known regulators of induction are diffusible proteins, some inducing proteins remain bound to the inducing cell surface. In one such pathway, cells expressing the Delta, Jagged, or Serrate proteins in their cell membranes activate neighboring cells that contain Notch protein in their cell membranes (see Artavanis-Tsakakonas and Muskavitch 2010). Notch extends through the cell membrane, and its external surface contacts Delta, Jagged, or Serrate proteins extending out from an adjacent cell. When complexed to one of these ligands, Notch undergoes a conformational change that enables a part of its cytoplasmic domain to be cut off by the presenilin-1 protease. The cleaved portion enters the nucleus and binds to a dormant transcription factor of the CSL family. When bound to the Notch protein, the CSL transcription factors activate their target genes (**FIGURE 4.43**; Lecourtois and Schweisguth 1998; Schroeder et al. 1998; Struhl and Adachi 1998). This activation is thought to involve the recruitment of histone acetyltransferases (Wallberg et al. 2002). Thus, Notch can be considered as a transcription

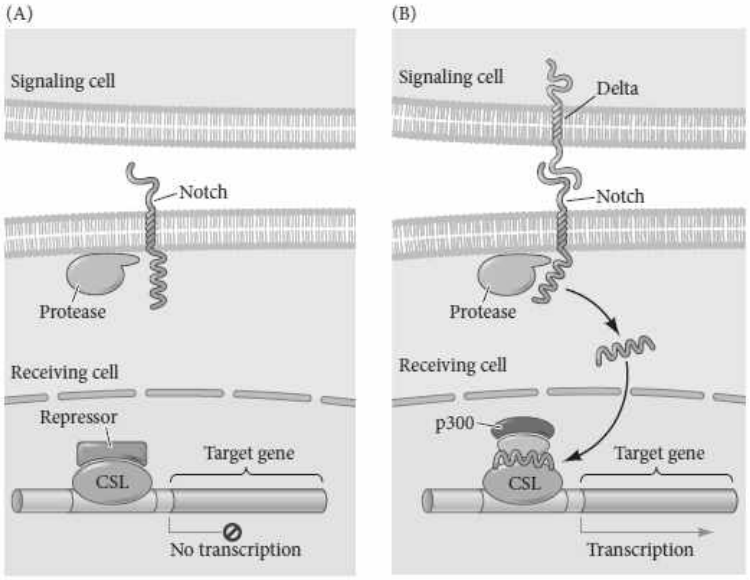


FIGURE 4.43 Mechanism of Notch activity. (A) Prior to Notch signaling, a CSL transcription factor (such as Suppressor of hairless or CBF1) is on the enhancer of Notch-regulated genes. The CSL binds repressors of transcription. (B) Model for the activation of Notch. A ligand (Delta, Jagged, or Serrate protein) on one cell binds to the extracellular domain of the Notch protein on an adjacent cell. This binding causes a shape change in the intracellular domain of Notch, which activates a protease. The protease cleaves Notch and allows the intracellular region of the Notch protein to enter the nucleus and bind the CSL transcription factor. This intracellular region of Notch displaces the repressor proteins and binds activators of transcription, including the histone acetyltransferase p300. The activated CSL can then transcribe its target genes. (After K. Koziol-Dube, personal communication.)

factor tethered to the cell membrane. When the attachment is broken, Notch (or a piece of it) can detach from the cell membrane and enter the nucleus (Kopan 2002).

Notch proteins are involved in the formation of numerous vertebrate organs—kidney, pancreas, and heart—and they are extremely important receptors in the nervous system. In both the vertebrate and *Drosophila* nervous systems, the binding of Delta to Notch tells the receiving cell not to become neural (Chitnis et al. 1995; Wang et al. 1998). In the vertebrate eye, the interactions between Notch and its ligands regulate which cells become optic neurons and which become glial cells (Dorsky et al. 1997; Wang et al. 1998).

WEB TOPIC 4.3 **NOTCH MUTATIONS** Humans have genes for more than one Notch protein and more than one ligand. Their interactions are critical in neural development, and mutations in Notch genes can cause nervous system abnormalities.

Induction does indeed occur on the cell-to-cell level, and one of the best examples is the formation of the vulva in the nematode worm *C. elegans*. Remarkably, the signal transduction pathways involved turn out to be the same as those used in the formation of retinal receptors in *Drosophila*; only the targeted transcription factors are different. In both cases, an epidermal growth-factor-like inducer activates the RTK pathway, leading to the differential regulation of Notch-Delta signaling.

Paracrine and juxtacrine signaling in coordination: Vulval induction in C. elegans

Most *C. elegans* individuals are hermaphrodites. In their early development, they are male, and the gonad produces sperm, which are stored for later use. As they grow older, they develop ovaries. The eggs "roll" through the region of sperm storage, are fertilized inside the nematode, and then pass out of the body through the vulva (see Chapter 8; Barkoulas et al. 2013). The formation of the vulva occurs during the larval stage from six cells called the **vulval precursor cells** (**VPCs**). The cell connecting the overlying gonad to the vulval precursor cells is called the **anchor cell** (**FIGURE 4.44**). The anchor cell secretes LIN-3 protein, a paracrine factor (similar to mammalian epidermal growth factor, or EGF) that activates the RTK pathway (Hill and Sternberg 1992). If the anchor cell is destroyed (or if the *lin-3* gene is mutated), the VPCs will not form a vulva and instead become part of the hypodermis or skin (Kimble 1981).

The six VPCs influenced by the anchor cell form an **equivalence group**. Each member of this group is competent to become induced by the anchor cell and can assume any of three fates, depending on its proximity to the anchor cell. The cell directly beneath the anchor cell divides to form the central vulval cells. The two cells flanking that central cell divide to become the lateral vulval cells, whereas the three cells farther away from the anchor cell generate hypodermal cells. If the anchor cell is destroyed, all six cells of the equivalence group divide once and contribute to the hypodermal tissue. If the three central VPCs are destroyed, the three outer cells, which normally form hypodermis, generate vulval cells instead.

LIN-3 secreted from the anchor cell forms a concentration gradient, in which the VPC closest to the anchor cell (i.e., the P6.p cell) receives the highest concentration of LIN-3 and generates the central vulval cells. The two adjacent VPCs (P5.p and P7.p) receive lower amounts of LIN-3 and become the lateral vulval cells. VPCs farther away from the anchor cell do not receive enough LIN-3 to have an effect, so they become hypodermis (Katz et al. 1995).

NOTCH-DELTA AND LATERAL INHIBITION We have discussed the reception of the EGF-like LIN-3 signal by the cells of the equivalence group that forms the vulva. Before this induction occurs, however, an earlier interaction has formed the anchor cell. The formation of the anchor cell is mediated by *lin-12*, the *C. elegans* homologue of the *Notch* gene. In

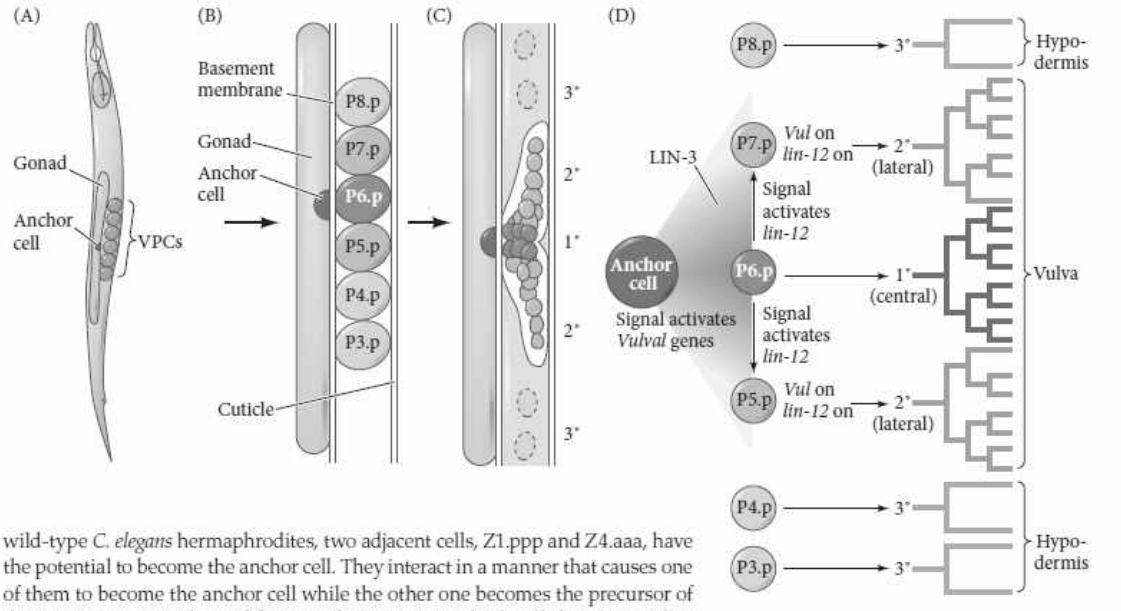


FIGURE 4.44 *C. elegans* vulval precursor cells (VPCs) and their descendants. (A) Location of the gonad, anchor cell, and VPCs in the second instar larva. (B,C) Relationship of the anchor cell to the six VPCs and their subsequent lineages. Primary (1°) lineages result in the central vulval cells, secondary (2°) lineages constitute the lateral vulval cells, and tertiary (3°) lineages generate hypodermal cells. (C) Outline of the vulva in the fourth instar larva. The circles represent the positions of the nuclei. (D) Model for the determination of vulval cell lineages in *C. elegans*. The LIN-3 signal from the anchor cell causes the determination of the P6.p cell to generate the central vulval lineage (dark purple). Lower concentrations of LIN-3 cause the P5.p and P7.p cells to form the lateral vulval lineages. The P6.p (central lineage) cell also secretes a short-range juxtacrine signal that induces the neighboring cells to activate the LIN-12 (Notch) protein. This signal prevents the P5.p and P7.p cells from generating the primary central vulval cell lineage. (After Katz and Sternberg 1996.)

wild-type *C. elegans* hermaphrodites, two adjacent cells, Z1.ppp and Z4.aaa, have the potential to become the anchor cell. They interact in a manner that causes one of them to become the anchor cell while the other one becomes the precursor of the uterine tissue. In loss-of-function *lin-12* mutants, both cells become anchor cells, whereas in gain-of-function mutations, both cells become uterine precursors (Greenwald et al. 1983). Studies using genetic mosaics and cell ablations have shown that this decision is made in the second larval stage, and that the *lin-12* gene needs to function only in that cell destined to become the uterine precursor cell. The presumptive anchor cell does not need it. Seydoux and Greenwald (1989) speculate that these two cells originally synthesize both the signal for uterine differentiation (the LAG-2 protein, homologous to Delta) and the receptor for this molecule (the LIN-12 protein, homologous to Notch; Wilkinson et al. 1994).

During a particular time in larval development, the cell that, by chance, is secreting more LAG-2 causes its neighbor to cease its production of this differentiation signal and to increase its production of LIN-12. The cell secreting LAG-2 becomes the gonadal anchor cell, while the cell receiving the signal through its LIN-12 protein becomes the ventral uterine precursor cell (**FIGURE 4.45**). Thus, the two cells are thought to determine each other prior to their respective differentiation events. When LIN-12 is used again during vulva formation, it is activated by the primary vulval lineage to stop the lateral vulval cells from forming the central vulval phenotype (see Figure 4.44). Thus, the anchor cell/ventral uterine precursor decision illustrates two important aspects of determination in two originally equivalent cells. First, the initial difference between the two cells is created by chance. Second, this initial difference is reinforced by feedback. This Notch-Delta mediated mechanism of restricting adjacent cell fates is called **lateral inhibition**.

Hippo: An integrator of pathways

Most of the signal transduction pathways that we have discussed are named for the players involved in the initial signaling event at the cell membrane. The Hippo signal transduction pathway does not have a dedicated ligand or receptor, however. Hippo stands for one of several important kinases that are critical for organ size control. It was first identified in *Drosophila*, where its loss resulted in a "hippopotamus"-shaped phenotype due to excessive growth (Hansen et al. 2015).

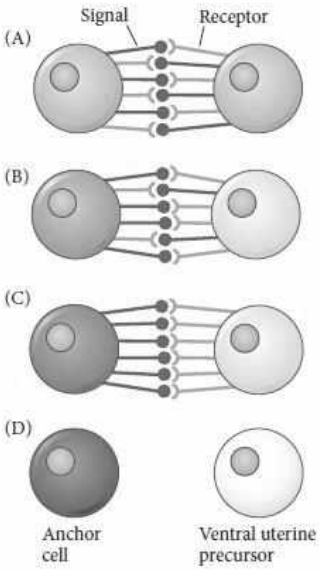


FIGURE 4.45 Model for the generation of two cell types (anchor cell and ventral uterine precursor cell) from two equivalent cells (Z1.ppp and Z4.aaa) in *C. elegans*. (A) The cells start off as equivalent, producing fluctuating amounts of signal and receptor. The *lag-2* gene is thought to encode the signal, and the *lin-12* gene is thought to encode the receptor. Reception of the signal turns down LAG-2 (Delta) production and upregulates LIN-12 (Notch). (B) A stochastic (chance) event causes one cell to produce more LAG-2 than the other cell at some particular critical time, which stimulates more LIN-12 production in the neighboring cell. (C) This difference is amplified because the cell producing more LIN-12 produces less LAG-2. Eventually, just one cell is delivering the LAG-2 signal, and the other cell is receiving it. (D) The signaling cell becomes the anchor cell, and the receiving cell becomes the ventral uterine precursor cell. (After Greenwald and Rubin 1992.)

Loss of Hippo (or overexpression of its main transcriptional effector, Yorkie) causes cells to divide significantly faster while slowing apoptosis (**FIGURE 4.46**; Justice et al. 1995; Xu et al. 1995; Huang et al. 2005).

The essential players in the Hippo signaling cascade begin at the cell membrane with cell-to-cell interactions involving cell adhesion molecules such as E-cadherin or Crumbs (see Figure 5.7B). These cell adhesion molecules interact with the F-actin binding protein angiomotin, which initiates activation of the Hippo kinase cascade (Hansen et al. 2015). The main kinase in this cascade is the Large tumor suppressor 1/2 (Lats1/2; Warts is the *Drosophila* homologue), which functions to phosphorylate Yorkie or its mammalian homologue Yap/Taz. When phosphorylated, Yap/Taz will either be retained in the cytoplasm or degraded, whereas lack of Hippo signaling frees Yap/Taz to enter the nucleus and function as a transcription co-activator of Tead (Scalloped homologue). There are a number of ways in which Hippo signaling components can regulate the pathways of other paracrine factors such as Wnts, EGF, TGF-β, and BMP. Likewise, these pathways can modulate Hippo signaling, typically operating through Yap/Taz. Thus, the Hippo pathway is emerging as a major crossroad for the biochemical pathways of the cell, heightening our attention to the long-unsolved problem of understanding how all these conceptually linear pathways are truly integrated.

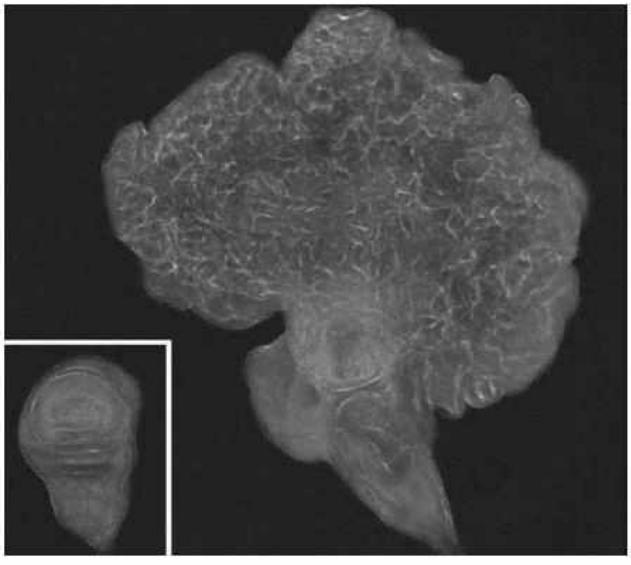
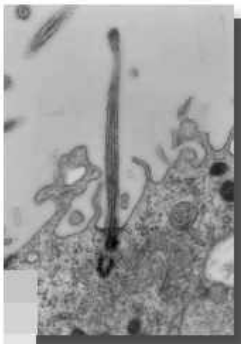


FIGURE 4.46 Hippo signaling is critical for controlling organ size. Overexpression of *yorkie* (the main transcriptional effector of the Hippo kinase) in *Drosophila* resulted in an extremely overgrown ("hippopotamus") wing imaginal disc compared to the same stage wild-type wing disc. (Photograph from Huang et al. 2005.)

Next Step Investigation

How do cells communicate, interact, and understand their place in the embryo? This chapter covered many of the mechanisms at play that facilitate cell-to-cell attachments, relay chemical signals, and respond to environmental cues. There are many exciting next steps to investigate, from the biophysics of morphogenesis to the role of cytonemes in morphogen gradients. These types of mechanisms are easy to comprehend on the scale of the cell and tissue, and we are sure that you can propose some logical and exciting experimental designs to further test such mechanisms. This field is lacking a significant understanding of how morphogenesis is coordinated on the scale of the entire embryo, however. How might you begin to apply your understanding of cell-to-cell communication toward a more comprehensive understanding of coordinated development across the embryo? Do you think there could be a kind of global oversight of timing, size, pattern, movement, and differentiation? Please know there are no correct answers to these questions at the back of the book, hiding in your professor's notes, or buried in Google search results. The answers reside in the completion of your own ideas and experiments.



Closing Thoughts on the Opening Photo

Is this a cell's antenna? If so, what is its purpose? It's for cells to communicate! This image shows a primary cilium on a neural stem cell in the brain, a structure that is in fact used like an antenna, enabling the cell to receive signals from its environment. We discussed the critical role of select signaling proteins that convey a myriad of information about position, adhesion, cell specification, and migration. New mechanisms of cell communication—such as the essential role of the primary cilium emphasized in this image; the potential reach of cytonemes, which may change our understanding of morphogen delivery; the modifying and potentially instructive roles of the extracellular matrix; and how the physical properties of cell adhesion can both sort different cells and regulate organ size—are rapidly emerging. (Photograph courtesy of Alvarez-Bullya et al. 1998.)

4 Snapshot Summary
Cell-to-Cell Communication

1. The sorting out of one cell type from another results from differences in the cell membrane.
2. The membrane structures responsible for cell sorting out are often cadherin proteins that change the surface tension properties of the cells. Cadherins can cause cells to sort by both quantitative (different amounts of cadherin) and qualitative (different types of cadherin) differences. Cadherins appear to be critical during certain morphological changes.
3. Cell migration occurs through changes in the actin cytoskeleton. These changes can be directed by internal instructions (from the nucleus) or by external instructions (from the extracellular matrix or chemoattractant molecules).
4. Inductive interactions involve inducing and responding tissues. The ability to respond to inductive signals depends on the competence of the responding cells. The specific response to an inducer is determined by the genome of the responding tissue.
   - Reciprocal induction occurs when the two interacting tissues are both inducers and are competent to respond to each other's signals.
   - Cascades of inductive events are responsible for organ formation.
   - Regionally specific inductions can generate different structures from the same responding tissue.
5. Paracrine interactions occur when a cell or tissue secretes proteins that induce changes in neighboring cells. Juxtacrine interactions are inductive interactions that take place between the cell membranes of adjacent cells or between a cell membrane and an extracellular matrix secreted by another cell.
6. Paracrine factors are secreted by inducing cells. These factors bind to cell membrane receptors in competent responding cells. Competence is the ability to bind and to respond to inducers, and it is often the result of a prior induction. Competent cells respond to paracrine factors through signal transduction pathways.
7. Morphogens are secreted signaling molecules that affect gene expression differently at different concentrations.
8. Signal transduction pathways begin with a paracrine or juxtacrine factor causing a conformational change in

its cell membrane receptor. The new shape can result in enzymatic activity in the cytoplasmic domain of the receptor protein. This activity allows the receptor to phosphorylate other cytoplasmic proteins. Eventually, a cascade of such reactions activates a transcription factor (or set of factors) that activates or represses specific gene activity.

9. The differentiated state can be maintained by positive feedback loops involving transcription factors, autocrine factors, or paracrine factors.
10. The extracellular matrix is both a source of signals and serves to modify how such signals may be secreted across cells to influence differentiation and cell migration.
11. Cells can convert from being epithelial to being mesenchymal and vice versa. The epithelial-mesenchymal transition (EMT) is a series of transformations involved in the dispersion of neural crest cells and the creation of vertebrae from somitic cells. In adults, EMT is involved in wound healing and cancer metastasis.
12. The cell surface is intimately involved with cell signaling. Proteoglycans and other membrane components can expand or restrict the diffusion of paracrine factors.
13. Specializations of the cell surface, including cilia and lamellipodia, may concentrate receptors for paracrine and extracellular matrix proteins. Newly discovered filopodia-like extensions called cytonemes can be involved in transferring morphogens between signaling and responding cells and may be a major component of cell signaling.
14. Juxtacrine signaling involves local protein interactions between receptors. Examples include Notch-Delta signaling that patterns cell fates through lateral inhibition and Hippo signaling that influences organ size.

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