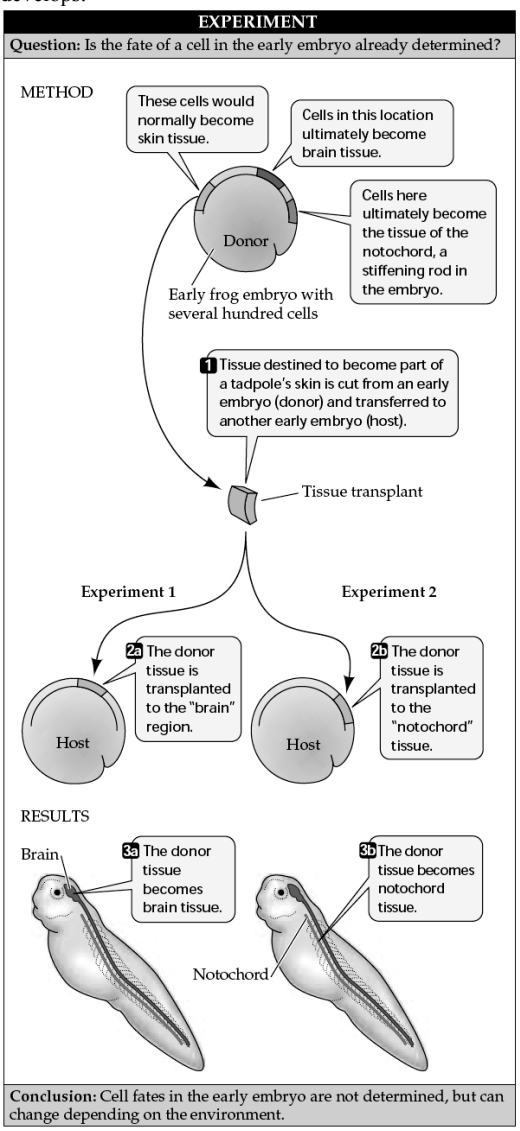
As development proceeds, cells become more and more specialized

Experiments in which specific cells of an early embryo were marked with stains have revealed which adult structures are derived from which parts of the embryo. These stained embryos produce what are known as *fate maps*. For instance, we know that the green-shaded area of the frog embryo shown in Figure 2 normally becomes part of the skin of the tadpole larva. However, if we cut out a piece from this region and transplant it to another location on another early frog embryo, it does not become skin. The type of tissue it does become is determined by its new environment. The developmental potential of these early embryonic cells—that is, their range of possible fates—is thus greater than their actual fate, which is limited to the cell type that normally develops.



2 Developmental Potential in Early Frog Embryos Cells that would be expected to form one kind of tissue can form completely different tissues when they are experimentally moved to another location. In this experiment, epithelial (skin) tissue from an early-stage frog embryo was transplanted from a donor to a host embryo. The tissue that developed in the host tadpole was not skin, but was consistent with the location to which the "skin graft" was transplanted.

Does embryonic tissue retain its broad developmental potential? Generally speaking, the answer is no. The developmental potential of cells becomes restricted fairly early in normal development. Tissue from a later-stage frog embryo, for example, if taken from a region fated to develop into the brain, becomes brain tissue even if transplanted to a part of an early-stage embryo destined to become another structure.

The cells of the later-stage embryo are thus said to be *determined:* Their fate has been sealed, regardless of their surroundings. By contrast, the cells of the younger tissue transplant in Figure 2 have not yet become determined.

Determination, the commitment of a cell to a particular fate, is a process influenced by the action of the extracellular function. This apparent contradiction results from regulation of the expression of various parts of the genome. When the embryo consists of only a few cells, each cell has the potential to develop in many different ways. As development proceeds, however, the possibilities available to individual cells gradually narrow, until each cell's fate is fully determined and the cell has differentiated.

Morphogenesis (literally, "creation of form") is the shaping of the multicellular body and its organs. Morphogenesis results from *pattern formation*, the organization of differentiated tissues into specific structures. In plant development, cells are constrained by cell walls and do not move around the body, so organized division and expansion of cells are the major processes that build the plant body. In animals, cell movements are very important in morphogenesis. And in both plants and animals, programmed cell death is essential to orderly development. Like differentiation, morphogenesis results ultimately from the regulated activities of genes and their products, as well as from the interplay of extracellular signals and their transduction in target cells.

As development proceeds, cells become more and more specialized

Experiments in which specific cells of an early embryo were marked with stains have revealed which adult structures are derived from which parts of the embryo. These stained embryos produce what are known as *fate maps*. For instance, we know that the green-shaded area of the frog embryo shown in Figure 2 normally becomes part of the skin of the tadpole larva. However, if we cut out a piece from this region and transplant it to another location on another early frog embryo, it does not become skin. The type of tissue it does become is determined by its new environment. The developmental potential of these early embryonic cells—that is, their range of possible fates—is thus greater than their actual fate, which is limited to the cell type that normally develops.

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Determination, the commitment of a cell to a particular fate, is a process influenced by the action of the extracellular environment and the contents of the cell on the cell's genome. Determination is not something that is visible under the microscope— cells do not change their appearance when they become determined. Determination is followed by *differentiation*, the actual changes in biochemistry, structure, and function that result in cells of different types. Differentiation often involves a change in appearance as well as function. Determination is a commitment; the final realization of that commitment is differentiation.

The Role of Differential Gene Expression in Cell Differentiation

Differentiated cells are recognizably different from one another, sometimes visually as well as in their protein products. For example, certain cells in our hair follicles continuously produce keratin, the protein that makes up hair, nails, feathers, and porcupine quills. Other cell types in the body do not produce keratin. In the hair follicle cells, the keratinencoding gene is transcribed; in most other cells in the body, that gene is not transcribed. Activation of the keratinencoding gene is a key step in the differentiation of hair follicle cells.

Generalizing from examples like this one, we may say that *differentiation results from differential gene expression*—that is, from the differential regulation of transcription, posttranscriptional events such as mRNA splicing, and translation in different cell types.

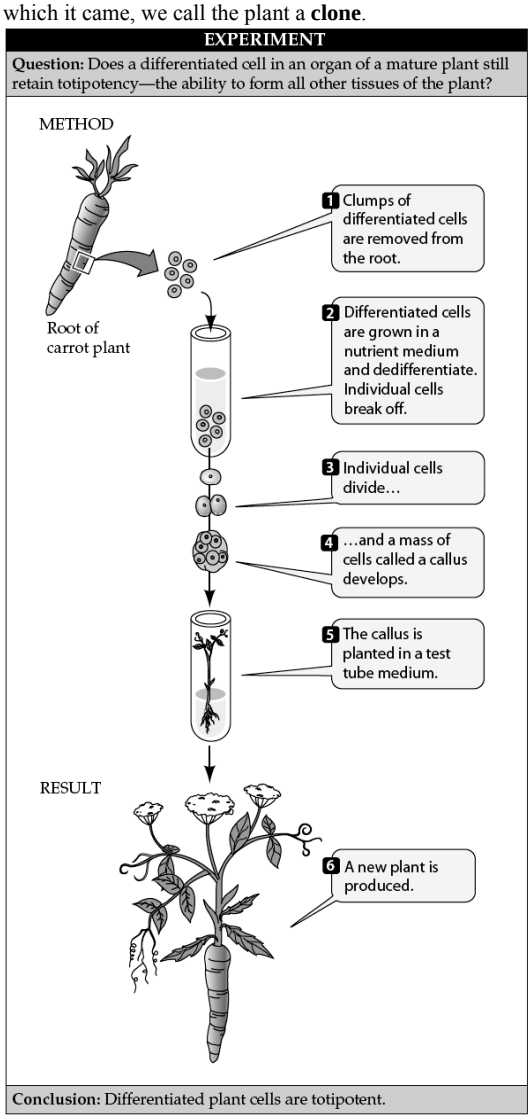
Because the fertilized egg, or **zygote**, has the ability to give rise to every type of cell in the adult body, we say it is **totipotent**. Its genome contains instructions for all of the structures and functions that will arise throughout the life cycle. Later in the development of animals, the cellular descendants of the zygote lose their totipotency and become determined. These determined cells then differentiate into specific types of specialized cells.

With differentiation, there is generally no irreversible change in the genome

Differentiation is irreversible in certain types of cells. Examples include the mammalian red blood cell, which loses its nucleus during development, and the tracheid, a water-conducting cell found in many plants. Tracheid development culminates in the death of the cell, leaving only the pitted cell walls that formed while the cell was alive.

In both of these extreme cases, the irreversibility of differentiation can be explained by the absence of a nucleus. Generalizing about the reversibility of differentiation in mature cells that retain functional nuclei is more difficult. We tend to think of plant differentiation as reversible and of animal differentiation as irreversible, but this is not a hard-andfast rule. Why is differentiation apparently reversible in some cases, such as a plant cutting, but not in others, such as a mammalian limb? At some stage of development, do changes within the nucleus permanently commit a cell to specialization? For both plants and animals, the answer appears to be no. Under the right environmental circumstances, differentiation is reversible in many cells.

TOTIPOTENCY IN PLANTS: A food storage cell in a carrot root normally faces a dark future. It cannot photosynthesize or give rise to new carrot plants. However, if we isolate that cell from the root, maintain it in a suitable nutrient medium, and provide it with appropriate chemical cues, we can "fool" the cell into acting as if it were a fertilized egg. It can divide and give rise to a mass of undifferentiated cells, called a *callus*, and eventually to a complete plant (Figure 3). Since the new plant is genetically identical to the somatic cell from which it came, we call the plant a **clone**.



3 Cloning a Plant Differentiated, specialized food storage cells from the root of a carrot can be induced by the chemical environment to dedifferentiate. These cells then act like early embryonic cells and form a new plant.

The ability of scientists to clone an entire carrot plant from a differentiated root cell indicates that the cell contains the entire carrot genome and that it can express the appropriate genes in the right sequence. Many types of cells from other plant species show similar behavior in the laboratory. This ability to generate a whole plant from a single cell has been invaluable in agricultural biotechnology.

TOTIPOTENCY IN EARLY EMBRYONIC ANIMAL CELLS: Experiments with plants have established that somatic cells are totipotent. Amore direct demonstration that all the genetic material is present in somatic cells has come from nuclear transplantation experiments. Such experiments were first done on frogs by Robert Briggs and Thomas King, who asked whether the nuclei of early frog embryos had lost the ability to do what the totipotent zygote nucleus could do. They first removed the nucleus from an unfertilized egg, thus forming an enucleated egg. Then, with a very fine glass tube, they punctured a cell from an early embryo and drew up part of its contents, including the nucleus, which they injected into the enucleated egg. They stimulated the eggs to divide, and many went on to form embryos, tadpoles, and eventually, frogs. These experiments led to two important conclusions:

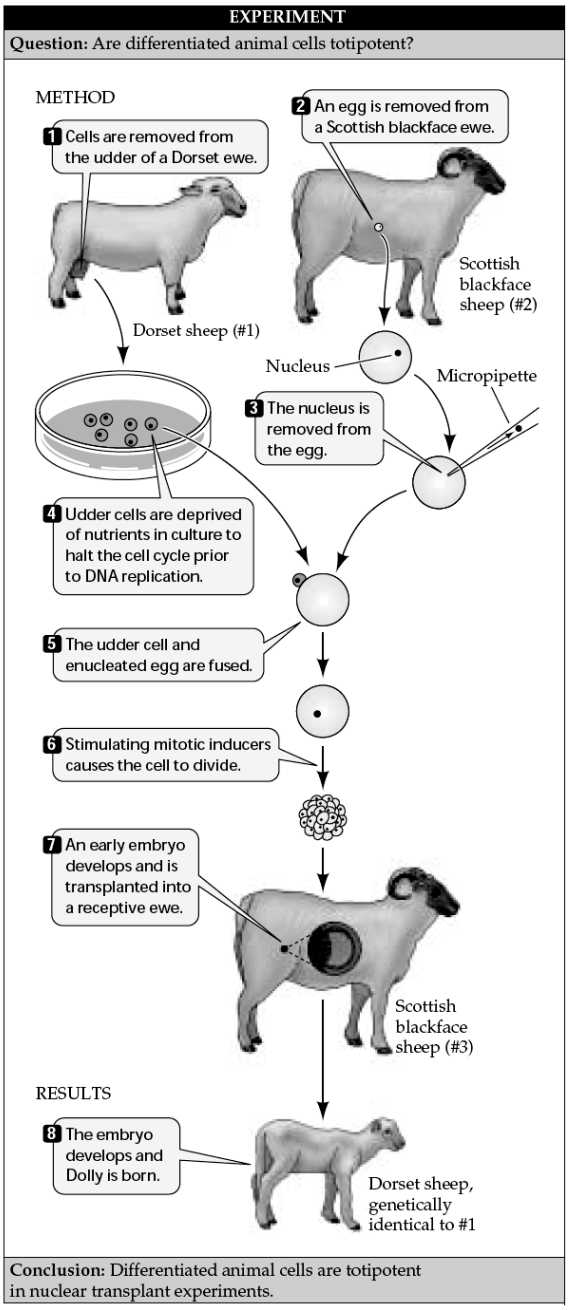
- No information is lost from the nuclei of cells as they pass through the early stages of embryonic development. This fundamental principle of developmental biology is known as **genomic equivalence**.
- The cytoplasmic environment around a nucleus can modify its fate.

Similar experiments have been performed on rhesus monkeys, in which a single cell can be removed from an 8-cell embryo and fused with an enucleated egg. This *cell fusion* technique causes the nucleus of the embryonic cell to enter the egg cytoplasm. The resulting cell acts like a zygote, forming an embryo, which can be implanted into a foster mother, who ultimately gives birth to a normal monkey. Each of the remaining 7 cells from the original embryo can similarly give rise to offspring by the same cell fusion technique.

In humans, the totipotency of early embryonic cells permits both genetic screening and in vitro fertilization. An 8-cell human embryo can be isolated in the laboratory and a single cell removed and examined to determine whether a harmful genetic condition is present. Each remaining cell, being totipotent, can be stimulated to divide and form an embryo, which can be implanted into the mother's uterus, where it develops into an infant.

TOTIPOTENCY IN ADULT SOMATIC CELLS: Successful cloning of animals was very difficult until the late 1990s, when Ian Wilmut and his colleagues at a biotechnology company in Scotland used the cell fusion procedure to clone sheep (Figure 4). Previous attempts to produce mammals by this method had worked, as in the rhesus monkey case, only if the donor nucleus was from an early embryo. Apparently, when mammalian donor cells were in the G2 phase of the cell cycle and were fused with the cytoplasm of eggs that were also in G2, some extra DNAreplication took place that created havoc with the cell cycle in the egg when it attempted to divide.

4 A Clone and Her Offspring In 1996, the experimental procedure described here produced the first cloned mammal, a Dorset sheep named Dolly. Dolly died in 2003 from lung disease, but in her lifetime she mated and gave birth to a "normal" offspring (the lamb on the right in the photo), proving the genetic viability of cloned mammals.

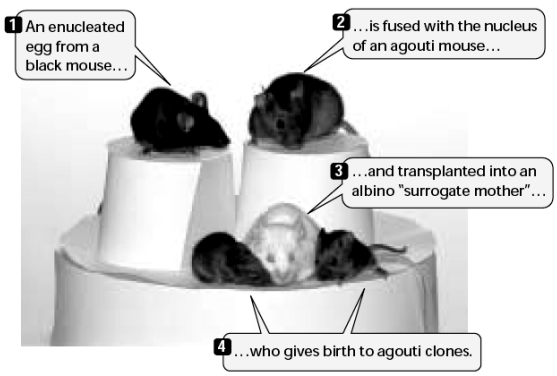


Wilmut took differentiated cells from a ewe's udder and starved them of nutrients for a week, thus halting the cells in G1 phase of the cell cycle. One of these cells was fused with an enucleated egg from a different breed of ewe. When mitotic inducers in the egg cytoplasm were stimulated, the donor nucleus entered S phase, and the rest of the cell cycle proceeded normally. After several cell divisions, the resulting early embryo was transplanted into the womb of a surrogate mother. Out of 277 successful attempts to fuse adult cells with

enucleated eggs, one lamb, named Dolly, survived to be born. DNA analyses confirmed that Dolly was genetically identical to the ewe from whose udder the donor nucleus had been obtained.

A major goal of Wilmut's experiment was to develop a method of cloning sheep that would produce products such as pharmaceuticals in their milk. The cloning procedure could make multiple, identical copies of transgenic sheep that are reliable producers of drugs such as α1-antitrypsin, which is used to treat people with emphysema or cystic fibrosis.

The trick of starving donor cells for cloning has been applied to other mammals. Mice have been cloned using the somatic cells surrounding the egg as a source of donor nuclei (Figure 5). Cattle have been cloned to preserve a rare breed in New Zealand. Genetically engineered goats have been cloned to produce several useful proteins in their milk. And, as we described at the beginning of this chapter, cloning is being done to preserve and expand endangered species. A private company has been set up that will clone your pet by nuclear transfer. This flurry of cloning has touched off a flurry of controversy, but cloning is not a new scientific concept. The idea of totipotency was accepted long before Dolly was born, but achieving it is an impressive technical achievement.



5 Cloned Mice Because so much is known about mouse genetics and molecular biology, cloned mice may be useful in studies of basic biology.

An example of nuclear totipotency gone awry occurs in a human tumor called a *teratocarcinoma*. Here, a differentiated cell dedifferentiates to form an unspecialized cell. Then it divides, forming a tumor, as occurs in most cancers. But some cells in the tumor redifferentiate to form specialized tissue arrangements. So the tumor can form a large mass of cells inside the abdomen, with some of the cells forming kidney tubules, others hair, and still others teeth! How this redifferentiation occurs is not clear.

Stem cells can be induced to differentiate by environmental signals

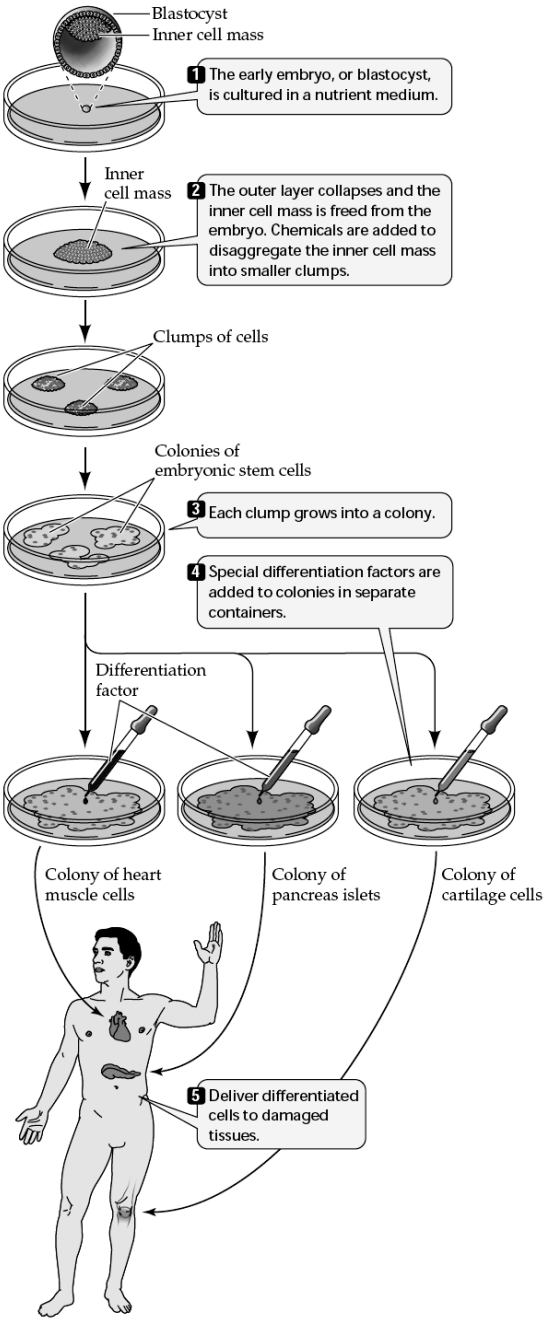
Genomic equivalence implies that a differentiated cell stays specialized because of its environment, not because of its genes, and that appropriate environmental changes could result in a new pattern of differentiation. In normal development, a complex series of signals results in the patterns of differentiation we see in a newborn organism. If these signals could be described in enough detail, we should be able to understand how any cell type becomes any other.

In plants, the growing regions at the tips of the roots and stems contain **meristems**, which are clusters of undifferentiated, rapidly dividing cells. These cells can give rise to the specialized cell types that make up roots and stems, respectively. Plants have many fewer (15–20) cell types than animals (as many as 200). Most plant cell types differ in the structure of their cell walls, whereas most animal cell types have specific cytoplasmic characteristics and many cell-specific proteins.

In mammals, **stem cells** are found in adult tissues that need frequent cell replacement, such as the skin, the inner lining of the intestine, and the blood system. As they divide, stem cells produce cells that differentiate to replace dead cells and maintain tissues. In the body, stem cells have limited abilities to differentiate. The stem cells in bone marrow, for example, produce only the various types of red and white blood cells, while the stem cells in the nervous system produce only the various types of nerve cells.

Can one kind of stem cell be manipulated by its environment to produce cells that differentiate into cells of another tissue type? The answer appears to be yes. For example, when stem cells from the brain were transplanted into the bone marrow of mice whose bone marrow stem cells had been depleted, they proceeded to act like bone marrow stem cells, producing blood cells. In the reverse experiment, bone marrow stem cells were implanted into the brains of mice, where they formed nerve cells. These experiments indicate that some component of the environment—presumably acting through intercellular signals—determines what a stem cell will do.

The stem cell populations that are closest to totipotency are not the ones found in adults, but those of the early embryo. In mice, these embryonic stem cells can be removed from an early embryo (called a *blastocyst*) and then induced to differentiate in some particular way. Normally, these cells are formed a few days after fertilization, and their fate in the developing embryo is soon determined. Before that time, however, they are virtually totipotent. Such cells can be grown indefinitely in the laboratory and, when injected back into a mouse blastocyst, will mix with the resident cells and differentiate to form all the cell types of the mouse. This kind of experiment shows that blastocyst cells do not lose any of their developmental potential while growing in the laboratory. Embryonic stem cells growing in the laboratory can be induced to differentiate if the right signal is provided (Figure 6). For example, treatment of mouse embryonic stem cells with a derivative of vitamin A causes them to form nerve cells, while other growth factors induce them to form blood cells, again demonstrating their developmental potential and the roles of environmental signals. This finding raises the possibility of using stem cell cultures as sources of differentiated cells for clinical medicine. Akey advance toward this use has been the ability to grow human embryonic stem cells in the laboratory.



6 The Potential Use of Embryonic Stem Cells in Medicine
Human embryonic stem cells can be cultured in the laboratory and induced to differentiate. Their use as transplants to replace damaged tissue is under intensive investigation.

A source for embryonic stem cells could be human embryos made for in vitro fertilization. This medical procedure is used by couples who want a child but cannot conceive naturally. Up to ten eggs are taken from the mother's ovaries and exposed to the father's sperm, with the hope that some early embryos will form. A few of these embryos are then implanted into the mother's uterus for development. Any remaining embryos not used for implantation could be a source of stem cells.

But a problem arises if these embryonic stem cells are induced to differentiate to form a tissue for transplantation— say, pancreatic tissue for a patient with diabetes. The cells and the recipient are genetically different, so the recipient's immune system may reject the transplanted cells . This problem has led to the proposal of **therapeutic cloning**, in which nuclear transplantation and stem cell technologies would be combined. This procedure would require several steps:

- Eggs are removed from a female donor.
- An egg is enucleated.
- A cell is removed from the recipient.
- The entire cell, or its nucleus only, is fused with the enucleated egg.
- The egg is stimulated to divide.
- Embryonic stem cells form; these cells are genetically the recipient's.
- The stem cells are induced to differentiate into the desired tissue for transplantation.

Progress with this ambitious program has been slow but steady. The age of custom-made cells to replace those lost to disease or injury is rapidly approaching.

Genes are differentially expressed in cell differentiation

Nuclear transplantation, cell fusion, and plant cell cloning have demonstrated genomic equivalence in somatic cells of an organism. Molecular experiments have provided even more convincing evidence. For example, the gene for β-globin, one of the protein components of hemoglobin, is present and expressed in red blood cells as they form in the bone marrow of mammals. Is the same gene also present—but unexpressed—in nerve cells in the brain, which do not make hemoglobin?

Nucleic acid hybridization can provide an answer. A probe for the □-globin gene can be applied to DNAfrom both brain cells and immature red blood cells (recall that mature red blood cells lose their nuclei and DNA). In both cases, the probe finds its complement, showing that the β-globin gene is present in both types of cells. On the other hand, if the probe is applied to cellular mRNA, rather than cellular DNA, it finds β-globin mRNA only in the red blood cells, and not in the brain cells. This result shows that the gene is expressed in only one of the two tissues. Many similar experiments have shown convincingly that differentiated cells lose none of the genes that were present in the fertilized egg.

What leads to this differential gene expression? One wellstudied example of differentiation is the conversion of undifferentiated muscle precursor cells, called *myoblasts*, into the large, multinucleated *muscle fibers* that make up mammalian skeletal muscles. The key event that starts this conversion is the expression of *MyoD1* (*myo*blast-*d*etermination gene 1). The protein product of this gene is a transcription factor (MyoD1) with a helix-loop-helix domain, which not only binds to the promoters of muscle-determining genes to stimulate their transcription, but also acts on its own promoter to keep its levels high in the myoblasts and in their descendants.

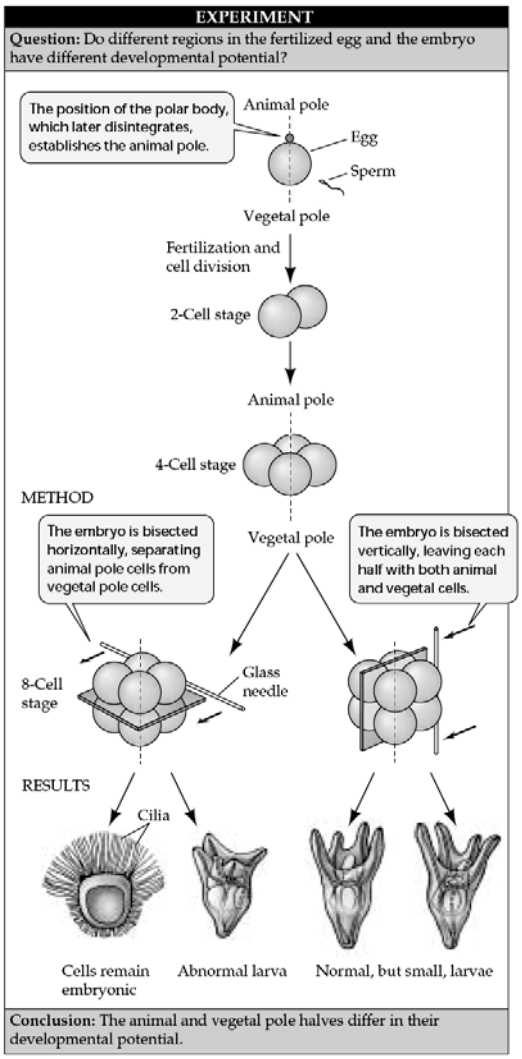
Strong evidence for the controlling role of *MyoD1* in muscle fiber differentiation comes from experiments in which a sequence containing an active promoter adjacent

to *MyoD1* is transfected into the precursors of other cell types. For example, if this sequence is added to fat cell precursors, the fat cells are reprogrammed to become muscle cells. Genes such as *MyoD1* that direct fundamental decisions in development, often by regulating genes on other chromosomes, usually encode transcription factors.

The Roles of Cytoplasmic Segregation and Induction in Cell Determination

What initially stimulates the *MyoD1* promoter to begin transcription is not clear, but chemical signals are clearly involved in cell differentiation. In general, two overall mechanisms for producing such signals have been found:

- **Cytoplasmic segregation**. A factor within an egg, zygote, or precursor cell is unequally distributed in thecytoplasm. After cell division, the factor ends up in some daughter cells or regions of cells, but not others.
- **Induction**. A factor is actively produced and secreted by certain cells to induce other cells to differentiate.



7 Asymmetry in the Early Embryo The upper (animal) and lower (vegetal) halves of the sea urchin egg differ in the cytoplasmic determinants they contain. Cells from both halves are necessary to produce a normal larva.

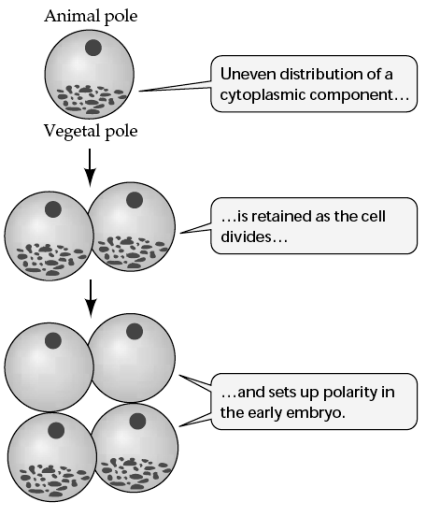
Polarity results from cytoplasmic segregation

As we learned from the cloning experiments described above, cell nuclei do not undergo irreversible changes during early development, so we must look for explanations of some embryological events in the *cytoplasmic* differences between cells. The development of **polarity**—the difference between one end of an organism and the other—is one such phenomenon. Polarity is obvious throughout development.

Our heads are distinct from our feet, and the distal ends of our arms (wrists and fingers) differ from the proximal ends (shoulders). An animal's polarity may develop early, even in the egg itself, in which yolk and other factors may be distributed asymmetrically.

An experiment with sea urchins demonstrates the effects of cytoplasmic segregation on development (Figure 7). Very early development in this species occurs by equal mitotic divisions of the fertilized egg; there is no increase in size at this stage. If an 8-cell embryo is cut vertically, both halves develop normally. On the other hand, if the embryo is cut horizontally, the top half does not develop at all and the bottom half develops into a small, abnormal embryo.

Clearly, then, there must be at least one factor essential for development that is segregated in the bottom half of the egg, such that the bottom cells of the embryo have it, but the top ones do not. This and many other experiments have established that certain materials, called **cytoplasmic determinants**, are distributed unequally in the egg cytoplasm, and that these materials play a role in directing the embryonic development of many organisms (Figure 8).

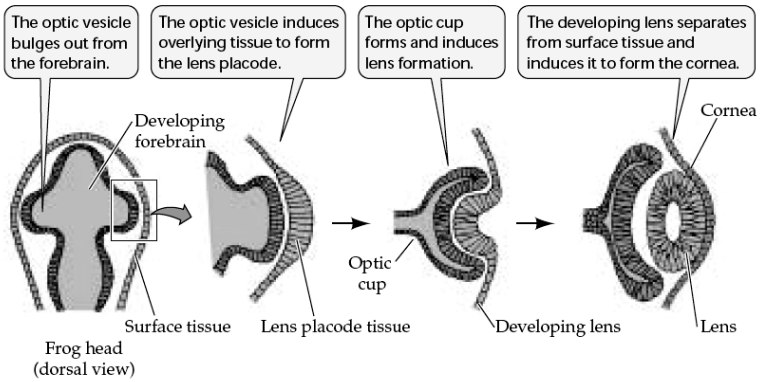


8 The Principle of Cytoplasmic Segregation The distribution of a cytoplasmic substance may determine cell fate.

Tissues direct the development of their neighbors by secreting inducers

Experimental work on developing embryos has clearly established that in many cases, the fates of particular tissues are determined by interactions with other specific tissues in the embryo. In developing animal embryos there are many such instances of induction, in which one

tissue causes an adjacent tissue to develop in a particular manner. These effects are mediated by intercellular biochemical communication—that is, by chemical signals and signal transduction mechanisms. We will describe two examples of such induction: one in the developing vertebrate eye, and the other in a developing reproductive structure in the nematode *C. elegans*.



9 Embryonic Inducers in the Vertebrate Eye The eye of a frog develops as different tissues take their turns inducing one another.

The development of the lens of the vertebrate eye is a classic example of induction. In a frog embryo, the developing forebrain bulges out at both sides to form the *optic vesicles*, which expand until they come into contact with the cells at the surface of the head (Figure 9). The surface tissue in the region of contact with the optic vesicles thickens, forming a *lens placode*. The lens placode bends inward, folds over on itself, and ultimately detaches from the surface tissue to produce a structure that will develop into the lens. If the growing optic vesicle is cut away before it contacts the surface cells, no lens forms. Placing an impermeable barrier between the optic vesicle and the surface cells also prevents the lens from forming. These observations suggest that the surface tissue begins to develop into a lens when it receives a signal—an *embryonic inducer*—from the optic vesicle. The interaction of tissues in eye development is a two-way street: There is a "dialogue" between the developing optic vesicle and the surface tissue. The optic vesicle induces lens development, and the developing lens determines the size of the *optic cup* that forms from the optic vesicle. If head surface tissue from a frog species with small eyes is grafted over the optic vesicle of one with large eyes, both lens and optic cup will have an intermediate size.

The developing lens also induces the surface tissue over it to develop into a *cornea*, a specialized layer that allows light to pass through and enter the eye. Thus a chain of inductive interactions participates in the development of the parts required to make an eye. Embryonic inducers trigger a sequence of gene expression in the responding cells. Tissues do not induce themselves; rather, different tissues interact and induce one another.

Single cells can induce changes in their neighbors

The tiny nematode *Caenorhabditis elegans* is used as a model organism in many biological studies, but it is especially useful for studying development. It normally lives in the soil, where it feeds on bacteria, but can also grow in the laboratory if supplied with its food source. The process of development from fertilized egg to larva takes only about 8 hours, and the worm reaches the adult stage in just 3.5 days. The process is easily observed using a low-magnification dissecting microscope because the body covering is transparent (Figure 10*a*). For all these reasons, *C. elegans* is a favorite experimental organism. The development of *C. elegans* does not vary, so it has been possible to identify the source of each of the 959 somatic cells of the adult form.

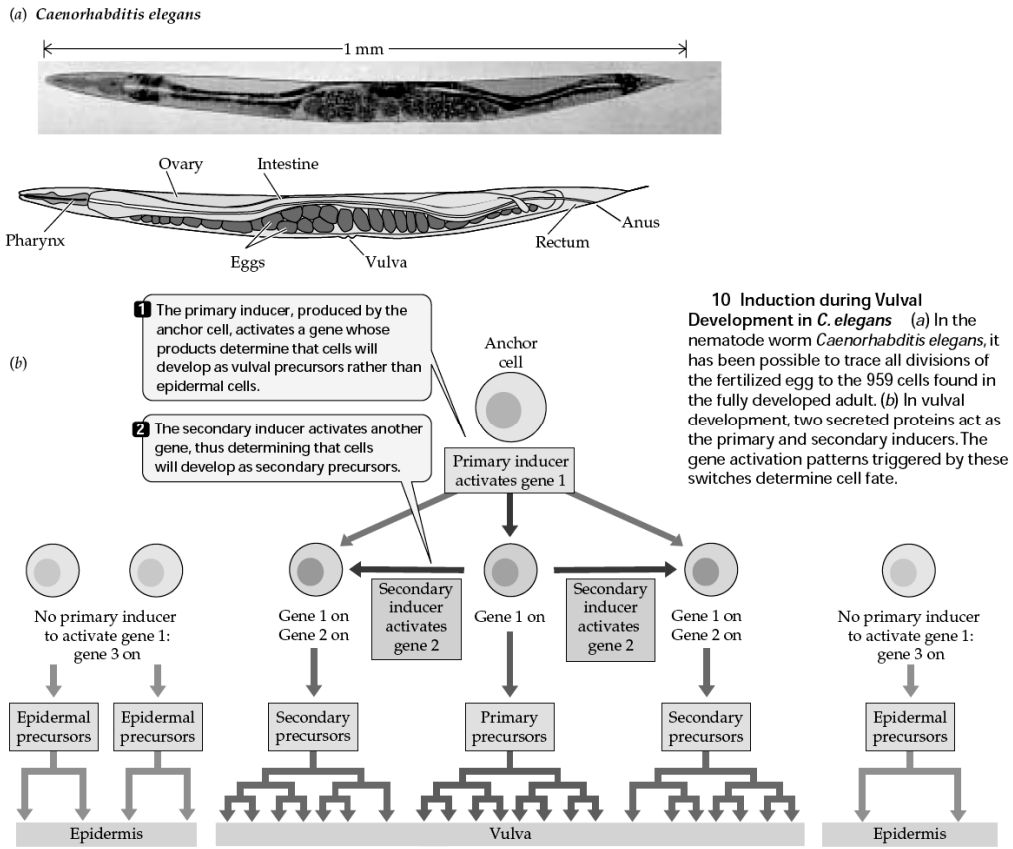
The adult nematode is *hermaphroditic*, containing both male and female reproductive organs. It lays eggs through a pore called the *vulva* on the ventral (belly) surface. During development, a single cell, called the *anchor cell*, induces the vulva to form. If the anchor cell is destroyed by laser surgery, no vulva forms. The eggs develop inside the parent, and a "bag of worms," which eventually consume the parent,
results.

The anchor cell controls the fates of six cells on the animal's ventral surface through two molecular switches. Each of these cells has three possible fates: It may become a pri mary vulval precursor cell, a secondary vulval precursor cell, or simply part of the worm's surface—an epidermal cell (Figure 10*b*).

The anchor cell produces an inducer that diffuses out of the cell and interacts with adjacent cells. Cells that receive enough of the inducer become vulval precursor cells; cells slightly farther from the anchor cell become epidermis. The first molecular switch, controlled by the inducer from the anchor cell, determines whether a cell takes the "track" toward becoming part of the vulva or the track toward becoming epidermis.

The cell closest to the anchor cell, having received the most inducer, differentiates into the primary vulval precursor cell. It produces its own inducer, which acts on the two neighboring cells and directs them to become secondary vulval precursor cells. Thus, the primary vulval precursor cell controls a second molecular switch, determining whether a vulval precursor will take the primary track or the secondary track. The two inducers control the activation or inactivationof specific genes in the responding cells.

There is an important lesson to draw from this example: *Much of development is controlled by molecular switches that allow a cell to proceed down one of two alternative tracks*. One challenge for the developmental biologist is to find these molecular switches and determine how they work. The primary inducer released by the *C. elegans* anchor cell appears to be a growth factor homologous to the mammalian epidermal growth factor (EGF). The nematode growth factor, called LIN-3, binds to a receptor on the surface of a potential vulval precursor cell. This binding sets in motion a signal transduction cascade involving the Ras protein and MAP kinases. The end result is increased transcription of the genes involved in the differentiation of vulval cells.



10 Induction during Vulval Development in *C. elegans* (*a*) In the nematode worm *Caenorhabditis elegans*, it has been possible to trace all divisions of the fertilized egg to the 959 cells found in the fully developed adult. (*b*) In vulval development, two secreted proteins act as the primary and secondary inducers. The gene activation patterns triggered by these switches determine cell fate.

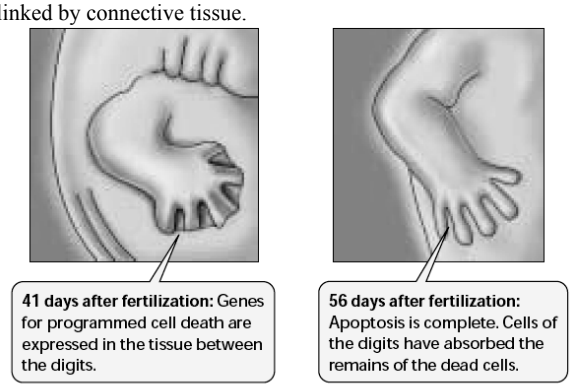
The Role of Pattern Formation in Organ Development

Pattern formation, the spatial organization of a tissue or organism, is inextricably linked to morphogenesis, the appearance of body form. The differentiation of cells is beginning to be understood in terms of molecular events, but how do molecular events contribute to the organization of multitudes of cells into specific body parts, such as a leaf, a flower, a shoulder blade, or a tear duct?

Some cells are programmed to die

Apoptosis is programmed cell death, a series of events caused by the expression of certain genes. Many of these "death genes" have been identified, and related ones have been found in organisms as diverse as nematodes and humans.

Apoptosis is vital to the normal development of all animals. For example, the nematode *C. elegans* produces precisely 1,090 somatic cells as it develops from a fertilized egg to an adult (see Figure 10). But 131 of these cells die. The sequential expression of two genes, called *ced-4* and *ced-3* (for *c*ell *d*eath) appears to control this process. In the nervous system, for example, there are 302 nerve cells that come from 405 precursors; thus 103 cells undergo apoptosis. If the protein encoded by either *ced-3* or *ced-4* is nonfunctional, all 405 cells form nerve cells, and disorganization results. A third gene, *ced-9*, codes for an inhibitor of apoptosis; that is, its protein blocks the function of the *ced-4* gene. So, where cell death is required, *ced-3* and *ced-4* are active and *ced-9* is inactive; where cell death does not occur, the reverse is true. Asimilar system of cell death genes acts in humans. During early development, human hands and feet look like tiny paddles: The fingers and toes are linked by connective tissue.



11 Apoptosis Removes the Tissue between Fingers Early in the second month of human development, the tissue connecting the fingers is removed by apoptosis, freeing the individual fingers.

Between days 41 and 56 of development, the cells between the digits die, freeing the individual fingers and toes (Figure 11).

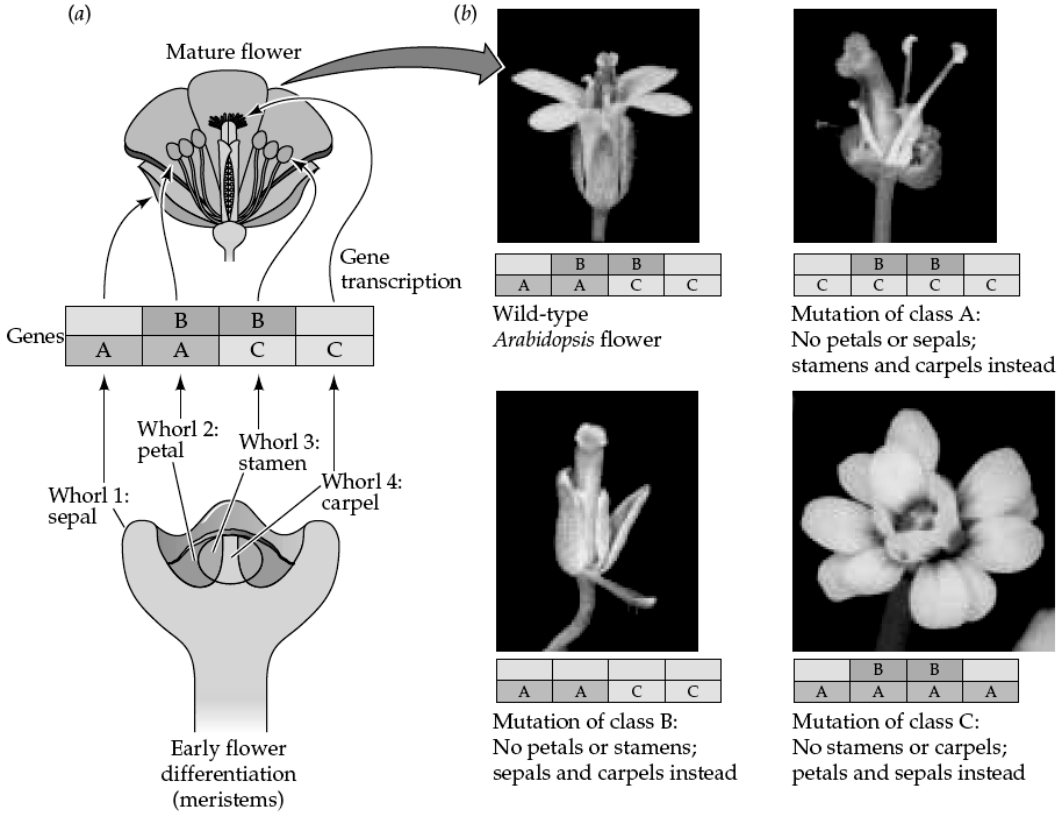
The protein—an enzyme called *caspase*—that stimulates this apoptosis is similar in amino acid sequence to the

protein encoded by *ced-3*, and a human protein (*bcl-2*) that inhibits apoptosis is similar to *ced-9*. So humans and nematodes, two creatures separated by more than 600 million years of evolutionary time, have similar genes controlling programmed cell death.

Apoptosis plays many other roles in your life. The dead cells that form the outermost layer of your skin and those from the uterine wall that are lost during menstruation have undergone apoptosis. White blood cells live only a few months in the circulation, then undergo apoptosis. In a form of cancer called *follicular large-cell lymphoma*, these white blood cells do not die, but continue to divide. This cancer results from a mutation that causes the overexpression of *bcl- 2*, the gene that inhibits cell death.

Plants have organ identity genes

Like animals, plants have organs—for example, leaves and roots. Many plants form flowers, and many flowers are composed of four types of organs: sepals, petals, stamens, and carpels. These floral organs occur in *whorls*, which are groups of each organ type stacked around a central axis. The whorls develop from meristems in the shape of domes, which develop at growing points on the stem (Figure 12*a*). How is the identity of a particular whorl determined? The answer appears to lie in the activities of a group of genes.



12 Organ Identity Genes in Arabidopsis Flowers (a) The four organs of a flower—carpel, stamens, petals, and sepals—grow in whorls that develop from meristems. (b) When a mutation in one of three organ identity genes occurs, one type of organ replaces another. Such mutations helped scientists decipher the pattern of gene expression that gives rise to normal flowers.

These genes have been best described in *Arabidopsis thaliana*. This plant is very useful for studies of development because of its small size (about 25 cm), abundant seed production (over 1,000 seeds per plant), rapid development (from seed to plant to seed in 6 weeks), and small genome. Finally, it is easy to produce mutations in this plant by treating the seeds with mutagens.

The development of the flower begins with the meristem, which contains undifferentiated cells. Within this seemingly homogeneous cell population, individual cells "sense" their position and differentiate into the whorls.

This happens through the expression of three **organ identity genes**, which code for proteins that act in combination with one another:

- Gene Ais expressed in whorls 1 and 2 (which form sepals and petals, respectively).
- Gene B is expressed in whorls 2 and 3 (which form petals and stamens, respectively).
- Gene C is expressed in whorls 3 and 4 (which form stamens and carpels, respectively).

There are two lines of experimental evidence for this model (Figure 12*b*):

- *Loss of function mutations:* For example, a mutation in gene Aresults in no sepals or petals.
- *Gain of function mutations:* For example, the promoter for gene C can be coupled to gene A. In this case, Ais expressed in all four whorls, resulting in only sepals and petals.

Genes A, B, and C code for subunits of transcription factors, which are active as dimers. Gene regulation in these cases is *combinatorial*—that is, the composition of the dimer determines which other genes will be activated by the transcription factor. For example, a dimer made up only of transcription factor A would activate transcription of the genes that make sepals; a dimer made up of Aand B would result in petals, and so forth.

A common feature of the A, B, and C proteins, as well as many other plant transcription factors, is a DNA-binding domain called the **MADS box** (named for homologous regions found in four genes in yeast and in two in plants and humans, that all encode a similar amino acid sequence). These 200-amino acid proteins also have domains for interaction with other proteins.

In addition to being fascinating to biologists, plant organ identity genes have caught the eye of horticultural and agricultural scientists. Flowers filled with petals instead of stamens and carpels often have mutations of the C genes. Many of the foods that make up the human diet, such as the grains of wheat, rice, and corn, come from fruits and seeds. These fruits and seeds form from the carpels (the female reproductive organs) of the flower. Genetically modifying the number of carpels on a particular plant could increase the amount of grain a crop could produce.

A gene called *leafy* codes for a protein that controls the transcription of the ABC genes. Plants with a mutation that causes the underexpression of *leafy* are just that—they make leaves, but no flowers. The protein product of this gene acts as a transcription factor stimulating genes A, B, and C so that they produce flowers (Figure 13).

Wild-type *Leafy* mutant

This finding, too, has practical applications. It usually takes 6–20 years before a citrus tree produces flowers, and thus the fruits we eat. Scientists have made an orange tree transgenic for *leafy* coupled to a strongly expressed promoter, which flowers and fruits years earlier than a normal tree.

Morphogen gradients provide positional information

During development, cells often need to "know" where they are with respect to the body as a whole, as in the case of the whorls in the flowers just described. This spatial "sense" is called **positional information**. Positional information usually comes in the form of a signal, called a **morphogen**, that diffuses from one group of cells down a body axis, setting up a concentration gradient. There are two requirements for a signal to be considered a morphogen:

- It must directly affect target cells, rather than triggering a secondary signal that affects target cells.
- Different concentrations of the signal must cause different effects.

The development of the vertebrate limb provides us with an example of a morphogen in action. The limb develops from a round bud. The cells that become the bones and muscles of the limb must receive positional information. If they do not, the limbs will be totally disorganized (imagine fingers growing out of your shoulders). Agroup of cells at the posterior base of the bud, just where it joins the body wall, makes a morphogen called BMP2, whose gradient determines the anterior–posterior ("thumb to little finger") axis of the developing limb. Cells getting the highest dose of BMP2 make the thumb, and the smallest dose results in the little finger.

The different concentrations of morphogens act through differential regulation of gene expression in their target cells. The model organism often used for studying this process has been the fruit fly.

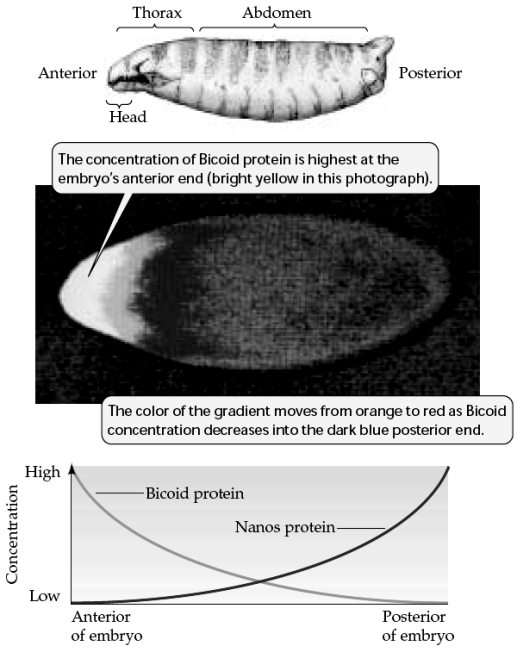
The Role of Differential Gene Expression in Establishing Body Segmentation

Insects such as the fruit fly *Drosophila melanogaster* develop a highly modular body composed of different types of segments. Complex interactions of different sets of genes underlie the pattern formation of segmented bodies. Unlike the body segments of segmented worms such as earthworms, which are all essentially alike, the segments of the *Drosophila* body are clearly different from one another. The adult fly has an anterior head (composed of several fused segments), three different thoracic segments, and eight abdominal segments at the posterior end. In the *Drosophila* larva, the thoracic and abdominal segments all appear to be similar, but they have already received their instructions to form these specialized adult segments. Several types of genes are expressed sequentially in the embryo to define these segments. The first step in this process is to establish the polarity of the embryo.

Maternal effect genes encode morphogens that determine polarity

Like those of the sea urchin, *Drosophila* eggs and larvae are characterized by unevenly distributed cytoplasmic determinants. These molecules, which include both mRNAs and proteins, are the products of specific **maternal effect genes**. The maternal effect genes are transcribed in the mother's ovarian cells, which surround and nurture the developing egg and deliver the gene products to specific regions of the egg as it forms. Maternal effect genes exert their effects on the embryo regardless of the genotype of the father. Their products establish the dorsal–ventral (back–belly) and anterior–posterior (head–tail) axes of the embryo.

The fact that these morphogens specify these axes was established by the results of experiments in which cytoplasm was transferred from one egg to another. Females that are homozygous for a particular mutation of the maternal effect gene *bicoid* produce larvae with no head and no thorax. However, if the eggs of these females are inoculated at the anterior end with cytoplasm from the anterior region of a wildtype egg, the treated eggs develop into normal larvae.



14 Bicoid and Nanos Protein Gradients Provide Positional Information The anterior–posterior axis of *Drosophila* arises from morphogens produced by the maternal effect genes *bicoid* and *nanos*. The gradients of these morphogens control the developing body's polarity.

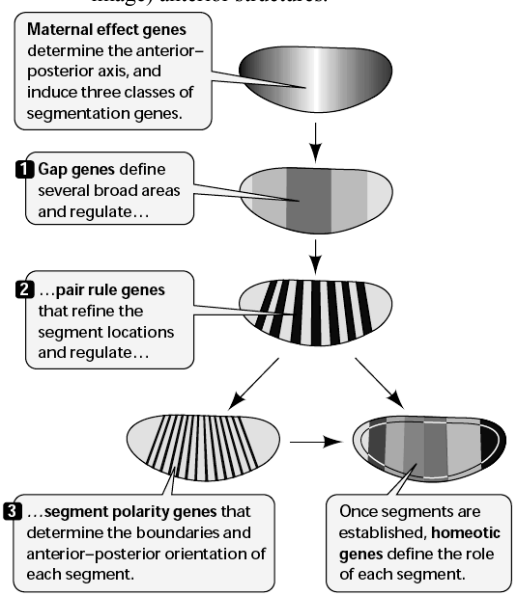
Conversely, removal of 5 percent or more of the cytoplasm from the anterior end of a wild-type egg results in an abnormal larva that looks like a *bicoid* mutant larva. Another maternal effect gene, *nanos*, plays a comparable role in the development of the posterior end of the larva. Eggs from homozygous *nanos* mutant females develop into larvae with missing abdominal segments. Injecting cytoplasm from the posterior region of a wild-type egg into a *nanos* mutant egg allows normal development. These findings show that, in wild-type larvae, the overall framework of the anterior–posterior axis is laid down by the activity of these two maternal effect genes (Figure 14).

After the axes of the embryo are determined, the next step in pattern formation is the determination of the larval segments.

Segmentation and homeotic genes act after the maternal effect genes

The number, boundaries, and polarity of the larval segments are determined by proteins encoded by the **segmentation genes**. These genes are expressed when there are about 6,000 nuclei in the embryo. These nuclei all look the same, but in terms of gene expression, they are not. The products of the maternal effect genes set the segmentation genes in motion. Three classes of segmentation genes act, one after the other, to regulate finer and finer details of the segmentation pattern (Figure 15):

- **Gap genes** organize broad areas along the anterior–posterior axis. Mutations in gap genes result in gaps in the body plan—the omission of several larval segments.
- **Pair rule genes** divide the embryo into units of two segments each. Mutations in pair rule genes result in embryos missing every other segment.
- **Segment polarity genes** determine the boundaries and anterior–posterior organization of the segments. Mutations in segment polarity genes can result in segments in which posterior structures are replaced by reversed (mirror-image) anterior structures.



15 A Gene Cascade Controls Pattern Formation in the *Drosophila* Embryo Gap, pair rule, and segment polarity genes are collectively referred to as the segmentation genes. The shading shows the locations of their gene products in the embryo.

Finally, after the basic pattern of segmentation has been established by the segmentation genes, differences between the segments are mediated by the activities of **homeotic genes**. These genes are expressed in different combinations along the length of the body and tell each segment what to become. Homeotic genes are analogous to the organ identity genes of plants. The maternal effect, segmentation, and homeotic genes interact to "build" a *Drosophila* larva step by step, beginning with the unfertilized egg.

Drosophila development results from a transcriptionally controlled gene cascade

One of the most striking and important observations about development in *Drosophila*—and in other animals—is that it results from a sequence of changes, with each change triggering the next. This sequence, or cascade, is largely controlled at the levels of transcription and translation. Most unfertilized eggs are storehouses of mRNAs, which are supplied by the mother to support protein synthesis during the early stages of embryonic development. Indeed, zygotes and early embryos do not

carry out transcription. Only after several cell divisions does transcription begin, forming the mRNAs needed for later development.

Cytoplasmic segregation of the prefabricated mRNAs in the egg provides positional information. Before the *Drosophila* egg is fertilized, mRNA for the Bicoid protein is localized at the end that is destined to become the anterior end of the fly. After the egg is fertilized and laid, nuclear divisions begin. (In *Drosophila*, cytokinesis does not begin right away; until the thirteenth nuclear division, the embryo is a single, multinucleated cell called a *syncytium*.) At this early point, *bicoid* mRNAis translated, forming Bicoid protein, which diffuses away from the anterior end, establishing a gradient. At the posterior end, the Nanos protein forms a gradient in the other direction. Thus each nucleus in the developing embryo is exposed to a different concentration ratio of Bicoid and Nanos proteins. The two morphogens regulate the expression of the gap genes, although in different ways. The Bicoid protein affects their transcription, while the Nanos protein affects their translation. The high concentrations of Bicoid protein in the anterior portion of the egg turn on a gap gene called *hunchback*,
while simultaneously turning off another gap gene, *Krüppel*. Nanos at the posterior end reduces the translation of *hunchback*, so a difference in the concentration of these two gap genes' products at the two ends is established.

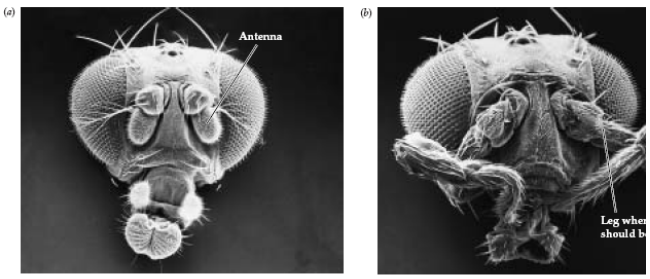
The proteins encoded by the gap genes control the expression of the pair rule genes. Many pair rule genes, in turn, encode transcription factors that control the expression of the segment polarity genes, giving rise to a complex, striped pattern (see Figure 15) of expression that foreshadows the segmented body plan of *Drosophila*. By this point, each nucleus of the embryo has been exposed to a distinct set of transcription factors. The segmented body pattern of the larva has been established even before any sign of segmentation is visible. When the segments do appear, they are not all identical, because the homeotic genes specify the different structural and functional properties of each segment. Each homeotic gene is expressed over a characteristic portion of the embryo. Let's turn now to the homeotic genes and see how their mutation can alter the course of development.

Homeotic mutations produce changes in segment identity

Two bizarre homeotic mutations in *Drosophila* are the *Antennapedia* mutation, in which legs grow in place of antennae (Figure 16), and the *bithorax* mutation, in which an extra pair of wings grows in a thoracic segment.

Edward Lewis at the California Institute of Technology found that *Antennapedia* and *bithorax* were mutations not of isolated genes, but of two adjacent clusters of genes that determine the identity of body segments. Moreover, the genes in these clusters were lined up along the chromosome in the same order as the segments they determined.

From left to right, genes in the first cluster specified anterior body segments, starting with genes for the different head segments and ending with thoracic segments. The second cluster began with a gene specifying the last thoracic segment, followed by a gene for the anterior abdominal segments, and ended with a gene for the posterior abdominal segments. Lewis hypothesized that all of these genes might have come from the duplication of a single gene in an ancestral, unsegmented organism. Molecular biologists confirmed Lewis's hypothesis using nucleic acid hybridization. Several scientists found that a probe for a sequence in one of the genes of one cluster bound not only to its own gene, but also to adjacent genes in its cluster and to genes in the other homeotic cluster. In other words, this DNA sequence is common to all the homeotic genes in both clusters.



16 A Homeotic Mutation in Drosophila Mutations of the homeotic g cause body parts to form on inappropriate segments. (a) A wild-typ fly. (b) An Antennapedia mutant fruit fly.

Homeobox-containing genes encode transcription factors

The 180-base-pair DNA sequence that is common to the bithorax and Antennapedia gene clusters is called the **homeobox**. It encodes a 60-amino acid sequence, called the *homeodomain*, that binds to DNA. The homeodomain turns out to be present in other proteins involved in *Drosophila* pattern formation, such as Bicoid. In all cases, the homeodomain portion of the protein has a helix-turn-helix motif (see Figure 15). Each type of homeodomain recognizes a specific DNA sequence in the promoter of its target genes. The Bicoid homeodomain, for example, recognizes TCCTAATCCC. What do homeodomain proteins do when they recognize their target sequence in DNA? Not surprisingly, they are transcription factors. The Bicoid protein, for example, binds to promoters of the gap gene *hunchback*, activating its transcription. The Hunchback protein is also a transcription factor, which binds to enhancers of genes involved in head and thorax formation. In this way, the homeodomain proteins produce the cascade of events that controls *Drosophila* development. Homeobox genes are found in many animals, including humans. They play a role in development similar to the role the MADS box genes play in plants.

B. Gametogenesis, fertilization and early development:

Sexual Reproduction

A large portion of the time and energy budgets of sexually reproducing animals goes into mating, which exposes them to predation, can result in physical damage, and detracts from other useful activities, such as feeding and caring for existing offspring. Furthermore, mating requires that resources be used to maintain a large population of males that do not bear offspring. In spite of all of these disadvantages, there is an overwhelming evolutionary advantage to sexual reproduction: It produces genetic diversity.

Sexual reproduction requires the joining of two haploid sex cells to form a diploid individual. These haploid cells, or *gametes*, are produced through **gametogenesis**, a process that involves meiotic cell divisions. Two events in meiosis contribute to genetic diversity: crossing over between homologous chromosomes and the independent assortment of chromosomes.

Sexual reproduction itself also contributes to genetic diversity. The genetic variation among the gametes of a single individual and the genetic variation between any two parents produce an enormous potential for genetic variation between any two offspring of a sexually reproducing pair of individuals. This genetic diversity is the raw material for natural selection.

Sexual reproduction in animals consists of three fundamental steps:

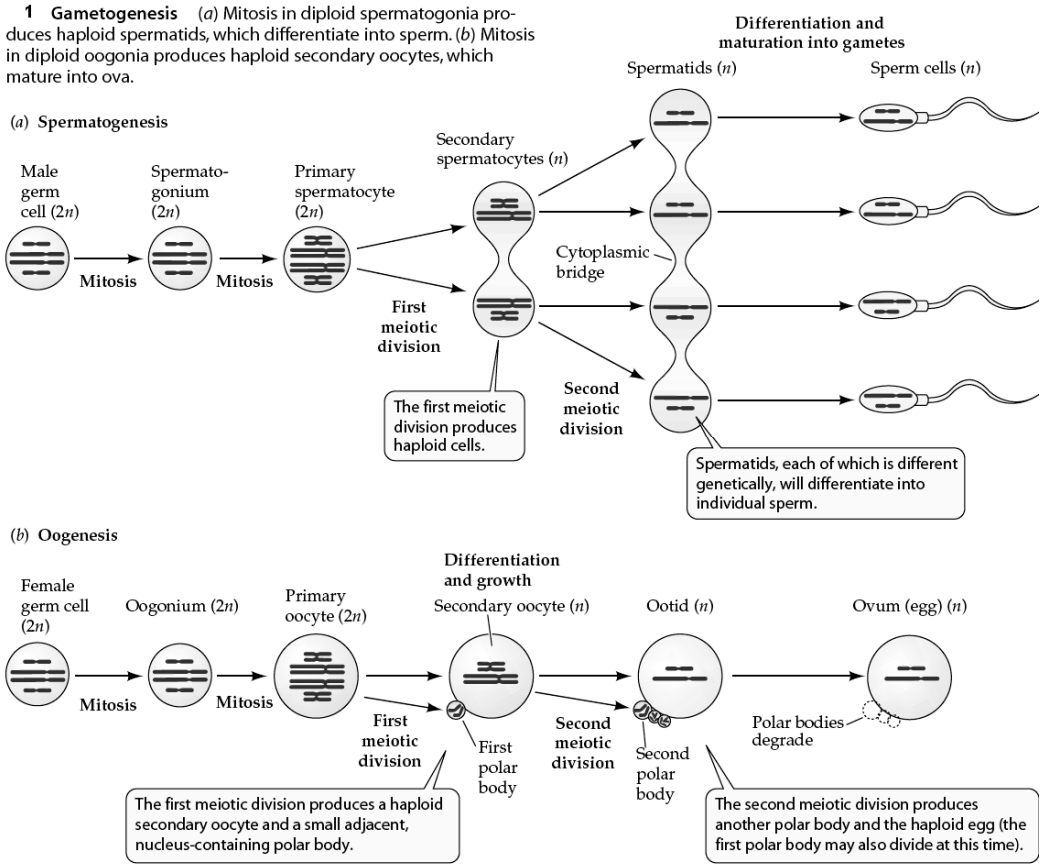
- *Gametogenesis* (making gametes)
- *Mating* (g etting gametes together)
- *Fertilization* (getting gametes to fuse)

There is not a great deal of diversity in gametogenesis when we compare different groups of animals. Processes of fertilization are also rather similar in widely different species. Therefore, although the discussion of gametogenesis that follows focuses primarily on mammals, and our discussion of fertilization mainly deals with sea urchins, the facts would not be dramatically different if we focused on a different group of animals. Adaptations for mating, on the other hand, show incredible anatomical, physiological, and behavioral diversity.

Eggs and sperm form through gametogenesis

Gametogenesis occurs in the **gonads**, which are **testes** (singular, testis) in males and **ovaries** in females. The tiny gametes of males, called **sperm**, move by beating their flagella. The larger gametes of females, called **eggs** or **ova** (singular, ovum), are nonmotile (see Figure 1).

1 Gametogenesis (*a*) Mitosis in diploid spermatogonia produces haploid spermatids, which differentiate into sperm. (*b*) Mitosis in diploid oogonia produces haploid secondary oocytes, which mature into ova.



Gametes are produced from **germ cells**, which have their origin in the earliest cell divisions of the embryo and remain distinct from the rest of the body. All other cells of the embryo are called *somatic cells*. Germ cells are sequestered in the body of the embryo until its gonads begin to form. The germ cells then migrate to the gonads, where they take up residence and proliferate by mitosis, producing **oogonia** (singular, oogonium) in females and **spermatogonia** (singular, spermatogonium) in males. Oogonia and spermatogonia, which are diploid, also multiply by mitosis, eventually producing **primary oocytes** and **primary spermatocytes**, which are still diploid cells.

Meiosis, the next step in gametogenesis, reduces the chromosomes to the haploid number, and the resulting haploid cells eventually mature into sperm and ova. Although the steps of meiosis are very similar in males and females, there are some significant differences in gametogenesis between the sexes.

SPERMATOGENESIS PRODUCES SPERM: Primary spermatocytes undergo the first meiotic division to form **secondary spermatocytes**, which are haploid. The second meiotic division produces four haploid **spermatids** for each primary spermatocyte that entered meiosis (Figure 1*a*). In mammals, these cells remain connected by cross-bridges of cytoplasm after each division.

The reason that mammalian spermatocytes remain in cytoplasmic contact throughout their development probably is the asymmetry of sex chromosomes in males. Half the secondary spermatocytes receive an X chromosome, the other half a Y chromosome. The Y chromosome contains fewer genes than the X chromosome, and apparently some of the products of genes found only on the X chromosome are essential for spermatocyte development. By remaining in cytoplasmic contact, all four spermatocytes can share the gene products of the X chromosomes, even though only half of them have an X chromosome.

A spermatid bears little resemblance to a mature sperm. Through further differentiation, however, it will become compact, streamlined, and motile.

OOGENESIS PRODUCES EGGS: Oogonia, like spermatogonia, proliferate through mitosis. The resulting primary oocytes immediately enter prophase of the first meiotic division. In many species, including humans, the development of the oocyte is arrested at this point and may remain so for days, months, or years. In contrast, there is no arrest during male gametogenesis, which goes steadily to completion once the primary spermatocyte has differentiated. In the human female, as we will see, some primary oocytes may remain in arrested prophase I for up to 50 years!

During this prolonged prophase I, or shortly before it ends, the primary oocyte undergoes its major growth phase. It grows larger due to increased production of ribosomes, RNA, cytoplasmic organelles, and energy stores. At this time, the primary oocyte acquires all the energy, raw materials, and RNA that the egg will need to survive its first cell divisions after fertilization. In fact, the nutrients in the egg will have to maintain the embryo until it is either nourished by the maternal system or can feed on its own.

When a primary oocyte resumes meiosis, its nucleus completes the first meiotic division near the surface of the cell. The daughter cells of this division receive grossly unequal shares of cytoplasm. This asymmetry represents another major difference from spermatogenesis, in which cytoplasm is apportioned equally. The daughter cell that receives almost all the cytoplasm becomes the **secondary oocyte**, and the one that receives almost none forms the *first polar body* (Figure 1*b*). The second meiotic division of the large secondary oocyte is also accompanied by an asymmetrical division of the cytoplasm.

One daughter cell forms the large, haploid **ootid**, which eventually differentiates into a mature ovum, and the other forms the *second polar body*. Polar bodies degenerate, so the end result of oogenesis is only one mature egg for each primary oocyte that entered meiosis. However, that egg is a very large, well-provisioned cell. A second period of arrested development occurs after the first meiotic division forms the secondary oocyte. The egg may be expelled from the ovary in this condition. In many species, including humans, the second meiotic division is not completed until the egg is fertilized by a sperm.

Fertilization is the union of sperm and egg

The union of the haploid sperm and the haploid egg creates a single diploid cell, called a **zygote**, which will develop into an embryo. Fertilization does more, however, than just restore the full genetic complement of the animal. The events and processes associated with fertilization help eggs and sperm get together, prevent the union of sperm and eggs of different species, guarantee that only one sperm will enter an egg, and activate the egg metabolically. Fertilization involves a complex series of events:

- The sperm and the egg recognize each other.
- The sperm is activated so that it is capable of gaining access to the plasma membrane of the egg.
- The plasma membranes of the sperm and the egg fuse.
- The egg blocks entry by additional sperm.
- The egg is metabolically activated and stimulated to start development.
- The egg and sperm nuclei fuse to create the diploid nucleus of the zygote.

SPECIFICITY IN SPERM–EGG INTERACTIONS:

Specific recognition molecules mediate interactions between sperm and eggs. These molecules ensure that the activities of sperm are directed toward eggs and not other cells, and they help prevent eggs from being fertilized by sperm from the wrong species. The latter function is particularly important in aquatic species that release eggs and sperm into the surrounding water. The sea urchin is such a species, and its mechanisms of fertilization have been well studied.

The eggs of sea urchins and various other marine invertebrates release chemical attractants that increase

the motility of sperm and cause them to swim toward the egg. These chemical attractants are species-specific. For example, eggs of one species of sea urchin release a peptide consisting of 14 amino acids. As this peptide diffuses from the egg, it binds to receptors on the sperm the same species. The sperm respond by increasing their mitochondrial respiration and their motility. Before exposure to the peptide, the sperm swim in tight little circles, but after binding the peptide, they swim energetically up the concentration gradient of the peptide until they reach the egg that is releasing it. The peptide released by eggs of one species of sea urchin does not bind to receptors on sperm of other species.

When sperm reach an egg, they must get through two protective layers before they can fuse with the egg plasma membrane. The eggs of sea urchins are covered with a **jelly coat**, which surrounds a proteinaceous **vitelline envelope**. The sperm's assault on these protective layers depends on a membrane-enclosed structure called an **acrosome** containing enzymes andother proteins. The acrosome is located at the front of the sperm head, where it forms a cap over the nucleus (Figure 2).

When the sperm makes contact with an egg of its own species, substances in the jelly coat trigger an *acrosomal reaction*, which begins with the breakdown of the plasma membrane covering the sperm head and the underlying acrosomal membrane. The acrosomal enzymes are released, and they digest a hole through the jelly coat. Next, a structure called an *acrosomal process* extends out of the head of the sperm. The acrosomal process forms from globular actin proteins behind the acrosome, which polymerize when the acrosomal membrane breaks down.

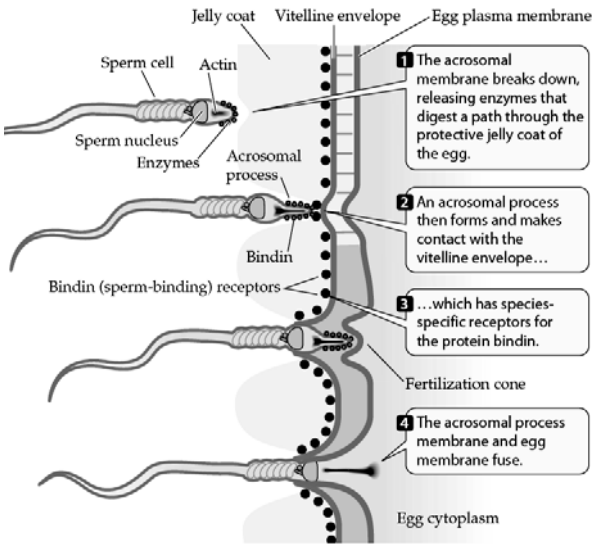
The acrosomal process extends through the remainder of the jelly coat to make contact with the vitelline envelope. The acrosomal process is coated with a membrane-bound protein called *bindin*. Different species have different kinds of bindin molecules. The plasma membrane of the egg has species-specific bindin receptors that extend through the vitelline envelope. The reaction of acrosomal bindin with these receptors stimulates the egg plasma membrane to form a *fertilization cone* that engulfs the sperm head, bringing it into the egg cytoplasm.

In animals that practice internal fertilization, mating behaviors help guarantee species specificity, but egg–sperm recognition mechanisms still exist. The mammalian egg, for example, is surrounded by a thick layer called the **cumulus**, which consists of a loose assemblage of maternal cells in a gelatinous matrix (Figure 2). Beneath the cumulus is a glycoprotein envelope called the **zona pellucida**, which is functionally similar to the vitelline envelope of sea urchin eggs. When mammalian sperm are deposited in the female reproductive tract, they become metabolically activated and are made capable of an acrosomal reaction if they should meet an egg. An activated sperm can penetrate the cumulus and interact with the zona pellucida.

Unlike the jelly coat of sea urchin eggs, the cumulus of mammalian eggs does not trigger the acrosomal reaction. When sperm make contact with the zona pellucida, a species-specific glycoprotein in the zona binds to recognition molecules on the head of the sperm. This binding triggers the acrosomal reaction, releasing acrosomal enzymes that digest a path through the zona. When the sperm head reaches the egg plasma membrane, other proteins cause the adhesion of sperm to egg plasma membrane and facilitate fusion of sperm and egg.

The importance of the zona pellucida and its sperm-binding molecules as a species-specific recognition mechanism was revealed in experiments on mammalian eggs and sperm in culture dishes. When the zona was stripped from human eggs and they were exposed to hamster sperm, fertilization took place, resulting in a hamster–human hybrid zygote. The hybrid zygote did not survive its first cell division because of chromosomal incompatibilities, but the experiment demonstrated that the recognition mechanism in mammalian species resides in the zona.

BLOCKS TO POLYSPERMY AND EGG ACTIVATION: The fusion of the sperm and egg plasma membranes and the entry of the sperm into the egg initiate a programmed sequence of events. The first responses to sperm entry are *blocks to polyspermy*—that is, mechanisms that prevent more than one sperm from entering the egg. If more than one sperm enters the egg, the resulting embryo is unlikely to survive. Blocks to polyspermy have been studied extensively in sea urchin eggs, which can be fertilized in a dish of seawater. Within seconds after a sperm enters a sea urchin egg, there is an influx of sodium ions, which changes the electric charge difference across the egg's plasma membrane. This *fast block to polyspermy* prevents the fusion of other sperm with the egg plasma membrane.

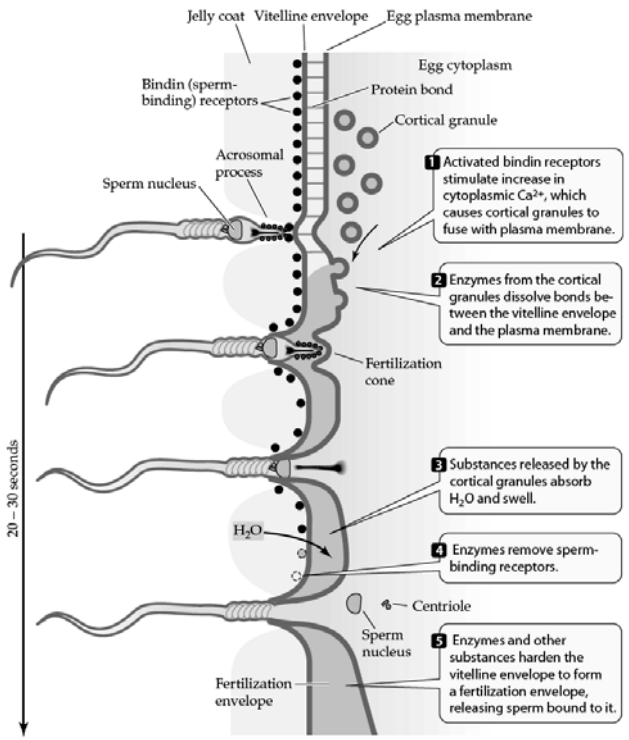


2 The Acrosomal Reaction The acrosomal reaction allows a sea urchin sperm to recognize an egg of the same species and pass through its protective layers.

The *slow block to polyspermy* takes about a minute and results from the release of calcium (Figure 3). Before fertilization, the vitelline envelope is bonded to the egg plasma membrane. Just under the plasma membrane are vesicles called *cortical granules*, which contain enzymes and other proteins. The sea urchin egg, like all animal cells, contains calcium ions that are sequestered in the endoplasmic reticulum. Sperm binding to the sea urchin egg stimulates the release of calcium from the egg's endoplasmic reticulum. The increase in cytoplasmic

calcium causes the egg's cortical granules to fuse with the plasma membrane and release their contents. The cortical granule enzymes break the bonds between the vitelline envelope and the plasma membrane, and other proteins released from the cortical granules attract water into the space between them. As a result, the vitelline envelope rises to form a *fertilization envelope*. Cortical granule enzymes also degrade sperm-binding molecules on the surface of the fertilization envelope and cause it to harden. The fertilization envelope prevents additional sperm from contacting the egg. The slow block to polyspermy in the sea urchin is mediated by the phosphatidyl inositol-bisphosphate (PIP2) second messenger system.

Activation of the bindin receptors activates



3. The Slow Block to Polyspermy Enzymes from the sea urchin egg's cortical granules trigger the slow block to polyspermy.

phospholipase C, which cleaves PIP2 in the egg plasma membrane, releasing inositol triphosphate (IP3) into the egg cell cytoplasm. IP3 diffuses to the endoplasmic reticulum, where it opens calcium channels. In mammals, sperm entry does not seem to cause a rapid change in membrane potential, but it triggers the PIP2 second messenger system, resulting in several events. Calcium is released from the endoplasmic reticulum, and as in the sea urchin, the increased calcium causes the cortical granules to fuse with the egg plasma membrane. A fertilization envelope does not form around the mammalian egg, but the cortical granule enzymes destroy the molecules in the zona pellucida that bind sperm. The rise in cytoplasmic calcium also activates the egg's metabolism and signals it to complete meiosis. The pH of the egg's cytoplasm increases, its oxygen consumption rises, protein synthesis increases, and DNA synthesis is initiated. The stage is set for the first cell division.

Anatomical and behavioral adaptations bring eggs and sperm together

As we have just seen, sexual reproduction requires the production of haploid gametes (gametogenesis) and the joining together of those gametes to form a diploid zygote(fertilization). Mating, the step in between these two processes, gets eggs and sperm close enough together so that fertilization can occur. The simplest distinction in mating systems is whether fertilization occurs externally or internally.

EXTERNAL FERTILIZATION: In an aquatic environment, animals can bring their gametes together by simply releasing them into the water. This practice, called *external fertilization*, is common among simple aquatic animals that are not very mobile. Such animals may produce huge numbers of gametes. A female oyster, for example, may produce 100 million eggs in a year, and the number of sperm produced by a male oyster is astronomical.

But numbers alone do not guarantee that gametes will meet. The reproductive activities of the males and females of a population must be synchronized, since released gametes have a limited life span. Seasonal breeders may use day length, changes in temperature, or changes in weather to time their production and release of gametes. Social stimulation is also important. Sexual activity by one member of a population can stimulate others to engage in it.

Behavior can play an important role in bringing gametes together even when fertilization is external. Many species travel great distances to congregate with potential mates and release their gametes at the same time in a suitable environment. Salmon are an extreme example, traveling hundreds of miles to spawn in the stream where they hatched.

INTERNAL FERTILIZATION: Terrestrial animals cannot simply release their gametes into the environment. Sperm can move only through liquid, and delicate gametes released into air would dry out and die. Terrestrial animals avoid these problems by releasing sperm directly into the female reproductive tract. This practice is called *internal fertilization*. Animals have evolved an incredible diversity of behavioral and anatomical adaptations for internal fertilization. As we saw above, gametogenesis occurs in the gonads, which are the *primary sex organs*. All of the additional anatomical components of an animal's reproductive system are called *accessory sex organs*. An obvious accessory sex organ in the males of many species is the **penis**, which enables the male to deposit sperm in the female's reproductive tract. Accessory sex organs include a variety of glands, tubules, ducts, and other structures.

Copulation is the physical joining of male and female accessory sex organs. Transfer of sperm in internal fertilization can also be indirect. Males of many invertebrate species (e.g., mites and scorpions) and a few vertebrates (e.g., salamanders) deposit *spermatophores*—packets of sperm—in the environment. When a female mite encounters a spermatophore from a potential mate, she straddles it and opens a pair of plates in her abdomen

so that the tip of the spermatophore enters her reproductive tract and allows the sperm to enter.

Male squids and spiders play a more active role in spermatophore transfer. The male spider secretes a drop containing sperm onto a bit of web, then uses a special structure on his foreleg to pick up the sperm-containing web and insert it through the female's genital opening. Male squids use one specialized tentacle to pick up a spermatophore and insert it into the female's genital opening.

Most male insects copulate and transfer sperm to the female's vagina through a penis. The **genitalia**—external sex organs—of insects often have species-specific shapes that match in a lock-and-key fashion. This mechanism ensures a tight, secure fit between the mating pair during the prolonged period of sperm transfer. In some insect species in which females mate with more than one male, the males have elaborate structures on their penises that can scoop sperm deposited by other males out of a female's reproductive tract, replacing it with their own.

A single body can function as both male and female

In most species, gametes are produced by individuals that are either male or female. Species that have separate male and female members are called **dioecious** species (from the Greek for "two houses"). In some species, however, a single individual may produce both sperm and eggs. Such species are called **monoecious** ("one house") or **hermaphroditic** species.

Almost all invertebrate groups contain some hermaphroditic species. An earthworm is an example of a *simultaneous hermaphrodite*, meaning that it is both male and female at the same time. When two earthworms mate, they exchange sperm, and as a result, the eggs of each are fertilized. Some vertebrates, such as the anemonefish are *sequential hermaphrodites*, meaning that an individual may function as a male or as a female at different times in its life.

What is the selective advantage of hermaphroditism? Some simultaneous hermaphrodites, such as parasitic tapeworms, have a low probability of meeting a potential mate. Even though a tapeworm may be large and cause lots of trouble for its host, it may be the only tapeworm in the host. Tapeworms can fertilize their own eggs. Most simultaneous hermaphrodites must mate with another individual, but since each member of the population is both male and female, the probability of encountering a possible mate is double what it would be in monoecious species. In some sequential hermaphrodites, all siblings are either male or female at the same time, thus reducing the incidence of inbreeding.

The evolution of vertebrate reproductive systems parallels the move to land

The earliest vertebrates evolved in aquatic environments. The closest living relatives of those earliest vertebrates are modern- day fishes. They remain exclusively aquatic animals, and most practice external fertilization. The most primitive of the fishes, the lampreys and hagfishes, simply release their gametes into the environment. In most fishes, however, mating behaviors bring females and males into close proximity at the time of gamete release. In some sharks and rays, fins have evolved into claspers that hold the male and female together and enable sperm to be transferred directly into the female reproductive tract.

Amphibians were the first vertebrates to live in terrestrial environments. They dealt with the challenge of a dry environment by returning to water to reproduce, as most amphibians still do today.

Reptiles were the first vertebrate group to solve the problem of reproduction in the terrestrial environment. Their solution, the **amniote egg**, is shared with the birds. Agood example is the chicken egg, which contains a supply of food (yolk) and water for the developing embryo. A hard shell protects the embryo and impedes water loss while allowing the diffusion of oxygen and carbon dioxide. The eggshell creates an obvious problem for fertilization, however: Sperm cannot penetrate the shell, so they have to reach the egg before the shell forms. Hence internal fertilization and the evolution of accessory sex organs were necessary for the evolution of the amniote egg. Male snakes and lizards have paired *hemipenes*, which can be filled with blood and thereby extruded from the male's body. Only one hemipene is inserted into the female's reproductive tract at a time. It is usually rough or spiny at the end to achieve a secure hold while sperm are transferred down a groove on its surface. Retractor muscles pull the hemipene back into the male's body when mating is completed. Some birds, mostly more primitive species, have erectile penises that channel sperm along a groove into the female's reproductive tract. Bird species with more recent evolutionary origins, however, do not have erectile penises; instead, the male and female simply bring their genital openings close together to transfer sperm. Usually this involves the male standing on the female's back.

All mammals practice internal fertilization, but except for the monotremes, they have done away with the shelled egg. They retain the developing embryo in the female reproductive tract, at least through the early stages of development. Mammalian species vary enormously as to the developmental stage of their offspring at the time of birth.

Reproductive systems are distinguished by where the embryo develops

Two patterns of care and nurture of the embryo have evolved in animals: oviparity (egg laying) and viviparity (live bearing). **Oviparous** animals lay eggs in the environment, and their embryos develop outside the mother's body. Oviparity is possible because eggs are stocked with abundant nutrients to supply the needs of the embryo.

Oviparous terrestrial animals, such as insects, reptiles, and birds, protect their eggs from desiccation with tough, waterproof membranes or shells. Some oviparous animals engage in various forms of parental behavior to protect their eggs, but until the eggs hatch, the embryos depend entirely on the nutrients stored in the egg. The only oviparous mammalian species are the monotremes: the echidnas and the duck-billed platypus.

Viviparous animals retain the embryo within the mother's body during its early developmental stages. All mammals (except monotremes) are viviparous. There are examples of viviparity in all other vertebrate groups except the crocodiles, turtles, and birds. Even some sharks retain fertilized eggs in their bodies and give birth to free-living offspring. But there is a big difference between viviparity in mammals and in other species. Mammals have a specialized portion of the female reproductive tract, called the **uterus** or *womb*, that holds the embryo and interacts with it to produce a **placenta**, which enables the exchange of nutrients and wastes between the blood of the mother and that of the embryo. Non-mammalian viviparous animals simply retain fertilized eggs in the mother's body until they hatch. These embryos still receive nutrition from stores in the egg, so this reproductive adaptation is called **ovoviviparity**.

Development Begins with Fertilization

Fertilization is the union of a haploid sperm and a haploid egg to produce a diploid zygote. Fertilization does more, however, than just restore a full complement of maternal and paternal genes. The entry of a sperm into an egg activates the egg metabolically and initiates the rapid series of cell divisions that produce a multicellular embryo. Also, in many species, the point of entry of the sperm creates an asymmetry in the radially symmetrical egg. This asymmetry is the initiating event that enables a bilateral body plan to emerge from the radial symmetry of the egg. We take a closer look at the cellular and molecular interactions of sperm and egg that result in the first steps of development.

The sperm and the egg make different contributions to the zygote

Nearly all of the cytoplasm of the zygote comes from the egg. Egg cytoplasm is well stocked with nutrients, ribosomes, and a variety of molecules, including mRNAs. Because the sperm's mitochondria degenerate, all of the mitochondria (and therefore all of the mitochondrial DNA) in the zygote come from the mother. In addition to its haploid nucleus, the sperm makes one other important contribution to the zygote in some species: a centriole. This centriole becomes the centrosome of the zygote, which produces the mitotic spindles for subsequent cell divisions.

It had long been assumed that the one thing that sperm and egg contributed equally to the zygote was their haploid nuclei. However, we now know that even though they are equivalent in terms of genetic material, mammalian sperm and eggs are not equivalent in terms of their roles in development.

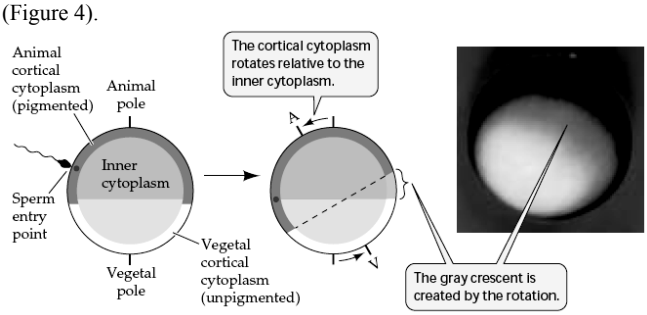
In the laboratory, it is possible to construct zygotes in which both haploid nuclei come from the mother or both come from the father. In neither case does development progress normally. Apparently, in mammals at least, certain genes involved in development are active only if they come from a sperm, and others are active only if they come from an egg. This phenomenon, called *genomic imprinting*.

Fertilization causes rearrangements of egg cytoplasm

The entry of the sperm into the egg stimulates changes in and rearrangements of the egg cytoplasm that establish the polarity of the embryo. The nutrients and molecules in the cytoplasm of the zygote are not homogeneously distributed, and therefore, they are not divided equally among all daughter cells when cell divisions begin. This unequal distribution of cytoplasmic factors sets the stage for the signal transduction cascades that orchestrate the sequential steps of development: determination, differentiation, and morphogenesis. Let's examine these earliest developmental events in the frog, an organism in which they have been well studied.

The rearrangements of egg cytoplasm in some frog species are easily observed because of pigments in the egg cytoplasm. The nutrient molecules in an unfertilized frog egg are dense, and they are therefore concentrated by gravity in the lower half of the egg, which is called the *vegetal hemisphere*. The haploid nucleus of the egg is located at the opposite end of the egg, in the *animal hemisphere*. The outermost (*cortical*) cytoplasm of the animal hemisphere is heavily pigmented, and the underlying cytoplasm has more diffuse pigmentation. The vegetal hemisphere is not pigmented.

The surface of the frog egg has specific sperm-binding sites located only in the animal hemisphere, so sperm always enter the egg in that hemisphere. When a sperm enters, the cortical cytoplasm rotates toward the site of sperm entry. This rotation reveals a band of diffusely pigmented cytoplasm on the side of the egg opposite the site of sperm entry. This band, called the **gray crescent**, will be the site of important developmental events (Figure 4).

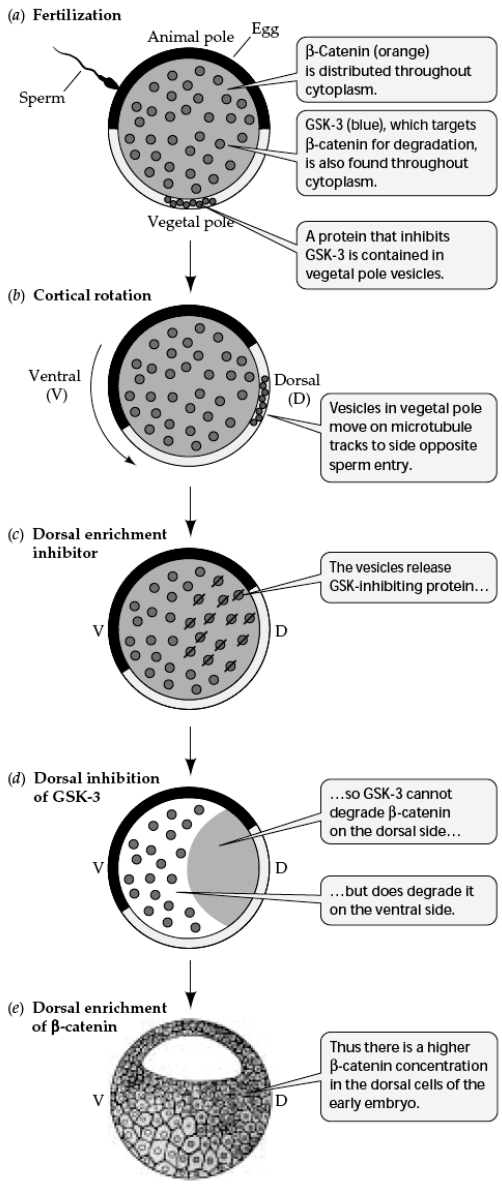


4 The Gray Crescent Rearrangements of the cytoplasm of frog eggs after fertilization create the gray crescent.

The cytoplasmic rearrangements that create the gray crescent bring different regions of cytoplasm into contact on opposite sides of the egg. Therefore, bilateral symmetry is imposed on what was a radially symmetrical egg. In addition to the up–down difference of the animal and vegetal hemispheres, the movement of the cytoplasm sets the stage for the creation of the anterior–posterior and left–right axes. In the frog, the site of sperm entry will become the *ventral* (belly) region of the embryo, and the gray crescent will become the *dorsal* (back) region. Since the gray crescent also marks the posterior end of the embryo, these relationships specify the anterior–posterior and left–right axes as well.

Rearrangements of egg cytoplasm set the stage for determination

The molecular mechanisms underlying the first steps in frog embryo formation are beginning to be understood. The sperm centriole rearranges the microtubules in the vegetal hemisphere cytoplasm into a parallel array that presumably guides the movement of the cortical cytoplasm. Organelles and certain proteins from the vegetal hemisphere move to the gray crescent region even faster than the cortical cytoplasm rotates. As a result of these movements of cytoplasm, proteins, and organelles, changes in the distribution of critical developmental signals occur.



5 Cytoplasmic Factors Set Up Signaling Cascades
Cytoplasmic movement changes the distributions of critical developmental signals. In the frog zygote, the interaction of the protein kinase GSK-3, its inhibitor, and the protein β-catenin are crucial in specifying the dorsal–ventral (back–belly) axis of the embryo.

A key transcription factor in early development is β-catenin, which is produced from maternal mRNA and is found throughout the cytoplasm of the egg. Also present throughout the egg cytoplasm is a protein kinase called GSK-3, which phosphorylates and thereby targets β-catenin for degradation. However, an inhibitor of GSK-3 is segregated in the vegetal cortex of the egg. After sperm entry, this inhibitor is moved along microtubules to the gray crescent, where it prevents the degradation of β-catenin. As a result, the concentration of β-catenin is higher on the dorsal side than on the ventral side of the developing embryo (Figure 5).

Evidence supports the hypothesis that β-catenin is a key player in the cell–cell signaling cascade that begins the process of cell determination and the formation of the embryo in the region of the gray crescent. But before there can be cell–cell signaling, there must be multiple cells, so let's turn first to the early series of cell divisions that transforms the zygote into a multicellular embryo.

Cleavage: Repackaging the Cytoplasm

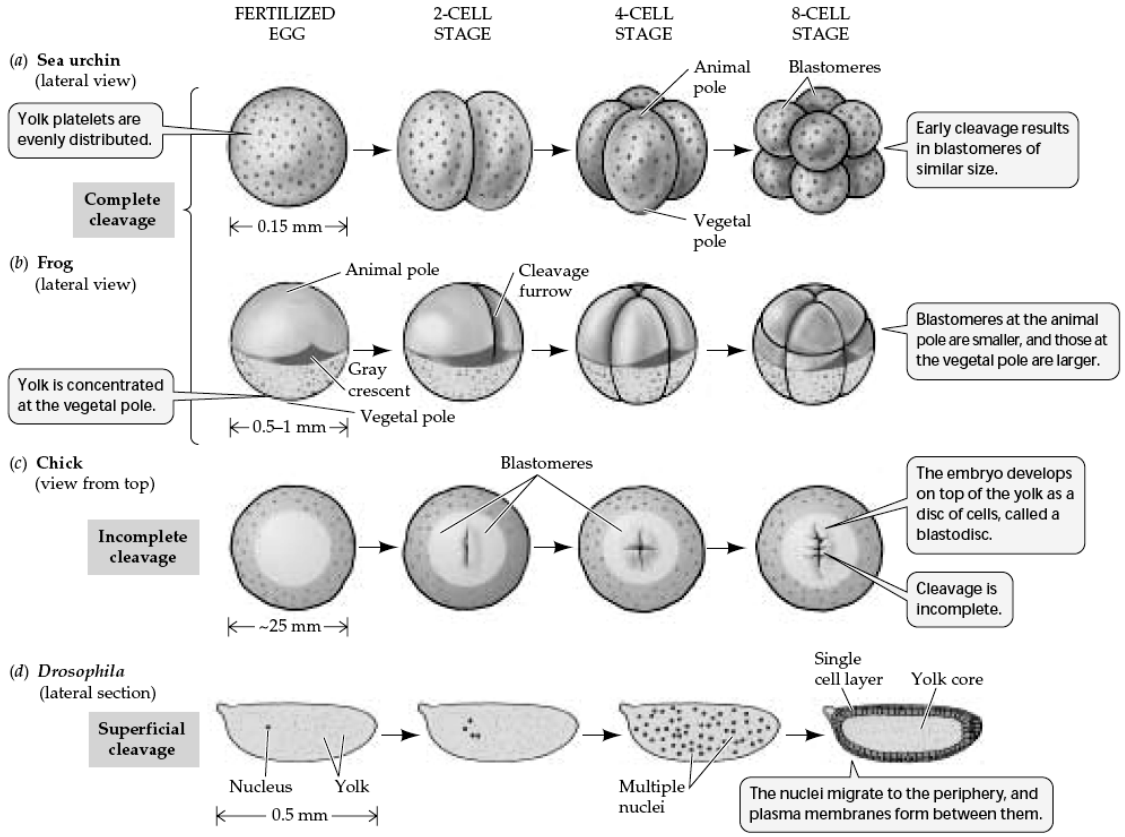
The transformation of the diploid zygote into a mass of cells occurs through a rapid series of cell divisions, called **cleavage**. Because the cytoplasm of the zygote is not homogeneous, these first cell divisions result in the differential distribution of nutrients and cytoplasmic determinants among the cells of the early embryo. In most animals, cleavage proceeds with rapid DNA replication and mitosis, but no cell growth and little gene expression. The embryo becomes a solid ball of smaller and smaller cells, called a *morula* (from the Latin word for "mulberry"). Eventually, this ball forms a central fluid-filled cavity called a **blastocoel**, at which point the embryo is called a **blastula**. Its individual cells are called **blastomeres**.

The pattern of cleavage, and therefore the form of the blastula, is influenced by two major factors. First, the amount of nutrient material, or **yolk**, stored in the egg differs among species. Yolk influences the pattern of cell divisions by impeding the pinching in of the plasma membrane to form a *cleavage furrow* between the daugh ter cells. Second, cytoplasmic determinants stored in the egg by the mother guide the formation of mitotic spindles and the timing of cell divisions.

The amount of yolk influences cleavage

In embryos with little or no yolk, there is little interference with cleavage furrow formation, and all the daughter cells are of similar size; the sea urchin egg provides an example (Figure 6*a*). More yolk means more resistance to cleavage furrow formation; therefore, cell divisions progress more rapidly in the animal hemisphere than in the vegetal hemisphere, where the yolk is concentrated. As a result, the cells derived from the vegetal hemisphere are fewer and larger; the frog egg provides an example of this pattern (Figure 6*b*). In spite of this difference between sea urchin and frog eggs, the cleavage furrows completely divide the egg mass in both cases; thus these animals are said to have *complete cleavage*. In contrast, in eggs that contain a lot of yolk, such as the chicken egg, the cleavage furrows do not penetrate the yolk. As a result, cleavage is incomplete, and the embryo forms as a disc of cells, called a **blastodisc**, on top of the yolk mass (Figure 6*c*). This type of incomplete cleavage, called *discoidal cleavage*, is

common in fishes, reptiles, and birds. Another type of incomplete cleavage, called *superficial cleavage*, occurs in insects such as the fruit fly (*Drosophila*). In the insect egg, the mass of yolk is centrally located (Figure 6*d*). Early in development, cycles of mitosis occur without cytokinesis. Eventually the resulting nuclei migrate to the periphery of the egg, and after several more mitotic cycles, the plasma membrane of the egg grows inward, partitioning the nuclei into individual cells.



6 Patterns of Cleavage in Four Model Organisms Differences in patterns of early embryonic development reflect differences in the way the egg cytoplasm is organized.

The orientation of mitotic spindles influences the pattern of cleavage

The positions of the mitotic spindles during cleavage are not random; rather, they are defined by cytoplasmic determinants that were produced from the maternal genome and stored in the egg. The orientation of the mitotic spindles determines the planes of cleavage and, therefore, the arrangement of the daughter cells.

If the mitotic spindles of successive cell divisions form parallel or perpendicular to the animal–vegetal axis of the zygote, the cleavage pattern is *radial*, as in the sea urchin and the frog. In these organisms, the first two cell divisions are parallel to the animal–vegetal axis and the third is perpendicular to it (Figure 6*a*,*b*). Another cleavage pattern, *spiral cleavage*, results when the mitotic spindles are at oblique angles to the animal–vegetal axis. Mollusks have spiral cleavage, and a visible expression of this is the coiling of snail shells.

Cleavage in mammals is unique

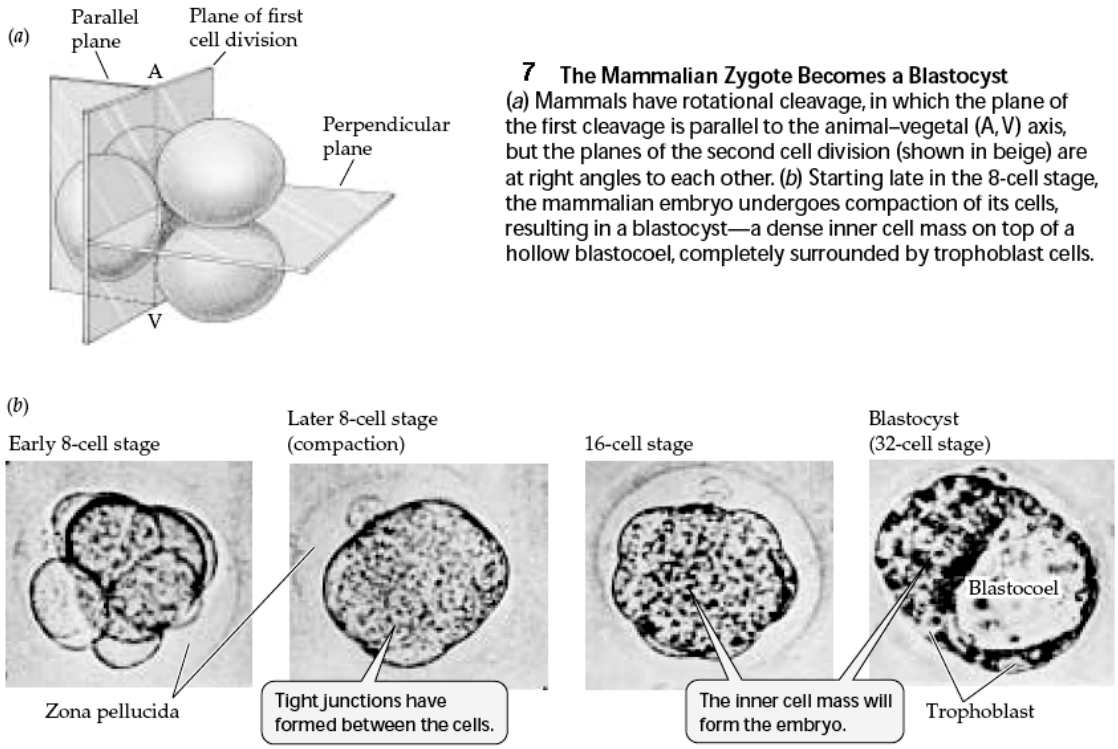
Several features of mammalian cleavage are very different from those seen in other animal groups. First, the pattern of cleavage in mammals is *rotational*: the first cell division is parallel to the animal–vegetal axis, yielding two blastomeres. The second cell division occurs at right angles: one blastomere divides parallel to the animal–vegetal axis, while the other divides perpendicular to it (Figure 7*a*).

Cleavage in mammals is very slow; cell divisions are 12–24 hours apart, compared with tens of minutes to a few hours in non-mammalian species. Also, the cell divisions of mammalian blastomeres are not in synchrony with each other. Because the blastomeres do not undergo mitosis at the same time, the number of cells in the embryo does not progress in the regular (2, 4, 8, 16, 32, etc.) progression typical of other species.

Another unique feature of the slow mammalian cleavage is that the products of genes expressed at this time play roles in cleavage. In species such as sea urchins and frogs, gene expression does not occur in the blastomeres, and cleavage is directed exclusively by molecules that were present in the egg prior to fertilization.

As in other animals that have complete cleavage, the early cell divisions in a mammalian zygote produce a loosely associated ball of cells. However, at about the 8-cell stage, the behavior of the mammalian blastomeres

changes. They change shape to maximize their surface contact with one another, form tight junctions, and become a very compact mass of cells (Figure7*b*).



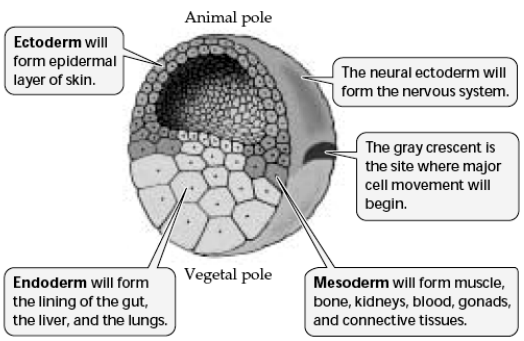
7 The Mammalian Zygote Becomes a Blastocyst
(*a*) Mammals have rotational cleavage, in which the plane of the first cleavage is parallel to the animal–vegetal (A, V) axis, but the planes of the second cell division (shown in beige) are at right angles to each other. (*b*) Starting late in the 8-cell stage, the mammalian embryo undergoes compaction of its cells, resulting in a blastocyst—a dense inner cell mass on top of a hollow blastocoel, completely surrounded by trophoblast cells.

At the transition from the 16-cell to the 32-cell stage, the cells separate into two groups. The **inner cell mass** will become the embryo, while the surrounding cells become an encompassing sac called the **trophoblast,** which will become part of the placenta. Trophoblast cells secrete fluid, creating a cavity (blastocoel) with the inner cell mass at one end (see Figure 7*b*). At this stage, the mammalian embryo is called a **blastocyst** to distinguish it from the blastulas of other animals. Fertilization in mammals occurs in the upper reaches of the mother's oviduct, and cleavage occurs as the zygote travels down the oviduct to the uterus. When the blastocyst arrives in the uterus, the trophoblast adheres to the *endometrium* (the uterine wall). This event begins the process of *implantation* that embeds the embryo in the wall of the uterus. In humans, implantation begins on about the sixth day after fertilization. As the blastocyst moves down the oviduct to the uterus, it must not embed itself in the oviduct wall, or the result will be an ectopic or tubal pregnancy—a very dangerous condition. Early implantation is normally prevented by an external proteinaceous layer called the *zona pellucida*, which surrounds the egg and remains around the cleaving ball of cells. At about the time the blastocyst reaches the uterus, it hatches from the zona pellucida, and implantation can occur.

Specific blastomeres generate specific tissues and organs

In all animal species, cleavage results in a repackaging of the egg cytoplasm into a large number of small cells surrounding a central cavity. Little cell differentiation occurs during cleavage, and in most nonmammalian species, none of the genome of the embryo is expressed. Nevertheless, cells in different regions of the resulting blastula possess different complements of the nutrients and cytoplasmic determinants that were present in the egg.

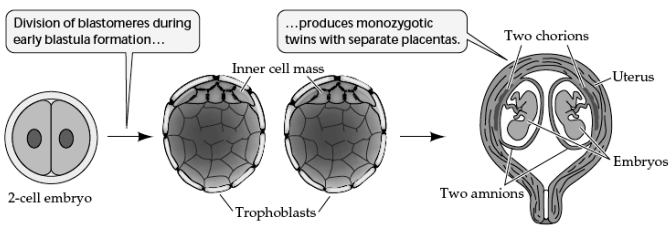
The blastocoel prevents cells from different regions of the blastula from interacting, but that will soon change. During the next stage of development, the cells of the blastula will move around and come into new associations with one another, communicate instructions to one another, and begin to differentiate. In many animals, these movements of the blastomeres are so regular and well orchestrated that it is possible to label a specific blastomere with a dye and identify the tissues and organs that form from its progeny. Such labeling experiments produce **fate maps** of the blastula (Figure 8).



8 Fate Map of a Frog Blastula The colors indicate the portions of the blastula that will form the three germ layers, and subsequently the frog's tissues and organs.

Blastomeres become **determined**—committed to specific fates—at different times in different species. In some species, such as roundworms and clams, blastomeres are determined by the 8-cell stage. If one of these blastomeres is experimentally removed, a particular portion of the embryo will not form. This type of development has been called **mosaic development** because each blastomere seems to contribute a specific set of "tiles" to the final "mosaic" that is the adult animal. In contrast, other species, such as sea urchins and vertebrates, have **regulative development**: The loss of some cells during cleavage does not affect the developing embryo because the remaining cells compensate for the loss.

If some blastomeres can change their fate to compensate for the loss of other cells during cleavage and blastula formation, are those cells capable of forming an entire embryo? To a certain extent, they are. During cleavage or early blastula formation in mammals, for example, if the blastomeres are physically separated into two groups, both groups can produce complete embryos (Figure 9). Since the two embryos come from the same zygote, they will be *monozygotic twins*—genetically identical. Non-identical twins occur when two separate eggs are fertilized by two separate sperm. Thus, while identical twins are always of the same sex, non-identical twins have a 50 percent chance of being the same sex.



9. Twinning in Humans. Because humans have regulative development, remaining cells can compensate when cells are lost in early cleavages.Monozygotic (identical) twins can result when cells in the early blastula become physically separated and each group of cells goes on to produce a separate embryo.

Gastrulation: Producing the Body Plan

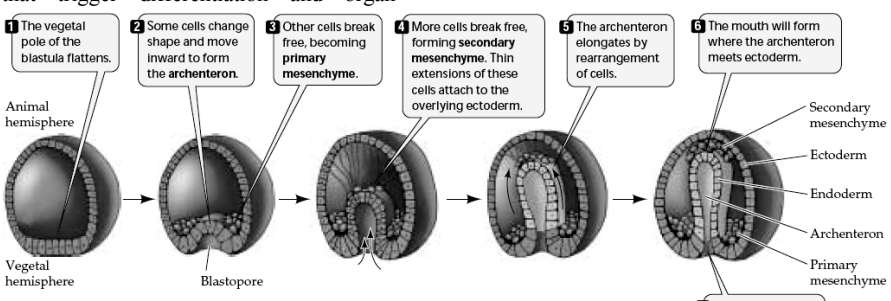
The blastula is typically a fluid-filled ball of cells. How does this simple ball of cells become an embryo, made up of multiple tissue layers, with head and tail ends and dorsal and ventral sides? **Gastrulation** is the process whereby the blastula is transformed by massive movements of cells into an embryo with multiple tissue layers and visible body axes. The resulting spatial relationships between tissues make possible the inductive interactions that trigger differentiation and organ formation. During gastrulation, the animal body forms three **germ layers** (also called *cell layers* or *tissue layers*):

- Some blastomeres move together as a sheet to the inside of the embryo, creating an inner germ layer called the **endoderm**. The endoderm will give rise to the lining of the digestive tract, respiratory tract, and circulatory system and make up other internal tissues such as the pancreas and liver.
- The cells remaining on the outside of the embryo become the outer germ layer, the **ectoderm**. The ectoderm will give rise to the nervous system, the skin, hair, and nails, sweat glands, oil glands, and milk secretory ducts.
- Other cells migrate between the endoderm and the ectoderm to become the middle germ layer, or **mesoderm**.

The mesoderm will contribute tissues to many organs, including blood vessels, muscle, bones, liver, and heart. Some of the most challenging and interesting questions in animal development have concerned what directs the cell movements of gastrulation and what is responsible for the resulting patterns of cell differentiation and organ formation. In the past 25 years, scientists have answered many of these questions at the molecular level. In the discussion that follows, we'll consider the similarities and differences among gastrulation in sea urchins, frogs, reptiles, birds, and mammals.

Invagination of the vegetal pole characterizes gastrulation in the sea urchin

The sea urchin blastula is a simple, hollow ball of cells that is only one cell thick. The end of the blastula stage is marked by a dramatic slowing of the rate of mitosis, and the beginning of gastrulation is marked by a flattening of the vegetal hemisphere (Figure 10). Some cells at the vegetal pole bulge into the blastocoel, break free, and migrate into the cavity. These cells become *primary mesenchyme* cells—cells of the middle germ layer, the mesoderm. (Mesenchyme cells are unconnected to one another and act as independent units, in contrast to epithelial cells, which are tightly packed into sheets or tubes.) The flattening at the vegetal pole results from changes in the shape of the individual blastomeres. These cells shift from being rather cuboidal to become wedge-shaped, with constricted outer edges and expanded inner edges. As a result of these shape changes, the vegetal pole bulges inward, or *invaginates*, as if someone were poking a finger into a hollow ball.



10 Gastrulation in Sea Urchins During gastrulation, cells move to new positions and form the three germ layers from which differentiated tissues develop.

The cells that invaginate become the endoderm and form the primitive gut, the *archenteron*. At the tip of the archenteron more cells break free, entering the blastocoel to form more mesoderm, the *secondary mesenchyme*. The early invagination of the archenteron is due to the changes in cell shapes, but eventually it is pulled by the secondary mesenchyme cells. These cells, attached to the tip of the archenteron, send out extensions that adhere to the overlying ectoderm and contract. Where the archenteron eventually makes contact with the ectoderm, the mouth of the animal will form. The opening created by the invagination of the vegetal pole is called the **blastopore**; it will become the anus of the animal.

What mechanisms control the various cell movements of sea urchin gastrulation? The immediate answer is that specific properties of particular blastomeres change. For example, some vegetal cells migrate into the blastocoel to form the primary mesenchyme because they lose their attachments to neighboring cells. Once they bulge into the blastocoel, they move by extending long processes called *filopodia* along an extracellular matrix of proteins that is laid down by the ectodermal cells lining the blastocoel.

A deeper understanding of gastrulation requires that we discover the molecular mechanisms whereby certain blastomeres develop properties different from those of others. Cleavage divides up the cytoplasm of the egg in a very systematic way. The sea urchin blastula at the 64-cell stage is radially symmetrical, but it has polarity. It consists of tiers of cells. As in the frog blastula, the top is the animal pole and the bottom the vegetal pole.

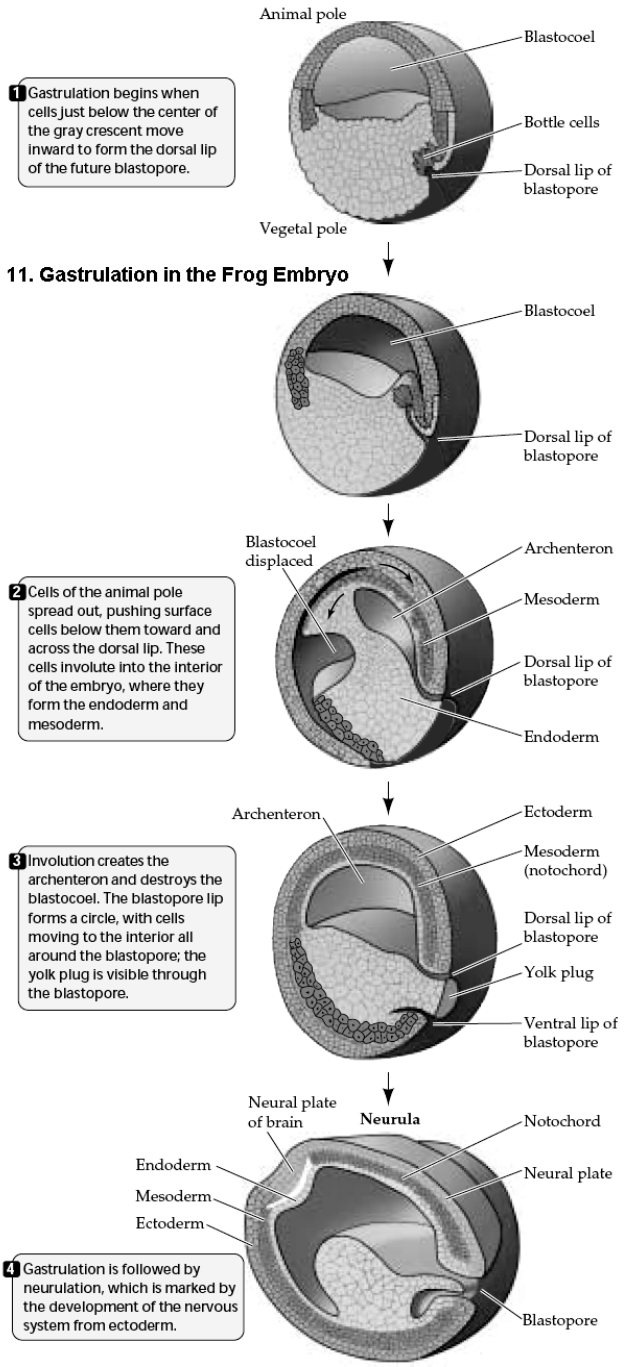
If different tiers of blastula cells are separated, they show different developmental potentials. Only cells from the vegetal pole are capable of initiating the development of a complete larva. It has been proposed that the reason for these differences is an uneven distribution of various transcriptional regulatory proteins in the egg cytoplasm. As cleavage progresses, these proteins end up in different combinations in different groups of cells. Therefore, specific sets of genes are activated in different cells, determining their different developmental capacities. Let's turn now to gastrulation in the frog, in which a number of key signaling molecules have been identified.

Gastrulation in the frog begins at the gray crescent

Amphibian blastulas have considerable yolk and are more than one cell thick; therefore, gastrulation is more complex in amphibians than in sea urchins. Furthermore, there is considerable variation among different species of amphibians. In this brief account, we will mix results from studies done on different species to produce a generalized picture of amphibian development.

Amphibian gastrulation begins when certain cells in the gray crescent change their shape and their cell adhesion properties. The main bodies of these cells bulge inward toward the blastocoel while they remain attached to the outer surface of the blastula by slender necks. Because of their shape, these cells are called *bottle cells*.

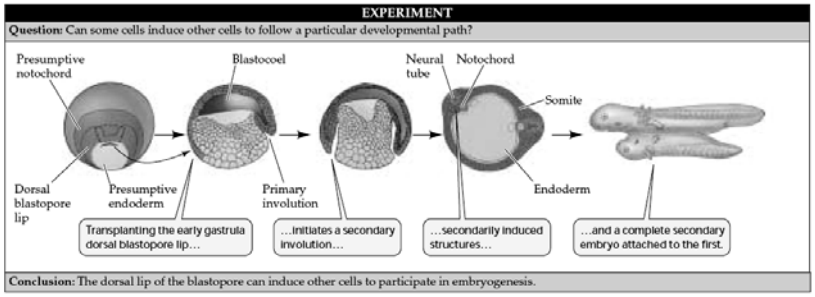
The bottle cells mark the spot where the **dorsal lip** of the blastopore will form (Figure 11). As the bottle cells move inward, they create this lip, over which successive sheets of cells will move into the blastocoel in a process called *involution*. The first involuting cells are those of the prospective endoderm, and they form the primitive gut, or archenteron. Closely following are the cells that will form the mesoderm. As gastrulation proceeds, cells from the animal hemisphere move toward the site of involution in a process called *epiboly*.



11. Gastrulation in the Frog Embryo

The blastopore lip widens and eventually forms a complete circle surrounding a "plug" of yolk-rich cells. As cells continue to move inward through the blastopore, the archenteron grows, gradually displacing the blastocoel.

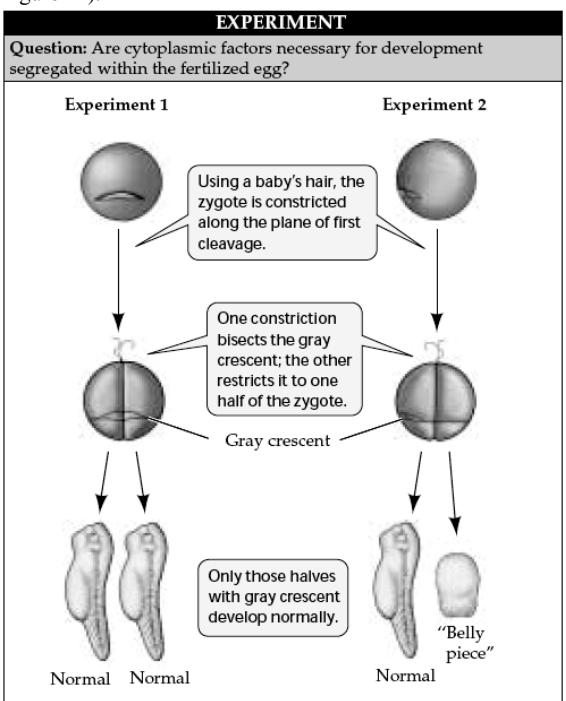
As gastrulation comes to an end, the amphibian embryo consists of three germ layers: ectoderm on the outside, endoderm on the inside, and mesoderm in the middle. The embryo also has a dorsal– ventral and anterior–posterior organization. Most importantly, however, the fates of specific regions of the endoderm, mesoderm, and ectoderm have been determined. The discovery of the events whereby determination takes place in the amphibian embryo is one of the most exciting stories in animal development.



13. The Dorsal Lip Induces Embryonic Organization In a famous experiment, Spemann and Mangold transplanted the dorsal lip of the blastopore.The transplanted tissue induced a second site of gastrulation and the formation of a second embryo.

The dorsal lip of the blastopore organizes embryo formation

In the 1920s, the German biologist Hans Spemann was studying the development of salamander eggs. He was interested in finding out whether the nuclei of blastomeres remain *totipotent*—capable of directing the development of a complete embryo. With great patience and dexterity, he formed loops from a single human baby hair to constrict fertilized eggs, effectively dividing them in half. When Spemann's loops bisected the gray crescent, both halves of the zygote gastrulated and developed into complete embryos (Experiment 1 in Figure 12).



12 Spemann's Experiment Spemann's research revealed that gastrulation and subsequent normal development in salamanders depended on cytoplasmic determinants localized in the gray crescent.

But when the gray crescent was on only one side of the constriction, only that half of the zygote developed into a complete embryo. The half lacking gray crescent material became a clump of undifferentiated cells that Spemann called the "belly piece" (Experiment 2 in Figure 13). Spemann thus hypothesized that cytoplasmic determinants in the region of the gray crescent are necessary for gastrulation and thus for the development of a normal organism.

To test his hypothesis, Spemann and his student Hilde Mangold conducted a series of delicate tissue transplantation experiments. They transplanted pieces of early gastrulas to various locations on other gastrulas. Guided by fate maps (see Figure 8), they were able to take a piece of ectoderm they knew would develop into skin and transplant it to a region that normally becomes part of the nervous system, and vice versa.

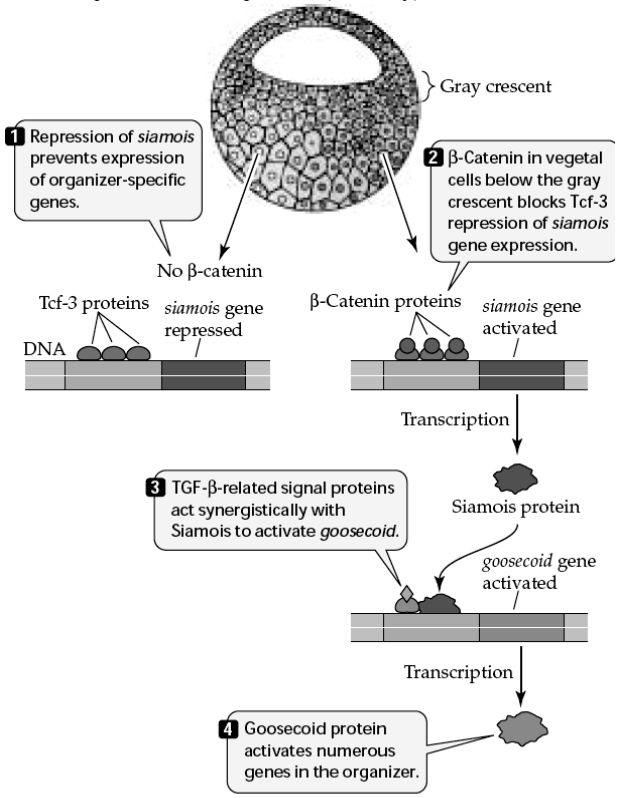
When they performed these transplants in early gastrulas, the transplanted pieces always developed into tissues that were appropriate for the location where they were placed. Donor presumptive epidermis (that is, cells destined to become skin in their original location) developed into host neural ectoderm (nervous system tissue), and donor presumptive neural ectoderm developed into host skin. Thus, the fates of the transplanted cells had not been determined before the transplantation.

In late gastrulas, however, the same experiment yielded opposite results. Donor presumptive epidermis produced patches of skin cells in the host nervous system, and donor presumptive neural ectoderm produced nervous system tissue in the host skin. Something had occurred during gastrulation to determine the fates of the embryonic cells. In other words, as Spemann and Mangold had hypothesized, the path of differentiation a cell would follow was determined during gastrulation.

Spemann and Mangold next did an experiment that produced momentous results: They transplanted the dorsal lip of the blastopore (Figure 13). When this small piece of tissue was transplanted into the presumptive belly area of another gastrula, it stimulated a second site of gastrulation, and second whole embryo formed belly-to-belly with the original embryo. Because the dorsal lip of the blastopore was apparently capable of inducing the formation of an entire embryo, Spemann and Mangold dubbed it the **primary embryonic organizer**, or simply the **organizer**.

MOLECULAR MECHANISMS OF THE ORGANIZER: In recent years, researchers have studied the primary embryonic organizer intensively to discover the molecular mechanisms involved in its action. The distribution of the transcription factor β-catenin in the late blastula corresponds to the location of the organizer in the early gastrula, so β-catenin is a candidate for the initiator of organizer activity. To prove that a protein is an inductive signal, it has to be shown that it is both

necessary and *sufficient* for the proposed effect. In other words, the effect should not occur if the candidate protein is not present (necessity), and the candidate protein should be capable of inducing the effect where it would otherwise not occur (sufficiency).



14 Molecular Mechanisms of the Primary Embryonic Organizer
The organizing potential of the gray crescent depends on the activity of the *goosecoid* gene, which in turn is activated by signaling pathways set up in the vegetal cells below the gray crescent.

The criteria of necessity and sufficiency have indeed been satisfied for the transcription factor β-catenin. If β-catenin mRNA transcripts are depleted by injections of antisense RNA into the egg, gastrulation does not occur. If β-catenin is experimentally overexpressed in another region of the blastula, it can induce a second axis of embryo formation, as the transplanted dorsal lip did in the Spemann– Mangold experiments. Thus, β-catenin appears to be both necessary and sufficient for the formation of the primary embryonic organizer—but it is only one component of a complex signaling process.

What follows is a summary of some of the critical early steps in this signaling cascade. This description may contain a confusing amount of detail. However, it is not the arcane names of the genes and gene products involved that are important to remember. Rather, we hope to provide a basic understanding of how these signaling molecules—their interactions and their gradients—can create and convey positional and temporal information. Studies of early gastrulas revealed that primary embryonic organizer activity is induced by signals emanating from vegetal cells just below the gray crescent. The protein β- catenin appears to play critical roles in generating these signals. One signal critical to stimulating the expression of organizer genes is the transcription factor Goosecoid. Expression of the *goosecoid* gene appears to depend on two signaling pathways, both of which involve β-catenin.

The first of these pathways involves a *goosecoid*-promoting transcription factor called Siamois. The *siamois* gene is normally repressed by a ubiquitous transcription factor called Tcf-3, but in cells where β-catenin is present, an interaction between Tcf-3 and β-catenin induces *siamois* expression (Figure 14). But Siamois protein alone is not sufficient for *goosecoid* expression.

Vegetal cells receive mRNA transcripts from the original egg cytoplasm for proteins in the TGF-β (transforming growth factor β) superfamily of cell signaling molecules. One or more of these proteins (candidates include Vg1 and Nodal) interact with Siamois protein by cooperatively binding to the promoter of the *goosecoid* gene and thereby controlling its transcription (see Figure 14). Thus it is a particular combination of factors that determine which cells become the primary organizer. Cells that receive other combinations of signaling molecules are induced to become different types of mesoderm.

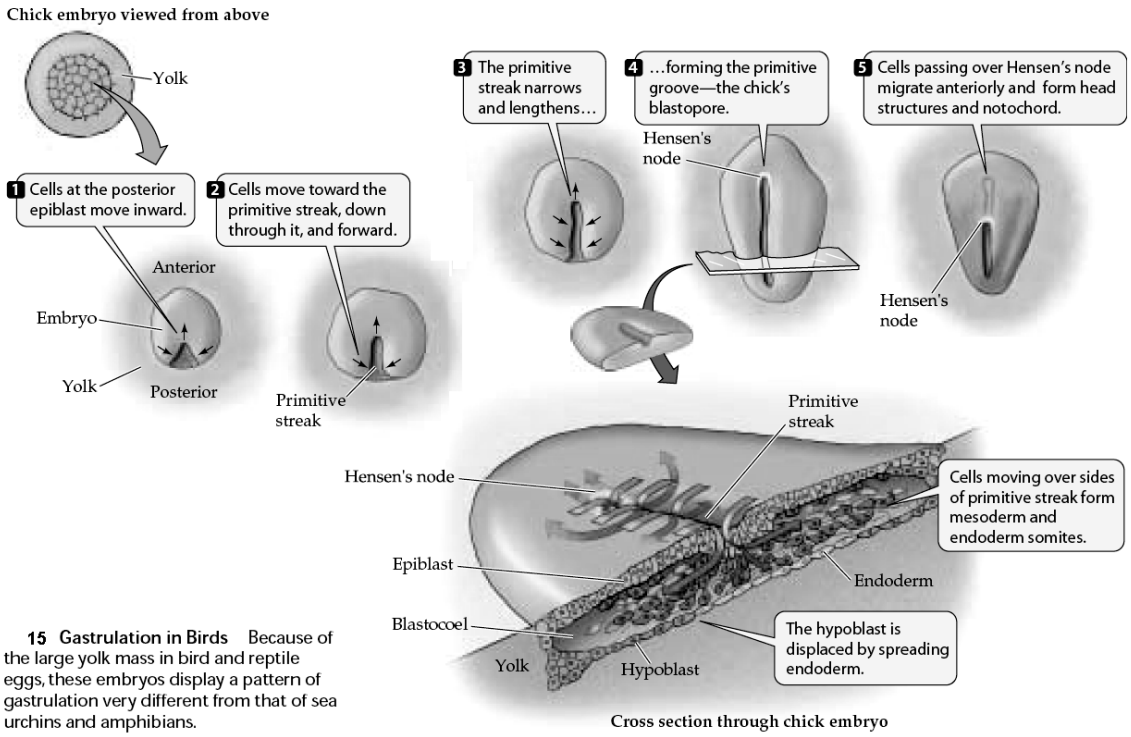
MOLECULAR MECHANISMS OF LEFT–RIGHT AXIS FORMATION: We have seen how the distribution of cytoplasmic determinants in the egg can set up a dorsal–ventral axis, and how the site of sperm entry can set up an anterior–posterior axis. What about the left–right body axis? After all, not everything in the animal is bilaterally symmetrical. The internal organs of a vertebrate have many left–right asymmetries: In humans, the heart is tilted to the right side of the body, the aorta comes off of the left side of the heart and the pulmonary artery comes off of the right side of the heart; the spleen is on the left side of the body; and the large intestine goes from right to left, to name only a few. We now know that there are a number of genes that are necessary for normal left–right organization of the body. If one of these genes is knocked out, it can randomize the left–right organization of the internal organs, with serious, even lethal, consequences.

What triggers the asymmetrical expression of these genes? We do not know the complete answer to this question, but it appears that the mechanism involves a left–right differential distribution of some of the transcription factors that act very early during gastrulation. For example, in frogs, one of the TGF-β proteins involved in organizer determination is also responsible for determining the left–right axis. In mammals, there are cilia that cause a differential flow of fluid in the yolk sac cavity. If these cilia are inactivated, the normal left–right asymmetries of the internal organs become random.

Reptilian and avian gastrulation is an adaptation to yolky eggs

The eggs of reptiles and birds contain a mass of yolk, and therefore the blastulas of these species develop as a disc of cells on top of the yolk (see Figure 6*c*). We will use the chicken egg to show how gastrulation proceeds in a flat disc of cells rather than in a ball of cells. Cleavage in the chick results in a flat, circular layer of cells called a

blastodisc. Between the blastodisc and the yolk mass is a fluid-filled space. Some cells from the blastodisc break free and move into this space. Other cells grow into this space from the posterior margin of the blastodisc. These cells come together to form a continuous layer called the **hypoblast**, which will later give rise to extraembryonic membranes that will support and nourish the developing embryo. The overlying cells make up the **epiblast**, which will form the embryo proper. Thus, the avian blastula is a flattened structure consisting of an upper epiblast and a lower hypoblast, which are joined at the margins of the blastodisc. The blastocoel is the fluid-filled space between the epiblast and hypoblast. Gastrulation begins with a thickening in the posterior region of the epiblast caused by the movement of cells toward the midline and then forward along the midline (Figure 15). The result is a midline ridge called the *primitive streak*. A depression called the *primitive groove* forms along the length of the primitive streak. The primitive groove functions as the blastopore, and cells migrate through it into the blastocoel to become endoderm and mesoderm.



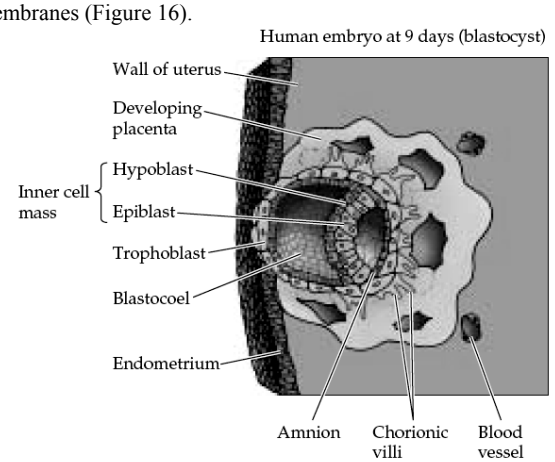
15 Gastrulation in Birds Because of the large yolk mass in bird and reptile eggs, these embryos display a pattern of gastrulation very different from that of sea urchins and amphibians.

In the chick embryo, no archenteron forms, but the endoderm and mesoderm migrate forward to form the gut and other structures. At the anterior end of the primitive groove is a thickening called **Hensen's node**, which is the equivalent of the dorsal lip of the amphibian blastopore. In fact, many signaling molecules that have been identified in the frog organizer are also expressed in Hensen's node. Cells that pass over Hensen's node become determined by the time they reach their final destination, where they differentiate into certain tissues and structures of the head and dorsal midline (but not the nervous system).

Mammals have no yolk, but retain the avian–reptilian gastrulation pattern

Mammals and birds both evolved from reptilian ancestors, so it is not surprising that they share patterns of early development, even though the eggs of mammals have no yolk. Earlier we described the development of the mammalian trophoblast and the inner cell mass, which is the equivalent of the avian epiblast.

As in avian development, the inner cell mass splits into an upper layer called the epiblast and a lower layer called the hypoblast, with a fluid-filled cavity between them. The embryo will form from the epiblast, and the hypoblast will contribute to the extraembryonic membranes (Figure 16).



16 A Human Blastocyst at Implantation Adehesion molecules and proteolytic enzymes secreted by trophoblast cells allow the blastocyst to burrow into the endometrium. Once implanted within the wall of the uterus, the trophoblast cells send out numerous projections—the chorionic villi—which increase the embyro's area of contact with the mother's bloodstream.

The epiblast also contributes to the extraembryonic membranes; specifically, it splits off an upper layer of

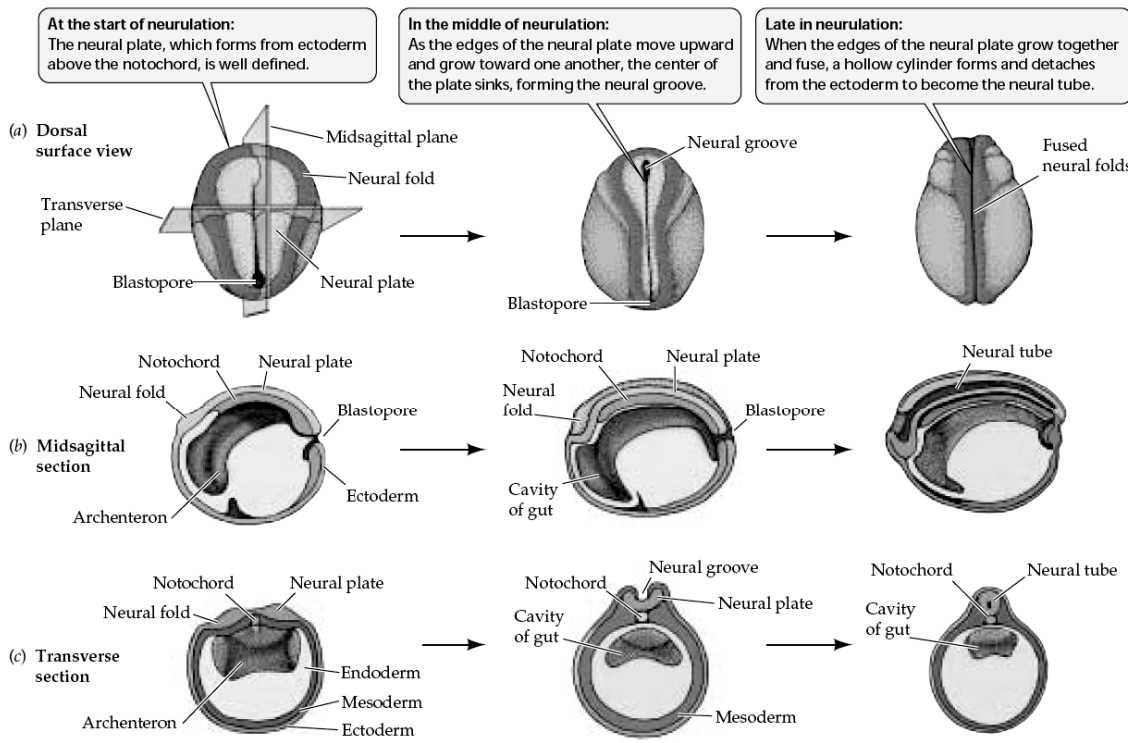
cells that will form the amnion. The amnion will grow to surround the developing embryo as a membranous sac filled with amniotic fluid. Gastrulation occurs in the mammalian epiblast just as it does in the avian epiblast. Aprimitive groove forms, and epiblast cells migrate through the groove to become layers of endoderm and mesoderm.

Neurulation: Initiating the Nervous System

Gastrulation produces an embryo with three germ layers that are positioned to influence one another through inductive interactions. During the next phase of development, called **organogenesis**, many organs and organ systems develop simultaneously and in coordination with one another. An early process of organogenesis that is directly related to gastrulation is **neurulation**, the initiation of the nervous system in vertebrates . We will examine this event in the amphibian embryo, but it occurs in a similar fashion in reptiles, birds, and mammals.

The stage is set by the dorsal lip of the blastopore

The first cells to pass over the dorsal lip of the blastopore move anteriorly and become the endodermal lining of the digestive tract. Following these first cells over the dorsal lip are those that will become mesoderm (see Figure 11). The dorsal mesoderm closest to the midline (the *chordomesoderm*) will become a rod of connective tissue called the **notochord**. The notochord gives structural support to the developing embryo; it is eventually replaced by the vertebral column. After gastrulation, the chordomesoderm induces the overlying ectoderm to begin forming the nervous system.



17 Neurulation in the Frog Embryo Continuing the sequence from Figures **8** and **11**, these drawings outline the development of the frog's neural tube.

Neurulation involves the formation of an internal neural tube from an external sheet of cells. The first signs of neurulation are flattening and thickening of the ectoderm overlying the notochord; this thickened area forms the *neural plate* (Figure 17). The edges of the neural plate that run in an anterior– posterior direction continue to thicken to form ridges or folds. Between these neural folds, a groove forms and deepens as the folds roll over it to converge on the midline. The folds fuse, forming both a cylinder, the **neural tube**, and a continuous overlying layer of epidermal ectoderm. The neural tube develops bulges at the anterior end, which become the major divisions of the brain; the rest of the tube becomes the spinal cord.

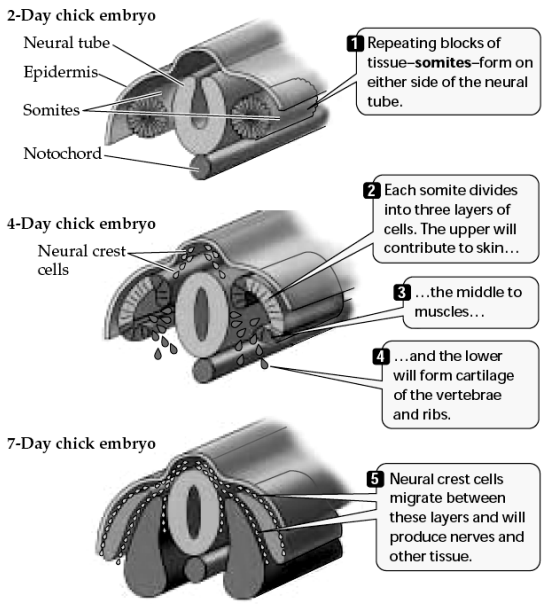
In humans, failure of the neural tube to develop normally can result in serious birth defects. If the neural folds fail to fuse in a posterior region, the result is a condition known as *spina bifida*. If they fail to fuse at the anterior end, an infant can develop without a forebrain—a condition called *anencephaly*. Whereas several genetic factors that can cause neural tube defects have been identified, there are also environmental factors, including dietary ones. The incidence of neural tube defects used to be about 1 in 300 live births, but we now know that this incidence can be cut in half if pregnant women have an adequate amount of folic acid (a B vitamin) in their diets.

Body segmentation develops during neurulation

Like the fruit flies whose development, vertebrates have a body plan consisting of repeating segments that are modified during development. These segments are most evident as the repeating patterns of vertebrae, ribs, nerves, and muscles along the anterior–posterior axis. As the neural tube forms, mesodermal tissues gather along the sides of the notochord to form separate blocks of cells called **somites** (Figure 18). The somites produce cells that will become the vertebrae, ribs, and muscles of the trunk and limbs.

The nerves that connect the brain and spinal cord with tissues and organs throughout the body are also arranged segmentally. The somites help guide the organization of these peripheral nerves, but the nerves are not of mesodermal origin. When the neural tube fuses, cells adjacent to the line of closure break loose and migrate inward between the epidermis and the somites and under the somites. These cells, called *neural crest cells*, give rise to a number of structures, including the peripheral nerves, which grow out to the body tissues and back into the spinal cord.

As development progresses, the segments of the body become different. Regions of the spinal cord differ, regions of the vertebral column differ in that some vertebrae grow ribs of various sizes and others do not, forelegs arise in the anterior part of the embryo, and hind legs arise in the posterior.

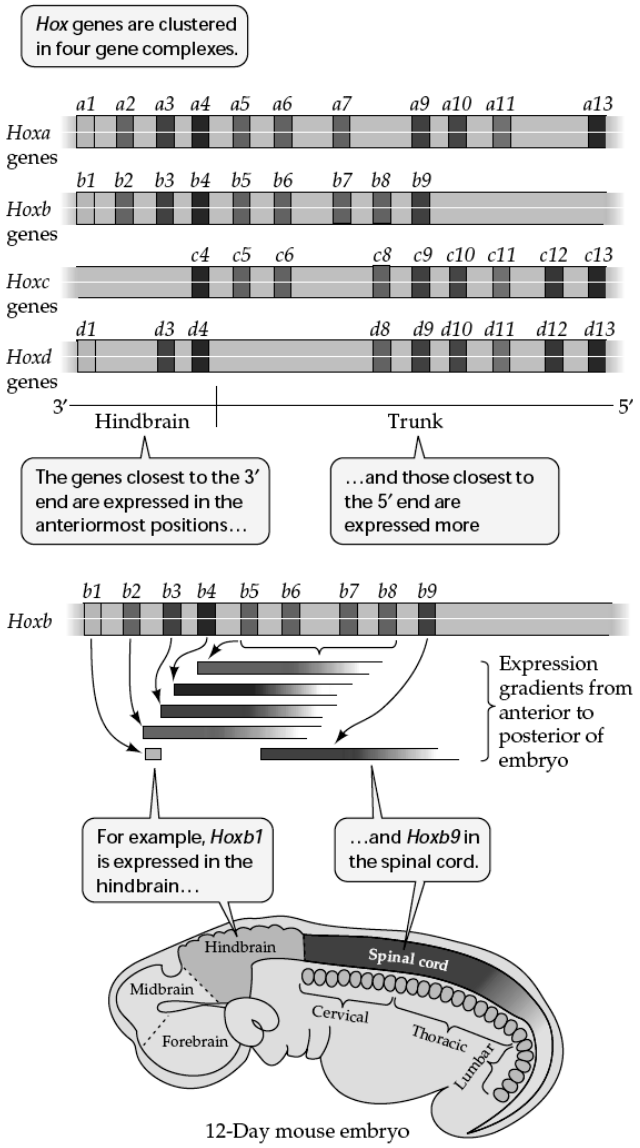


18 The Development of Body Segmentation Repeating blocks of tissue called somites form on either side of the neural tube. Skin, muscle, and bone form from the somites.

Hox genes control development along the anterior–posterior axis

Homeobox genes are central to the process of anterior–posterior determination and differentiation. Earlier, we saw how homeotic genes control body segmentation in *Drosophila*. In the mouse, four families of homeobox genes, called **Hox genes**, control differentiation along the anterior–posterior body axis.

Each mammalian Hox gene family resides on a different chromosome and consists of about 10 genes. What is remarkable is that the temporal and spatial expression of these genes follows the same pattern as their linear order on their chromosome. That is, the Hox genes closest to the 3′ end of each gene complex are expressed first and are expressed in the anterior of the embryo. The Hox genes closer to the 5′ end of the gene complex are expressed later and in a more posterior part of the embryo. As a result, different segments of the embryo receive different combinations of Hox gene products, which serve as transcription factors (Figure 19). What causes the linear, sequential expression of Hox genes is unclear.



19. Hox Genes Control Body Segmentation Hox genes are expressed along the anterior–posterior axis of the embryo in t same order as their arrangement between the 3′ and 5′ ends o gene complex.

Whereas Hox genes give cells information about their position on the anterior–posterior body axis, other genes give cells information about their dorsal–ventral position.

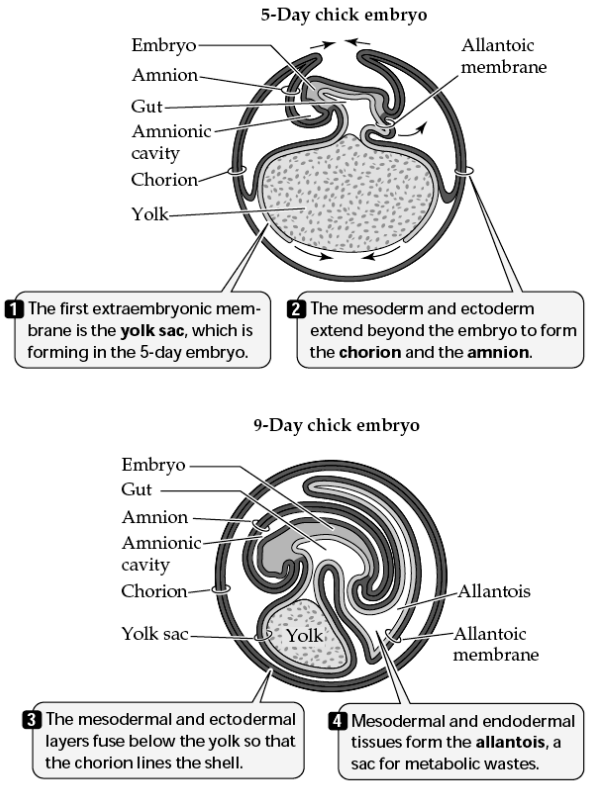
Tissues in each segment of the body differentiate according to their dorsal–ventral location. In the spinal cord, for example, sensory nerve connections develop in the dorsal region and motor nerve connections develop in the ventral region. In the somites, dorsal cells develop into skin and muscle and ventral cells develop into cartilage and bone (see Figure 18).

An example of a gene that provides dorsal–ventral information in vertebrates is *sonic hedgehog*, which is expressed in the mammalian notochord and induces cells in the overlying neural tube to have fates characteristic of ventral spinal cord cells. (As with the Hox genes, *sonic hedgehog* is homologous to a *Drosophila* gene, which is known simply as *hedgehog*.) One family of homeobox genes, the Pax genes, plays many roles in nervous system and somite development. One of these genes, *Pax3*, is expressed in those neural tube cells that will develop into dorsal spinal cord structures. *Sonic hedgehog* represses the expression of *Pax3*, and their interaction is one source of dorsal–ventral information for the differentiation of the spinal cord.

After the development of body segmentation, the formation of organs and organ systems progresses rapidly. The development of an organ involves extensive inductive interactions of the kind we saw in the example of the vertebrate eye. These inductive interactions are a current focus of study for developmental biologists. ***Extraembryonic Membranes*** There is more to a developing reptile, bird, or mammal than the embryo itself. The embryos of these vertebrates are surrounded by several **extraembryonic membranes**, which originate from the embryo but are not part of it. The extraembryonic membranes function in nutrition, gas exchange, and waste removal.

Extraembryonic membranes form with contributions from all germ layers

We will use the chicken to demonstrate how the extraembryonic membranes form from the germ layers created during gastrulation. The **yolk sac** is the first extraembryonic membrane to form, and it does so by extension of the endodermal tissue of the hypoblast layer along with some adjacent mesoderm. The yolk sac grows to encloses the entire body of yolk in the egg. It constricts at the top to create a tube that is continuous with the gut of the embryo. However, yolk does not pass through this tube. Yolk is digested by the endodermal cells of the yolk sac, and the nutrients are then transported to the embryo through blood vessels that form from the mesoderm and line the outer surface of the yolk sac. The **allantoic membrane** is also an outgrowth of the extraembryonic endoderm plus adjacent mesoderm. It forms the *allantois*, a sac for storage of metabolic wastes. Just as the endoderm and mesoderm of the hypoblast grow out from the embryo to form the yolk sac and the allantoic membrane, ectoderm and mesoderm combine and extend beyond the limits of the embryo to form the other extraembryonic membranes. Two layers of cells extend all along the inside of the eggshell, both over the embryo and below the yolk sac. Where they meet, they fuse, forming two membranes, the inner **amnion** and the outer **chorion**. The amnion surrounds the embryo, forming the amniotic cavity. The amnion secretes fluid into the cavity, providing a protective environment for the embryo. The outer membrane, the chorion, forms a continuous membrane just under the eggshell (Figure 20). It limits water loss from the egg and also works with the enlarged allantoic membrane to exchange respiratory gases between the embryo and the outside world.



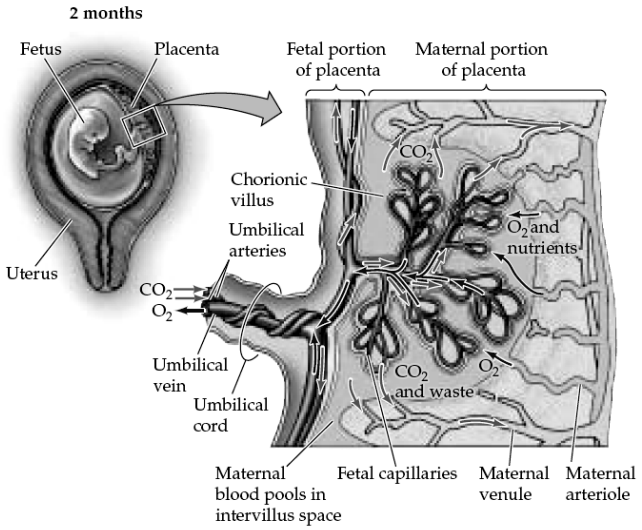
20. The Extraembryonic Membranes In birds, reptiles, and mammals, the embryo constructs four extraembryonic membranes. The yolk sac encloses the yolk, and the amnion and chorion enclose the embryo. Fluids secreted by the amnion fill the amniotic cavity, providing an aqueous environment for the embryo. The chorion, along with the allantois, mediates gas exchange between the embryo and its environment. The allantois stores the embryo's waste products.

Extraembryonic membranes in mammals form the placenta

In mammals, the first extraembryonic membrane to form is the trophoblast, which is already apparent by the fifth cell division (see Figure 7). When the blastocyst reaches the uterus and hatches from its encapsulating zona pellucida, the trophoblast cells interact directly with the endometrium. Adhesion molecules expressed on the surfaces of these cells attach them to the uterine wall. By excreting proteolytic enzymes, the trophoblast burrows into the endometrium, beginning the process of implantation (see Figure 16). Eventually, the entire trophoblast is within the wall of the uterus. The trophoblastic cells then send out numerous projections, or villi, to increase the surface area of contact with maternal blood.

Meanwhile, the hypoblast cells extend to form what in the bird would be the yolk sac. But there is no yolk in mammalian eggs, so the yolk sac contributes mesodermal

tissues that interact with trophoblast tissues to form the chorion. The chorion, along with tissues of the uterine wall, produces the **placenta**, the organ of nutrient, respiratory gas, and metabolic waste exchange between the mother and the embryo (Figure 21).



21. The Mammalian Placenta In most mammals, nutrients and wastes are exchanged between maternal and fetal blood in the placenta, which forms from the chorion and tissues of the uterine wall.The embryo is attached to the placenta by the umbilical cord. Embryonic blood vessels invade the placental tissue to form fingerlike chorionic villi.Maternal blood flows into the spaces surrounding the villi.

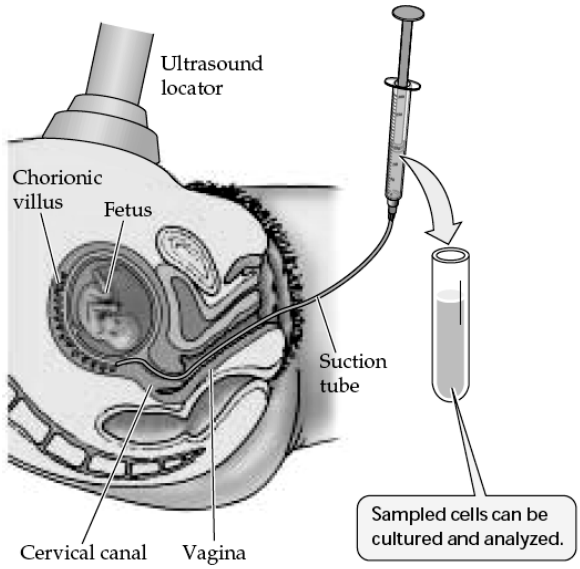
At the same time the yolk sac is forming from the hypoblast, the epiblast produces the amnion, which grows to enclose the entire embryo in a fluid-filled amniotic cavity. The rupturing of the amnion and chorion and the loss of the amniotic fluid ("water breaking") herald the onset of labor in humans.

An allantois also develops in mammals, but its importance depends on how well nitrogenous wastes can be transferred across the placenta. In humans the allantois is minor; in pigs it is important. In humans and other mammals, allantoic tissues contribute to the formation of the umbilical cord, by which the embryo is attached to the chorionic placenta. It is through the blood vessels of the umbilical cord that nutrients and oxygen from the mother reach the developing fetus and wastes, including carbon dioxide and urea, are removed (see Figure 21).

The extraembryonic membranes provide means of detecting genetic diseases

Cells slough off of the developing human embryo and float in the amniotic fluid that bathes it. Later in development, a small sample of the amniotic fluid may be sampled with a needle as the first step of a process called **amniocentesis**. Cells from the fluid can be cultured and used for biochemical and genetic analyses that can reveal the sex of the fetus, as well as genetic markers for diseases such as cystic fibrosis, Tay-Sachs disease, and Down syndrome.

If amniocentesis is performed, it is usually not until after the fourteenth week of pregnancy, and the tests require two weeks to complete. If abnormalities in the fetus are detected, termination of the pregnancy at that stage would put the mother's health at greater risk than would an earlier abortion. Therefore, a newer technique, called **chorionic villus sampling**, is now in common use. In this test, a small sample of the tissue from the surface of the chorion is taken (Figure 22). This test can be done as early as the eighth week of pregnancy, and the results are available in several days.



22 Chorionic Villus Sampling Information about genetic defects can be obtained from chorionic tissues. The fetus and placer ta are imaged by a sonogram to guide a catheter, which samples a chorionic villus.

Human Development

In humans, **gestation**, or pregnancy, lasts about 266 days, or 9 months. In smaller mammals gestation is shorter—for example, 21 days in mice—and in larger mammals it is longer—for example, 330 days in horses and 600 days in elephants. The events of human gestation can be divided into three periods of roughly 3 months each, called *trimesters*.

Intrauterine development can be divided into three trimesters

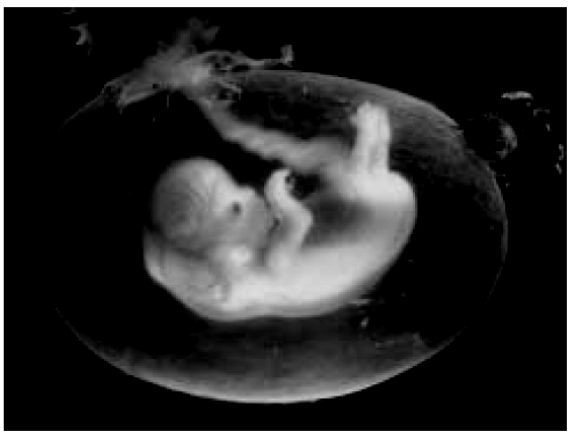
THE FIRST TRIMESTER: Implantation of the human blastocyst begins on about the sixth day after fertilization. After implantation, gastrulation occurs, the placenta forms, tissues differentiate, and organs begin to develop. The heart begins to beat in week 4, and limbs form by week 8 (Figure 23*a*). Most organs have started to form by the end of the first trimester. By that time, the embryo looks like a miniature version of the adult, and is called a **fetus**.

The first trimester is a time of rapid cell division and tissue differentiation. Signal transduction cascades and the resulting branching sequences of developmental processes are in their early stages. Therefore, the first trimester is the period during which the embryo is most sensitive to damage from radiation, drugs, chemicals, and pathogens that can cause birth defects. An embryo can be damaged before the mother even knows she is pregnant.

Aclassic and tragic case is that of thalidomide, a drug widely prescribed in Europe in the late 1950s to treat nausea. Women who took this drug in the fourth and fifth week of pregnancy, when the embryo's limbs are beginning to form, gave birth to children with severely malformed arms and legs. Hormonal changes cause major and noticeable responses in the mother during the first trimester, even though the fetus at the end of that time is still so small that it would fit into a teaspoon. Soon after the blastocyst implants itself, it begins to secrete human chorionic gonadotropin (hCG). This hormone stimulates the mother's ovary to continue to produce the hormones estrogen and progesterone, which help to maintain the pregnancy. These hormonal changes cause the well-known symptoms of pregnancy: morning sickness, mood swings, changes in the senses of taste and smell, and swelling of the breasts.

THE SECOND TRIMESTER: During the second trimester the fetus grows rapidly to a weight of about 600 g, and the moth- er's abdomen enlarges considerably. The limbs of the fetus elongate, and the fingers, toes, and facial features become well formed (Figure 23*b*). Fetal movements are first felt by the mother early in the second trimester, and they become progressively stronger and more coordinated. By the end of the second trimester, the fetus may suck its thumb.

(*a*)



(*b*)

23 Stages of Human Development (*a*) The organs and body structures of this 8-week-old embryo are forming rapidly, and it is visibly a human male. The embryo is approximately 4 cm long and weighs less than 10 g. The umbilical cord attaches the embryo to the placenta (upper left). (The dark red structure at the upper right is the remnant of the yolk sac.) (*b*) At 4 months, the fetus is about 14 cm long and weighs about 200 g. It has fully formed limbs and digits (fingers and toes) and moves freely within its protective amniotic cavity.

THE THIRD TRIMESTER: The fetus and the mother continue to grow rapidly during the third trimester. Even though the embryo is most susceptible to adverse effects of drugs, chemicals, and diseases during the first trimester, the potential for serious effects of exposure to many environmental factors continues throughout pregnancy. Severe protein malnutrition, alcohol consumption, and cigarette smoking are examples of factors that can result in low birth weight, mental retardation, and other developmental complications. As the third trimester approaches its end, many internal organs mature. The digestive system begins to function, the liver stores glycogen, the kidneys produce urine, and the brain undergoes cycles of sleep and waking.

Developmental changes continue throughout life

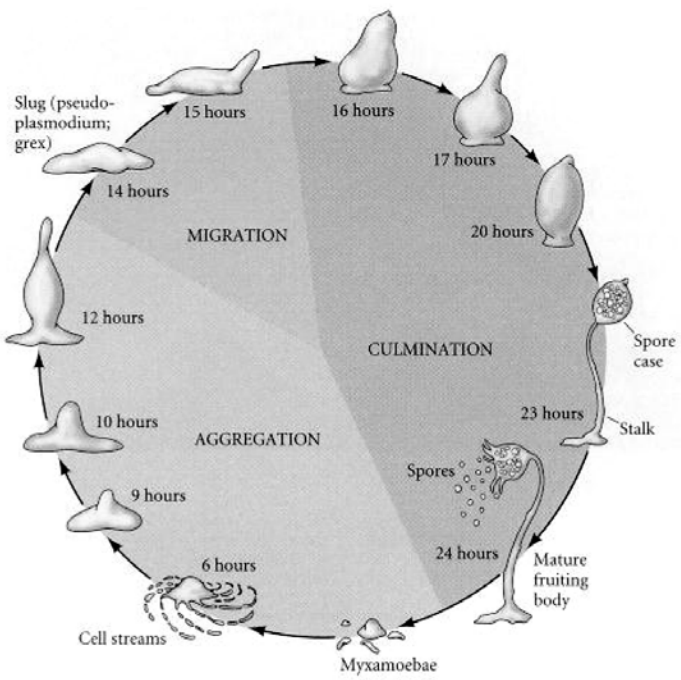
Development does not end with birth. Obviously, growth continues until adult size is reached, and even when growth stops, organs of the body continue to repair and renew themselves through cycles of cell replacement by the progeny of undifferentiated stem cells. In humans, in particular, enormous developmental changes occur in the brain in the years between birth and adolescence. Especially in the early years, there is a great deal of plasticity in the organization of the nervous system as patterns of connection between neurons develop. For example, if a child is born with its eyes misaligned, a condition known as *strabismus*, he or she will use mostly one eye. The connections to the brain from that eye will become strong, and connections from the other eye will become weak. The child will develop with reduced visual acuity and depth perception. If the eye alignment is corrected in the first 3 years of life, however, the connections between the eyes and the brain will correct themselves, and the child will develop normal vision. If the eye alignment is corrected after 3 years of age, the correct connections between the eyes and the brain will not develop, and the visual impairment will be irreversible. Thus, plasticity in the development of the visual system in humans continues for several years after birth. Avery exciting area of current research is the role of learning in stimulating the production and differentiation of new neurons in the brains of young and even adult animals.

C1. Cell aggregation and differentiation in *Dictyostelium*

Differentiation and Morphogenesis in Dictyostelium: Cell Adhesion

The life cycle of dictyostelium

Another type of multicellular organization derived from unicellular organisms is found in *Dictyostelium discoideum.** The life cycle of this fascinating organism is illustrated in Figure. In its asexual cycle, solitary haploid amoebae (called myxamoebae or "social amoebae" to distinguish them from amoeba species that always remain solitary) live on decaying logs, eating bacteria and reproducing by binary fission. When they have exhausted their food supply, tens of thousands of these myxamoebae join together to form moving streams of cells that converge at a central point. Here they pile atop one another to produce a conical mound called a tight aggregate. Subsequently, a tip arises at the top of this mound, and the tight aggregate bends over to produce the migrating slug (with the tip at the front). The slug (often given the more dignified title of pseudoplasmodium or grex) is usually 2-4 mm long and is encased in a slimy sheath. The grex begins to migrate (if the environment is dark and moist) with its anterior tip slightly raised. When it reaches an illuminated area, migration ceases, and the grex differentiates into a fruiting body composed of spore cells and a stalk. The anterior cells, representing 15-20% of the entire cellular population, form the tubed stalk. This process begins as some of the central anterior cells, the prestalk cells, begin secreting an extracellular coat and extending a tube through the grex. As the prestalk cells differentiate, they form vacuoles and enlarge, lifting up the mass of prespore cells that had made up the posterior four-fifths of the grex.



The stalk cells die, but the prespore cells, elevated above the stalk, become spore cells. These spore cells disperse, each one becoming a new myxamoeba.

In addition to this asexual cycle, there is a possibility for sex in *Dictyostelium.* Two myxamoebae can fuse to create a giant cell, which digests all the other cells of the aggregate.

When it has eaten all its neighbors, it encysts itself in a thick wall and undergoes meiotic and mitotic divisions; eventually, new myxamoebae are liberated.

Dictyostelium has been a wonderful experimental organism for developmental biologists because initially identical cells are differentiated into one of two alternative cell types, spore and stalk. It is also an organism wherein individual cells come together to form a cohesive structure composed of differentiated cell types, akin to tissue formation in more complex organisms. The aggregation of thousands of myxamoebae into a single organism is an incredible feat of organization that invites experimentation to answer questions about the mechanisms involved.

Aggregation is initiated as each of the cells begins to synthesize cAMP. There are no dominant cells that begin the secretion or control the others. Rather, the sites of aggregation are determined by the distribution of myxamoebae.

Neighboring cells respond to cAMP in two ways: they initiate a movement toward the cAMP pulse, and they release cAMP of their own . After this, the cell is unresponsive to further cAMP pulses for several minutes. The result is a rotating spiral wave of cAMP that is propagated throughout the population of cells. As each wave arrives, the cells take another step toward the center.

The differentiation of individual myxamoebae into either stalk (somatic) or spore (reproductive) cells is a complex matter. Raper (1940) and Bonner (1957) demonstrated that the anterior cells normally become stalk, while the remaining, posterior cells are usually destined to form spores. However, surgically removing the anterior part of the slug does not abolish its ability to form a stalk. Rather, the cells that now find themselves at the anterior end (and which originally had been destined to produce spores) now form the stalk. Somehow a decision is made so that whichever cells are anterior become stalk cells and whichever are posterior become spores. This ability of cells to change their developmental fates according to their location within the whole organism and thereby compensate for missing parts is called **regulation**. We will see this phenomenon in many embryos, including those of mammals.

Cell adhesion molecules in dictyostelium

How do individual cells stick together to form a cohesive organism? This problem is the same one that embryonic cells face, and the solution that evolved in the protists is the same one used by embryos: developmentally regulated cell adhesion molecules.

While growing mitotically on bacteria, *Dictyostelium* cells do not adhere to one another. However, once cell division stops, the cells become increasingly adhesive, reaching a plateau of maximum cohesiveness around 8 hours after starvation. The initial cell-cell adhesion is mediated by a 24,000-Da (24-kDa) glycoprotein that is absent in myxamoebae but appears shortly after division ceases. This protein is synthesized from newly transcribed mRNA and becomes localized in the cell membranes of the myxamoebae. If myxamoebae are treated with antibodies that bind to and mask this protein, they will not stick to one another, and all subsequent development ceases.

Once this initial aggregation has occurred, it is stabilized by a second cell adhesion molecule. This 80-kDa glycoprotein is also synthesized during the aggregation phase. If it is defective or absent in the cells, small slugs will form, and their fruiting bodies will be only about one-third the normal size. Thus, the second cell adhesion system seems to be needed for retaining a large enough number of cells to form large fruiting bodies. In addition, a third cell adhesion system is activated late in development, while the slug is migrating.

This protein appears to be important in the movement of the prestalk cells to the apex of the mound. Thus, *Dictyostelium* has evolved three developmentally regulated systems of cell-cell adhesion that are necessary for the morphogenesis of individual cells into a coherent organism. Metazoan cells also use cell adhesion molecules to form the tissues and organs of the embryo.

Dictyostelium is a "part-time multicellular organism" that does not form many cell types, and the more complex multicellular organisms do not form by the aggregation of formerly independent cells. Nevertheless, many of the principles of development demonstrated by this "simple" organism also appear in embryos of more complex phyla. The ability of individual cells to sense a chemical gradient (as in the myxamoeba's response to cAMP) is very important for cell migration and morphogenesis during animal development. Moreover, the role of cell surface proteins in cell cohesiveness is seen throughout the animal kingdom, and differentiation-inducing molecules are beginning to be isolated in metazoan organisms.

Differentiation in dictyostelium

Differentiation into stalk cell or spore cell reflects another major phenomenon of embryogenesis: the cell's selection of a developmental pathway. Cells often select a particular developmental fate when alternatives are available. A particular cell in a vertebrate embryo, for instance, can become either an epidermal skin cell or a neuron. In *Dictyostelium,* we see a simple dichotomous decision, because only two cell types are possible. How is it that a given cell becomes a stalk cell or a spore cell?

Although the details are not fully known, a cell's fate appears to be regulated by certain diffusible molecules. The two major candidates are differentiationinducing factor (DIF) and cAMP. DIF appears to be necessary for stalk cell differentiation. This factor, like the sex-inducing factor of *Volvox,* is effective at very low concentrations (10-10*M*); and, like the *Volvox* protein, it appears to induce differentiation into a particular type of cell.

When added to isolated myxamoebae or even to prespore (posterior) cells, it causes them to form stalk cells. The synthesis of this low molecular weight lipid is genetically regulated, for there are mutant strains of *Dictyostelium* that form only spore precursors and no stalk cells. When DIF is added to these mutant cultures, stalk cells are able to differentiate, and new prestalk-specific mRNAs are seen in the cell cytoplasm. While the mechanisms by which DIF induces 20% of the grex cells to become stalk tissue are still controversial, DIF may act by releasing calcium ions from intracellular compartments within the cell.

Although DIF stimulates myxamoebae to become prestalk cells, the differentiation of prespore cells is most likely controlled by the continuing pulses of cAMP. High concentrations of cAMP initiate the expression of pre-spore specific mRNAs in aggregated myxamoebae. Moreover, when slugs are placed in a medium containing an enzyme that destroys extracellular cAMP, the prespore cells lose their differentiated characteristics.

The biochemistry of this reaction involves a receptor that binds cAMP. When this binding occurs, specific gene transcription takes place, motility toward the source of the cAMP is initiated, and adenyl cyclase enzymes (which synthesize cAMP from ATP) are activated. The newly formed cAMP activates the cell's own receptors, as well as those of its neighbors. The cells in the area remain insensitive to new waves of cAMP until the bound cAMP is removed from the receptors by another cell surface enzyme, phosphodiesterase.

The mathematics of such oscillation reactions predict that the diffusion of cAMP should initially be circular. However, as cAMP interacts with the cells that receive and propagate the signal, the cells that receive the front part of the wave begin to migrate at a different rate than the cells behind them. The result is the rotating spiral of cAMP and migration. Interestingly, the same mathematical formulas predict the behavior of certain chemical reactions and the formation of new stars in rotating spiral galaxies.

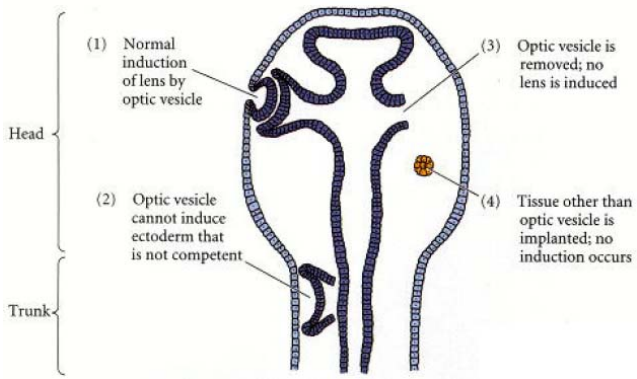
C2. Eye lens induction

Organs are complex structures composed of numerous types of tissues. In the vertebrate eye, for example, 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 this organ 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 fate. This kind of interaction at close range between two or more cells or tissues of different history and properties is called proximate interaction, or **induction**. 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. The second component, the tissue being induced, is the responder.

Not all tissues can respond to the signal being produced by the inducer. For instance, if the optic vesicle (presumptive retina) of *Xenopus laevis* is placed in an ectopic location (i.e., in a different place from where it normally forms) underneath the head ectoderm, it will induce that ectoderm to form lens tissue.

Only the optic vesicle appears to be able to do this; therefore, it is an inducer. However, if the optic vesicle is placed beneath ectoderm in the flank or abdomen of the same organism, that ectoderm will not be able to respond. Only the head ectoderm is competent to respond to the signals from the optic vesicle by producing a lens.

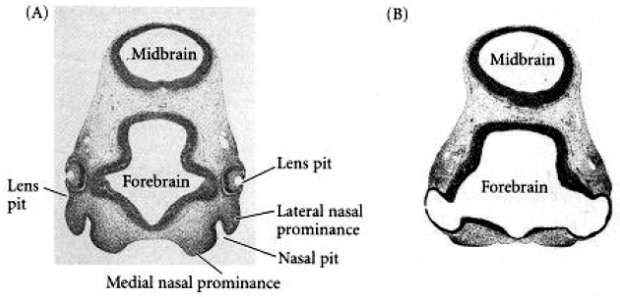


This ability to respond to a specific inductive signal is called **competence.**

Competence is not a passive state, but an actively acquired condition. For example, in the developing chick and mammalian eye, the Pax6 protein appears to be important in making the ectoderm competent to respond to the inductive signal from the optic vesicle.

Pax6 expression is seen in the head ectoderm, which can respond to the optic vesicle by forming lenses, and it is not seen in other regions of the surface ectoderm.

Moreover, the importance of Pax6 as a **competence factor** was demonstrated by recombination experiments using embryonic rat eye tissue. The homozygous Pax6-mutant rat has a phenotype similar to the homozygous Pax6-mutant mouse, lacking eyes and nose.



It has been shown that part of this phenotype is due to the failure of lens induction. But which is the defective component the optic vesicle or the surface ectoderm?

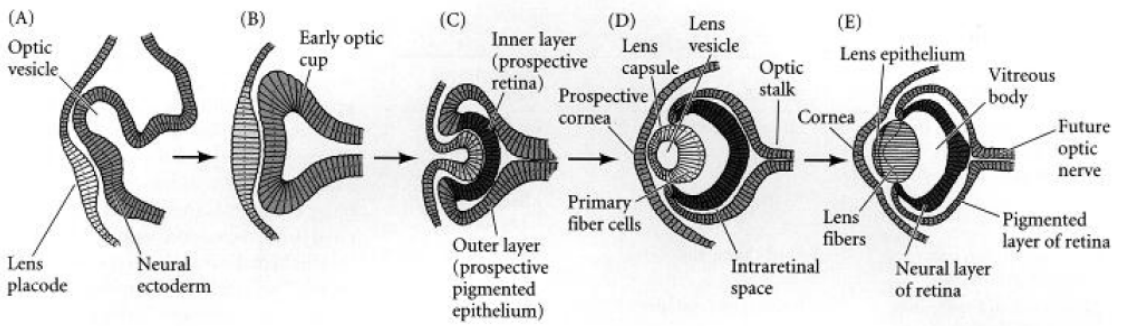
When head ectoderm from Pax6-mutant rat embryos was combined with a wild-type optic vesicle, no lenses were formed. However, when the head ectoderm from wild-type rat embryos was combined with a Pax6-mutant optic vesicle, lenses formed normally. Therefore, Pax6 is needed for the surface ectoderm to respond to the inductive signal from the optic vesicle.

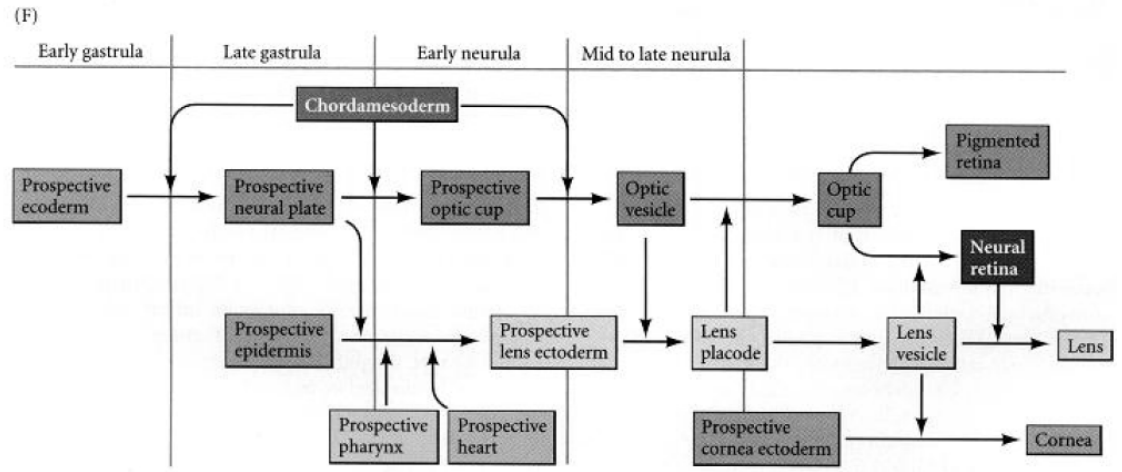
The inducing tissue does not need it. It is not known how Pax6 becomes expressed in the anterior ectoderm of the embryo, although it is thought that its expression is induced by the anterior regions of the neural plate. Competence to respond to the optic vesicle inducer can be conferred on ectodermal tissue by incubating it next to anterior neural plate tissue. Thus, there is no single inducer of the lens.

Studies on amphibians suggest that the first inducers may be the pharyngeal endoderm and heartforming mesoderm that underlie the lens-forming ectoderm during the early- and mid-gastrula stages. The anterior neural plate may produce the next signals, including a signal that promotes the synthesis of Pax6 in the anterior ectoderm. Thus, the optic vesicle appears to be *the* inducer, but the anterior ectoderm has already been induced by at least two other factors. (The situation is like that of the player who kicks *the* "winning goal" of a soccer match.)

Cascades of induction: Reciprocal and sequential inductive events

Another feature of induction is the reciprocal nature of many inductive interactions. Once the lens has formed, it can then induce other tissues. One of these responding tissues is the optic vesicle itself. Now 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. Such interactions are called **reciprocal inductions**.





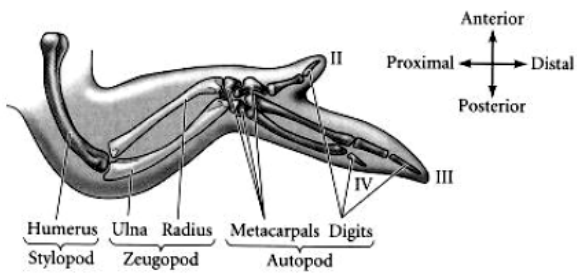
At the same time, the lens is also inducing the ectoderm above it to become the cornea. Like the lens-forming ectoderm, the cornea-forming ectoderm has achieved a particular competence to respond to inductive signals, in this case the signals from the lens.

Under the influence of the lens, the corneal ectodermal cells become columnar and secrete multiple layers of collagen. Mesenchymal cells from the neural crest use this collagen matrix to enter the area and secrete a set of proteins (including the enzyme hyaluronidase) that further differentiate the cornea. A third signal, the hormone thyroxine, dehydrates the tissue and makes it transparent. Thus, there are sequential inductive events, and multiple causes for each induction.

C3. Limb Development in Vertebrates

Pattern formation is the process by which embryonic cells form ordered spatial arrangements of differentiated tissues. The ability to carry out this process is one of the most dramatic properties of developing organisms, and one that has provoked a sense of awe in scientists and laypeople alike. How is it that the embryo is able not only to generate all the different cell types of the body, but also to produce them in a way that forms functional tissues and organs? It is one thing to differentiate the chondrocytes and osteocytes that synthesize the cartilage and bone matrices, respectively; it is another thing to produce those cells in a temporalspatial orientation that generates a functional bone. It is still another thing to make that bone a humerus and not a pelvis or a femur.

The ability of limb cells to sense their relative positions and to differentiate with regard to those positions has been the subject of intense debate and experimentation. How are the cells that differentiate into the embryonic bone specified so as to form an appendage with digits at one end and a shoulder at the other? (It would be quite a useless appendage if the order were reversed.) Here the cell types are the same, but the patterns they form are different.



The vertebrate limb is an extremely complex organ with an asymmetrical arrangement of parts. There are three major axes to consider, one of which is the proximal

(close) to distal (far) axis. The bones of the limb, be it wing, foot, hand, or flipper, consist of a proximal **stylopod** (humerus/femur) adjacent to the body wall, a **zeugopod** (radius-ulna/tibia-fibula) in the middle region, and a distal **autopod** (carpals-fingers/tarsals-toes). Originally, these structures are cartilaginous, but eventually, most of the cartilage is replaced by bone.

The positions of each of the bones and muscles in the limb are precisely organized. The second axis is the anterior (front) to posterior (back) axis. Our little fingers, for instance, mark the posterior side, while our thumbs are in the anterior. In humans, it is obvious that each hand develops as a mirror image of the other. One can imagine other arrangements to exist such as the thumb developing on the left side of both hands but these do not occur. The third axis is the dorsal-ventral axis. The palm (ventral) is readily distinguishable from the knuckles (dorsal).

In some manner, the three-dimensional pattern of the forelimb is routinely produced. The fundamental problem of morphogenesis how specific structures arise in particular places is exemplified in limb development. How is it that one part of the lateral plate mesoderm develops limb-forming capacities? How is it that the fingers form at one end of the limb and nowhere else?

How is it that the little finger develops at one edge of the limb and the thumb at the other? The basic "morphogenetic rules" for forming a limb appear to be the same in all tetrapods. Fallon and Crosby (1977) showed that grafted pieces of reptile or mammalian limb buds can direct the formation of chick limbs, and Sessions and co-workers (1989) found that regions of frog limb buds can direct the patterning of salamander limbs, and vice versa.

Moreover, the regeneration of salamander limbs appears to follow the same rules as developing limbs. But what are these morphogenetic rules?

The positional information needed to construct a limb has to function in a threedimensional coordinate system.* During the past decade, particular proteins have been identified that play a role in the formation of each of these limb axes. The proximal-distal (shoulder-finger; hip-toe) axis appears to be regulated by the fibroblast growth factor (FGF) family of proteins. The anterior-posterior (thumb-pinky) axis seems to be regulated by the Sonic hedgehog protein, and the dorsal-ventral (knuckle-palm) axis is regulated, at least in part, by Wnt7a.

The interactions of these proteins determine the differentiation of the cell types and also mutually support one another.

Formation of the Limb Bud

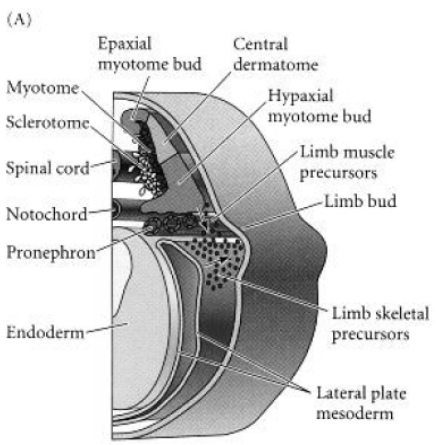
Specification of the limb fields: Hox genes and retinoic acid

Limbs will not form just anywhere along the body axis. Rather, there are discrete positions where limb fields are generated. Using the techniques, researchers have precisely localized the limb fields of many vertebrate species. Interestingly, in all land vertebrates, there are only four limb buds per embryo, and they are always opposite each other with respect to the midline. Although the limbs of different vertebrates differ with respect to which somite level they arise from, their position is constant with respect to the level of Hox gene expression along the anterior-posterior axis. For instance, in fishes (in which the pectoral and pelvic fins correspond to the anterior and posterior limbs, respectively), amphibians, birds, and mammals, the forelimb buds are found at the most anterior expression region of *Hoxc- 6,* the position of the first thoracic vertebra. The lateral plate mesoderm in the limb field is also special in that it will induce myoblasts to migrate out from the somites and enter the limb bud. No other region of the lateral plate mesoderm will do that.

Retinoic acid appears to be critical for the initiation of limb bud outgrowth, since blocking the synthesis of retinoic acid with certain drugs prevents limb bud initiation. Bryant and Gardiner (1992) suggested that a gradient of retinoic acid along the anterior-posterior axis might activate certain homeotic genes in particular cells and thereby specify them to become included in the limb field. The source of this retinoic acid is probably Hensen's node. The specification of limb fields by retinoic acid-activated Hox genes might explain a bizarre observation made by Mohanty-Hejmadi and colleagues (1992) and repeated by Maden (1993). When the tails of tadpoles were amputated and the stumps exposed to retinoic acid during the first days of regeneration, the tadpoles regenerated several legs from the tail stump. It appears that the retinoic acid caused a homeotic transformation in the regenerating tail by respecifying the tail tissue as a limb-forming pelvic region.

Induction of the early limb bud: Fibroblast growth factors

Limb development begins when mesenchyme cells proliferate from the somatic layer of the limb field lateral plate mesoderm (limb *skeletal* precursors) and from the somites (limb *muscle* precursors) These cells accumulate under the epidermal tissue to create a circular bulge called a **limb bud**. Recent studies on the earliest stages of limb formation have shown that the signal for limb bud formation comes from the lateral plate mesoderm cells that will become the prospective limb mesenchyme.

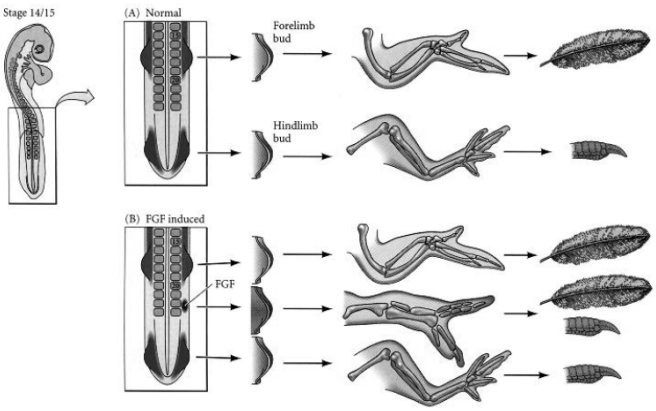


These cells secrete the paracrine factor FGF10. FGF10 is capable of initiating the limb-forming interactions between the ectoderm and the mesoderm. If beads containing FGF10 are placed ectopically beneath the flank ectoderm, extra limbs emerge.

Specification of forelimb or hindlimb: Tbx4 and Tbx5

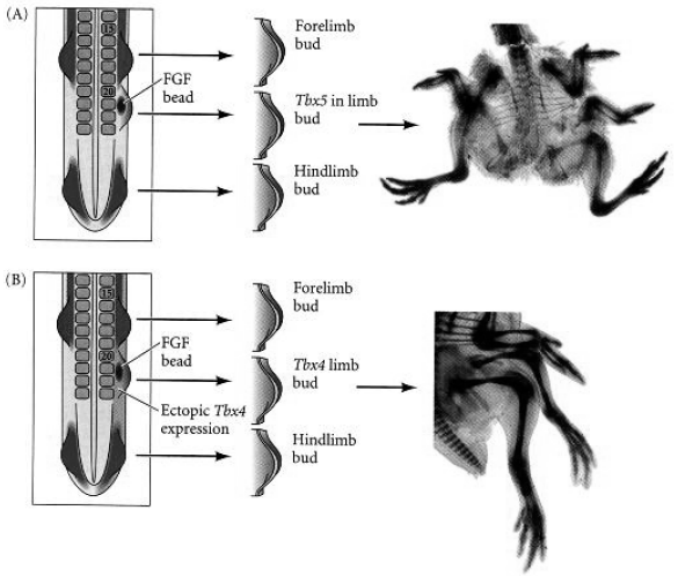
The limb buds have to be specified as being those of either the forelimb or the hindlimb. How are these distinguished? In 1996, Gibson-Brown and colleagues made a tantalizing correlation: The gene encoding the **Tbx5** transcription factor is transcribed in mouse *forelimbs,* while the gene encoding the closely related transcription factor **Tbx4** is expressed in *hindlimbs.*

Could these two transcription factors be involved in directing forelimb versus hindlimb specificity? The loss-of-function data were equivocal: humans heterozygous for the *TBX5* gene have Holt-Oram syndrome, characterized by abnormalities of the heart and upper limbs. The legs are not affected, but neither are the arms transformed into a pair of legs.



In 1998 and 1999, however, several laboratories provided gain-of-function evidence that Tbx4 and Tbx5 specify hindlimbs and forelimbs, respectively. First, if FGFsecreting beads were used to induce an ectopic limb between the chick hindlimb and forelimb buds, the type of limb produced was determined by the Tbx protein expressed. Those buds induced by placing FGF beads close to the hindlimb (opposite somite 25) expressed *Tbx4* and became hindlimbs. Those buds induced close to the forelimb (opposite somite 17) expressed *Tbx5* and developed as forelimbs (wings). Those buds induced in the center of the flank tissue expressed *Tbx5* in the anterior portion of the limb and *Tbx4* in the posterior portion of the limb.

These limbs developed as chimeric structures, with the anterior resembling a forelimb and the posterior resembling a hindlimb. Moreover, when a chick embryo was made to express *Tbx4* throughout the flank tissue (by infecting the tissue with a virus that expressed Tbx4), limbs induced in the anterior region of the flank often became legs instead of wings. Thus, Tbx4 and Tbx5 appear to be critical in instructing the limbs to become hindlimbs and forelimbs, respectively.



Induction of the apical ectodermal ridge

As mesenchyme cells enter the limb region, they secrete factors that induce the overlying ectoderm to form a structure called the **apical ectodermal ridge** (**AER**). This ridge runs along the distal margin of the limb bud and will become a major signaling center for the developing limb. Its roles include (1) maintaining the mesenchyme beneath it in a plastic, proliferating phase that enables the linear (proximal-distal) growth of the limb; (2) maintaining the expression of those molecules that generate the anteriorposterior (thumb-pinky) axis; and (3) interacting with the proteins specifying the anteriorposterior and dorsal-ventral axes so that each cell is given instructions on how to differentiate.

The factor secreted by the mesenchyme cells to induce the AER is probably FGF10. (Other FGFs, such as FGF2, FGF4, and FGF8, will also induce an AER to form; but FGF10 appears to be the FGF synthesized at the appropriate time and in the appropriate places.) FGF10 is capable of inducing the AER in the competent ectoderm between the dorsal and ventral sides of the embryo. This junction is important. In mutants in which the limb bud is dorsalized and there is no dorsal-ventral junction (as in the chick mutant *limbless*), the AER fails to form, and limb development ceases.

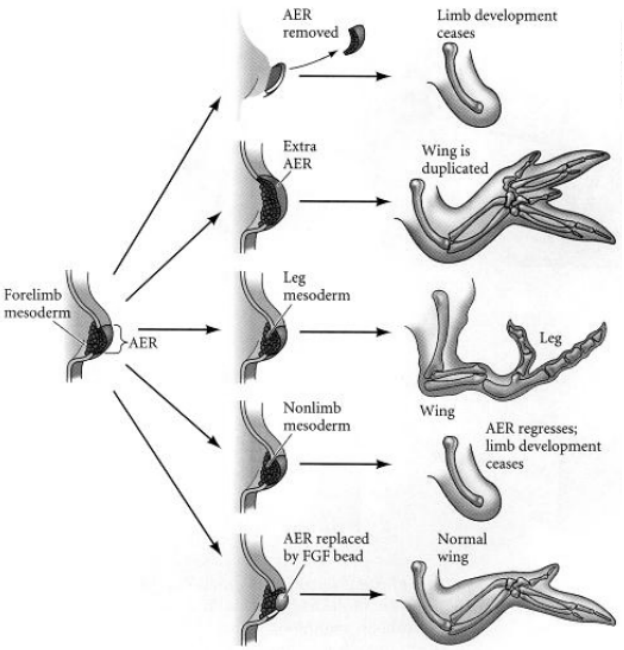
Generating the Proximal-Distal Axis of the Limb

The apical ectodermal ridge: The ectodermal component

The proximal-distal growth and differentiation of the limb bud is made possible by a series of interactions between the limb bud mesenchyme and the AER. These interactions were demonstrated by the results of several experiments on chick embryos:

1. If the AER is removed at any time during limb development, further development of distal limb skeletal elements ceases.

2. If an extra AER is grafted onto an existing limb bud, supernumerary structures are formed, usually toward the distal end of the limb.



3. If leg mesenchyme is placed directly beneath the wing AER, distal hindlimb structures (toes) develop at the end of the limb. (However, if this mesenchyme is placed farther from the AER, the hindlimb mesenchyme becomes integrated into wing structures.)
4. If limb mesenchyme is replaced by nonlimb mesenchyme beneath the AER, the AER regresses and limb development ceases.

Thus, although the mesenchyme cells induce and sustain the AER and determine the type of limb to be formed, the AER is responsible for the sustained outgrowth and development of the limb.

The AER keeps the mesenchyme cells directly beneath it in a state of mitotic proliferation and prevents them from forming cartilage. Hurle and co-workers (1989) found that if they cut away a small portion of the AER in a region that would normally fall between the digits of the chick leg, an extra digit emerged at that place.

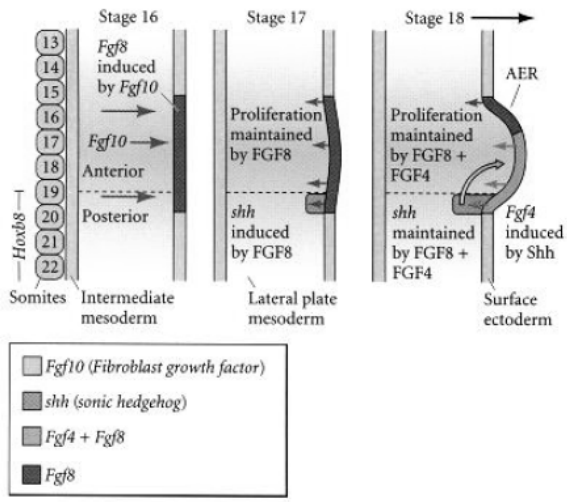
The progress zone: The mesodermal component

The proximal-distal axis is defined only after the induction of the apical ectodermal ridge by the underlying mesoderm. The limb bud elongates by means of the proliferation of the mesenchyme cells underneath the AER. This region of cell division is called the **progress zone**, and it extends about 200 μm in from the AER. Molecules from the AER are thought to keep the progress zone mesenchyme cells dividing, and it is now thought that FGFs are the molecules responsible. When the AER is removed from an early limb bud, only the most proximal parts of the stylopod are made. However, if an FGF-containing bead is placed in the hole left by the removal of the AER, a normal limb will form.

When the mesenchyme cells leave the progress zone, they differentiate in a regionally specific manner. The first cells leaving the progress zone form proximal (stylopod) structures; those cells that have undergone numerous divisions in the progress zone become the more distal structures. Therefore, if the AER is removed from an early-stage wing bud, the cells of the progress zone stop dividing, and only a humerus forms. If the AER is removed slightly later, humerus, radius, and ulna form.

Proximal-distal polarity resides in the mesodermal compartment of the limb. If the AER provided the positional information somehow instructing the undifferentiated mesoderm beneath it as to what structures to make then older AERs combined with younger mesoderm should produce limbs with deletions in the middle, while younger AERs combined with older mesoderm should produce duplications of structures. This was not found to be the case, however. Rather, normal limbs form in both experiments. But when the entire progress zone, including both the mesoderm and AER, from an early embryo is placed on the limb bud of a later-stage embryo, new proximal structures are produced beyond those already present. Conversely, when old progress zones are added to young limb buds, distal structures immediately develop, so that digits are seen to emerge from the humerus without the intervening ulna and radius.

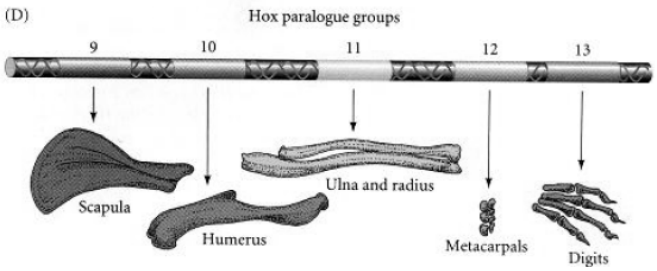
The mitotic state of the progress zone is maintained by interactions between the FGF proteins of the progress zone and of the AER. FGF10 secretion by the mesenchyme cells induces the AER, and it also induces the AER to express FGF8 . The FGF8 secreted by the AER reciprocates by maintaining the mitotic activity of the progress zone mesenchyme cells.



Hox genes and the specification of the proximal-distal axis

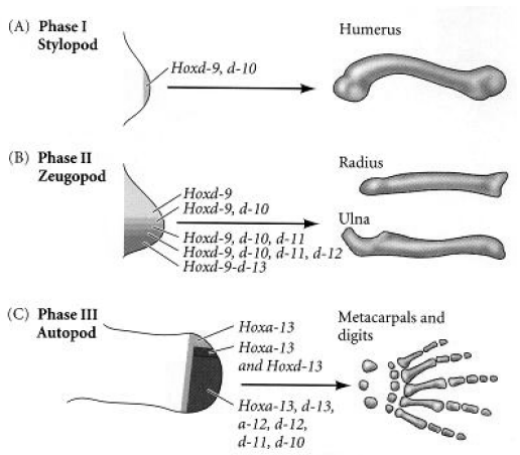
The type of structure formed along the proximal-distal axis is specified by the Hox genes. The products of the Hox genes have already played a role in specifying the place where the limbs will form. Now they will play a second role in specifying whether a particular mesenchymal cell will become stylopod, zeugopod, or autopod. The 5′ (AbdB-like) portions (paralogues 9 13)

of the *HoxA* and *HoxD* complexes appear to be active in the forelimb buds of mice. Based on the expression patterns of these genes, and on naturally occurring and gene knockout mutations, Davis and colleagues (1995) proposed a model wherein these Hox genes specify the identity of a limb region. For instance, when they knocked out all four loci for the paralogous genes *Hoxa-11* and *Hoxd-11,* the resulting mice lacked the ulna and radius of their forelimbs. Similarly, knocking out all four *Hoxa-13* and *Hoxd-13* loci resulted in loss of the autopod. Humans homozygous for a *HOXD13* mutation show abnormalities of the hands and feet wherein the



digits fuse, and human patients with homozygous mutant alleles of *HOXA13* also have deformities of their autopods. In both mice and humans, the autopod (the most distal portion of the limb) is affected by the loss of function of the most 5´ Hox genes.

The mechanism by which Hox genes could specify the proximal-distal axis is not yet understood, but one clue comes from the analysis of chicken *Hoxa-13.* Ectopic expression of this gene (which is usually expressed in the distal ends of developing chick limbs) appears to make the cells expressing it stickier. This, in turn, would cause the cartilaginous nodules to condense in specific ways.



As the limb grows outward, the pattern of Hox gene expression changes. When the stylopod is forming, *Hoxd-9* and *Hoxd-10* are expressed in the progress zone mesenchyme. When the zeugopod bones are being formed, the pattern shifts remarkably, displaying a nested sequence of *Hoxd* gene expression.

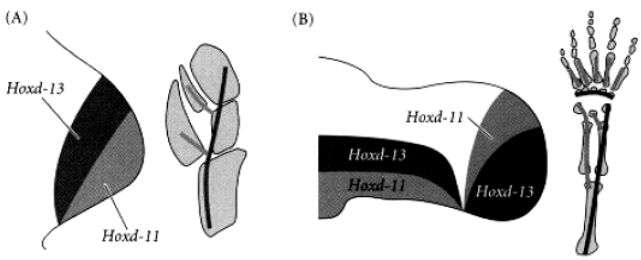
The posterior region expresses all the *Hoxd* genes from *Hoxd-9* to *Hoxd-13,* while only *Hoxd-9* is expressed anteriorly. In the third phase of limb development, when the autopod is forming, there is a further redeployment of Hox gene products. *Hoxd-9* is no longer expressed.

Rather, *Hoxa-13* is expressed in the anterior tip of the limb bud and in a band marking the boundary of the autopod. *Hoxd-13* products join those of *Hoxa-13* in the anterior region of the limb bud, while *Hoxa-12,Hoxa-11,* and *Hoxd-10 12* are expressed throughout the posterior twothirds of the limb bud.

Hox Genes and the Evolution of the Tetrapod Limb

Macroevolution, the generation of morphological novelties in the evolution of new species and higher taxa, results from alterations of development. One of the most obvious macroevolutionary changes is that from the fish fin to the amphibian leg. As Richard Owen (1849) pointed out, there is considerable homology between the bones of the fish fin and the tetrapod limb, the pectoral and pelvic fins of the fish being homologous to the tetrapod forelimb and hindlimb, respectively. While specific homologies were able to be made between the proximal elements of the fin and the limb, the homologies proposed between the autopod of the limb (the hand or foot at the distal end) and the rays of the fins "did not hold water." This was true even when one compared the tetrapod limb with the fins of the crossopterygian (lobe-finned) fishes thought to have been closely related to the ancestors of the amphibians. While there seems to be homology for the proximal and central elements of the limb, the autopod seems to be something new what evolutionary biologists call a neomorphic structure.

Recent studies have strongly suggested that the expression of the 5´ genes of the Hoxd group may be crucial in the change from fin to limb. Tetrapods and fishes share the first two phases of the Hox expression pattern in their appendages. Thus, both groups form stylopods and zeugopods. However, the phase III pattern of Hox gene expression is unique to tetrapods and is not found in fishes. Moreover, this change in Hox gene expression is mediated by a single enhancer element that is not found in fishes. This phase III change represents an inversion of gene expression, placing the most 5´ Hox gene products in the anterior of the limb bud. Instead of being restricted to the posterior of the limb bud, the expression of the 5´ Hox genes sweeps across the distal mesenchyme, just beneath the AER. This band of expression is coincident with the "digital arch" from which the digits form.



Thus, while the Hox gene expression pattern is homologous between fish and tetrapod limbs in the proximal regions, the expression pattern in the late-bud distal mesenchyme is new.

These studies also confirm the paleontological interpretations of Shubin and Alberch, who proposed that the path of digit formation was not (as previously believed) through the fourth digit (making the fin rays homologous to the other digits), but through an arch of distal wrist condensations (metapterygia) that begins posteriorly and turns anteriorly across the distal mesenchyme.

Thus, the border of *5´ HoxD* gene expression follows the metapterygial axis that Shubin and Alberch hypothesized as being the origin of digits. The foot and hand, then, appear to be new structures in evolution, and they appear to have been formed by the repositioning of *HoxD* gene expression during fin development. The use of the same enhancer to generate both fingers and toes also helps solve the problem of how these structures were evolved at the same time.

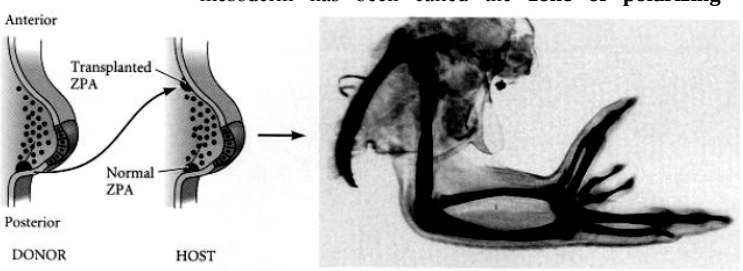
Specification of the Anterior-Posterior Limb Axis

The zone of polarizing activity

The specification of the anterior-posterior axis of the limb is the earliest change from the pluripotent condition. In chicks, this axis is specified shortly before a limb bud is recognizable.

Hamburger (1938) showed that as early as the 16-somite stage, prospective wing mesoderm transplanted to the flank area develops into a limb with the anterior-posterior and dorsal-ventral polarities of the donor graft, not those of the host tissue.

Although the differentiation of the proximal-distal structures is thought to depend on how many divisions a cell undergoes while in the progress zone, information instructing a cell as to its position on the anterior-posterior and dorsal-ventral axes must come from other sources. Several experiments suggest that the anterior-posterior axis is specified by a small block of mesodermal tissue near the posterior junction of the young limb bud and the body wall. This region of the mesoderm has been called the **zone of polarizing activity** (**ZPA**).When this tissue is taken from a young limb bud and transplanted into a position on the anterior side of another limb bud, the number of digits of the resulting wing is doubled. Moreover, the structures of the extra set of digits are mirror images of the normally produced structures. The polarity has been maintained, but the information is now coming from both an anterior and a posterior direction.



Sonic hedgehog defines the ZPA

The search for the molecule(s) conferring this polarizing activity on the ZPA became one of the most intensive quests in developmental biology. In 1993, Riddle and colleagues showed by in situ hybridization that *sonic hedgehog* (*shh*), a vertebrate homologue of the *Drosophila hedgehog* gene, was expressed specifically in that region of the limb bud known to be the ZPA.

As evidence that this association between the ZPA and *sonic hedgehog* was more than just a correlation, Rid56dle and co-workers (1993) demonstrated that the secretion of Sonic hedgehog protein is sufficient for ZPA activity.

They transfected embryonic chick fibroblasts (which normally would never synthesize this protein) with a viral vector containing the *shh* gene. The gene became expressed and translated in these fibroblasts, which were then inserted under the anterior ectoderm of an early chick limb bud.

Mirror-image digit duplications like those induced by ZPA transplants were the result. More recently, beads containing Sonic hedgehog protein were shown to cause the same duplications. Thus, Sonic hedgehog appears to be the active agent of the ZPA.

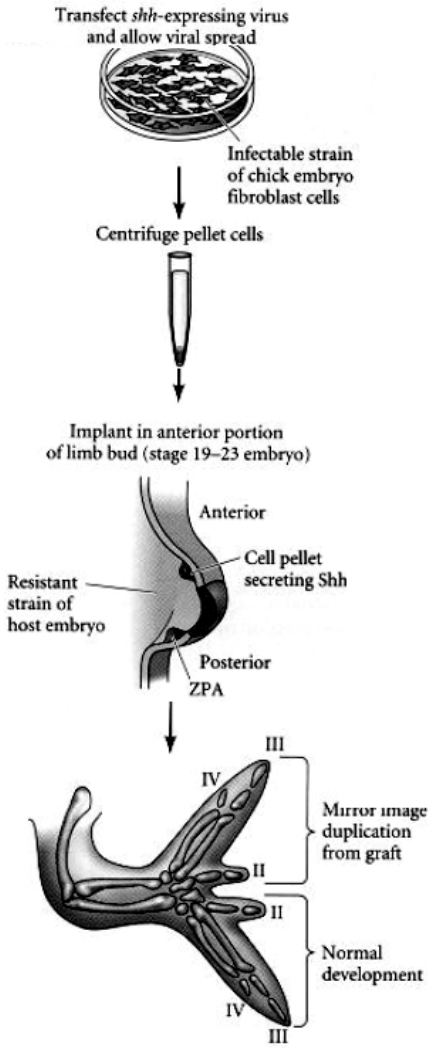
Specification of the posterior limb bud to express sonic Hedgehog

Two new questions emerged from this discovery: First, how does Sonic hedgehog become expressed only in the posterior region of the limb bud? And second, what does it do once it is expressed? We do not yet know what causes the activation of the *sonic hedgehog* genes specifically in the cells of the posterior limb bud and not in the cells located more anteriorly. The *sonic hedgehog* gene appears to be activated by an FGF protein coming from the newly formed apical ectodermal ridge.

FGF8 is secreted from the AER, and is capable of activating *sonic hedgehog*. But why doesn't FGF8 activate all the mesenchyme cells beneath the AER? The answer may reside in the differential competence of certain mesenchyme cells to respond to the FGF signal.

Charité and colleagues (1994) have suggested that the *Hoxb-8* protein may be critical in providing this restricted competence. They observed that the *Hoxb-8* gene is usually expressed in the posterior half of the mouse forelimb bud. They then constructed transgenic mice in which the *Hoxb-8* gene was placed under the control of a new promoter that would cause its expression throughout the forelimb buds. This resulted in the expression of *sonic hedgehog* in the anterior portion of the limb buds, the creation of a new ZPA, and mirror-image forelimb duplications.

This evidence suggests that the Hoxb-8 protein is involved in specifying the domain of *sonic hedgehog* expression and thus establishing the ZPA.



The action of sonic hedgehog

When Sonic hedgehog was first shown to define the ZPA, it was thought to act as a morphogen. In other words, it was thought to diffuse from the ZPA where it was being synthesized and to form a concentration gradient from the posterior to the anterior of the limb bud. However, recent research has provided evidence that Sonic hedgehog protein (or its active amino terminal region) does not diffuse outside the ZPA. It is now thought that Sonic hedgehog works by initiating and sustaining a cascade of other proteins, such as BMP2 and BMP7. A gradient of BMPs may emanate from the ZPA and specify the digits. However it works, Sonic hedgehog (directly or with help from the BMP cascade) regulates the expression of the *5´ HoxD* genes. The transition from phase I to phase II Hox expression patterns is coincident with Sonic hedgehog expression in the ZPA.

Moreover, transplantation of either the ZPA or other Sonic hedgehog-secreting cells to the anterior margin of the limb bud at this stage leads to the formation of mirror-image patterns of *HoxD* gene expression and results in mirror-image digit patterns.

The Generation of the Dorsal-Ventral Axis

The third axis of the limb distinguishes the dorsal limb (knuckles, nails) from the ventral limb (pads, soles). In 1974, MacCabe and co-workers demonstrated that the dorsal-ventral polarity of the limb bud is determined by the ectoderm encasing it. If the ectoderm is rotated 180° with respect to the limb bud mesenchyme, the dorsal-ventral axis is partially reversed; the distal elements (digits) are "upside down." This suggested that the late specification of the dorsalventral axis of the limb is regulated by its ectodermal component. One molecule that appears to be particularly important in specifying the dorsal-ventral polarity is Wnt7a. The *Wnt7a* gene is expressed in the dorsal (but not the ventral) ectoderm of the chick and mouse limb buds. In 1995, Parr and McMahon genetically deleted *Wnt7a* from mouse embryos. The resulting embryos had sole pads on both surfaces of their paws, showing that Wnt7a is needed for the dorsal patterning of the limb.

encodes a transcription factor that appears to be essential for specifying dorsal cell fates in the limb. If this factor is expressed in the ventral mesenchyme cells, they develop a dorsal phenotype. Mutants of *Lmx1* in humans and mice also show its importance for specifying dorsal limb fates. Knockouts of this gene in mice produce a syndrome in which the dorsal limb phenotype is lacking, and loss-of-function mutations in humans produce the nail-patella syndrome, a condition in which the dorsal sides of the limbs have been ventralized.

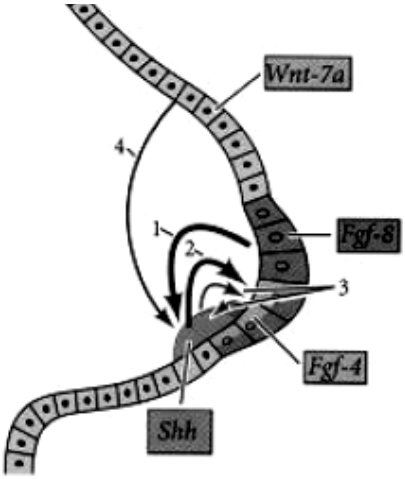
Coordination among the Three Axes

The three axes of the tetrapod limb are all interrelated and coordinated. Some of the principal interactions among the mechanisms specifying the axes.

Indeed, the molecules that define one of these axes are often used to maintain another axis. For instance, Sonic hedgehog in the ZPA activates the expression of the *Fgf4* gene in the posterior region of the AER. *Fgf4* expression is important in recruiting mesenchyme cells into the progress zone, and it is also critical in maintaining the expression of Sonic hedgehog in the ZPA. Therefore, the AER and the ZPA mutually support each other through the positive loop of Sonic hedgehog and FGF4 .

The *Wnt7a*-deficient mice mentioned above not only lacked dorsal limb structures; they also lacked posterior digits, suggesting that Wnt7a is also needed for the anterior-posterior axis.

Yang and Niswander (1995) made a similar set of observations in chick embryos. These investigators removed the dorsal ectoderm from the developing limb and found that such an operation resulted in the loss of posterior skeletal elements from the limbs. The reason that these limbs lacked posterior digits was that Sonic hedgehog expression was missing. Viral-induced expression of Wnt7a was able to replace the dorsal ectoderm and restore Sonic hedgehog expression and posterior phenotypes. These findings showed that the synthesis of Sonic hedgehog is stimulated by the combination of FGF4 and Wnt7a proteins

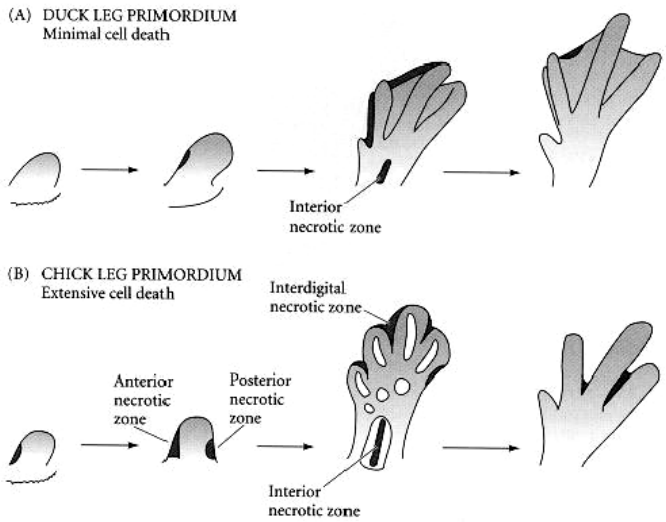


Cell Death and the Formation of Digits and Joints
Sculpting the autopod

Cell death plays a role in sculpting the limb. Indeed, it is essential if our joints are to form and if our fingers are to become separate. The death (or lack of death) of specific cells in the vertebrate limb is genetically programmed and has been selected for during evolution.

One such case involves the webbing or nonwebbing of feet. The difference between a chicken's foot and that of a duck is the presence or absence of cell death between the digits. Saunders and co-workers have shown that after a certain stage, chick cells between the digit cartilage are destined to die, and will do so even if transplanted to another region of the embryo or placed into culture. Before that time, however, transplantation to a duck limb will save them.

Between the time when the cell's death is determined and when death actually takes place, levels of DNA, RNA, and protein synthesis in the cell decrease dramatically.



In addition to the **interdigital necrotic zone**, there are three other regions that are "sculpted" by cell death. The ulna and radius are separated from each other by an **interior necrotic zone**, and two other regions, the **anterior** and **posterior necrotic zones**, further shape the end of the limb. Although these zones are said to be "necrotic," this term is a holdover from the days when no distinction was made between necrotic cell death and apoptotic cell death. These cells die by apoptosis, and the death of the interdigital tissue is associated with the fragmentation of their DNA.

The signal for apoptosis in the autopod is probably provided by the BMP proteins. BMP2, BMP4, and BMP7 are each expressed in the interdigital mesenchyme, and blocking BMP signaling (by infecting progress zone cells with retroviruses carrying dominant negative BMP receptors) prevents interdigital apoptosis. Since these BMPs are expressed throughout the progress zone mesenchyme, it is thought that cell death would be the "default" state unless there were active suppression of the BMPs. This suppression may come from the Noggin protein, which is made in the developing digits and in the perichondrial cells surrounding them. If *noggin* is expressed throughout the limb bud, no apoptosis is seen.

Forming the joints

The function originally ascribed to BMPs was the formation, not the prevention, of bone and cartilage tissue. In the developing limb, BMPs induce the mesenchymal cells either to undergo apoptosis or to become cartilage-producing chondrocytes depending on the stage of development. The same BMPs can induce death or differentiation, depending on the age of the target cell. This "context dependency" of signal action is a critical concept in developmental biology. It is also critical for the formation of joints. Macias and colleagues (1997) have shown that during early limb bud stages (before cartilage condensation), beads secreting BMP2 or BMP7 cause apoptosis. Two days later, the same beads cause the limb bud cells to form cartilage.

In the normally developing limb, BMPs use both these properties to form joints. BMP7 is made in the perichondrial cells surrounding the condensing chondrocytes and promotes cartilage formation. Two other BMP proteins, BMP2 and GDF5, are expressed at the regions between the bones, where joints will form. Mouse mutations have suggested that the function of these proteins in joint formation is critical. Mutations of *Gdf5* produce brachypodism, a condition characterized by a lack of limb joints. In mice homozygous for loss-of-function alleles of *noggin*, no joints form, either. It appears that the BMP7 in these *noggin*-defective embryos is able to recruit nearly all the surrounding mesenchyme into the digits. The roles of BMP2 and GDF5 are more controversial. They may either be destroying mesenchymal cells to form the joint or inducing them to rapidly differentiate and join one or the other cartilaginous nodule. In either way, a space is made between the nodules, and a joint can form.

Limb development is an exciting meeting place for developmental biology, evolutionary biology, and medicine. Within the next decade, we can expect to know the bases for numerous congenital diseases of limb formation, and perhaps we will understand how limbs are modified into flippers, wings, hands, and legs.

C4. Key Points of Developmental Biology

A. PRINCIPLES OF DEVELOPMENT: DEVELOPMENTAL ANATOMY

1. Organisms must function as they form their organs. They have to use one set of structures while constructing others.
2. The main question of development is, How does the egg becomes an adult? This question can be broken down into the component problems of differentiation (How do cells become different from one another and from their precursors?), morphogenesis (How is ordered form is generated?), growth (How is size regulated?), reproduction (How does one generation create another generation?), and evolution (How do changes in developmental processes create new anatomical structures?).
3. Epigenesis happens. New organisms are created de novo each generation from the relatively disordered cytoplasm of the egg.
4. Preformation is not in the anatomical structures, but in the instructions to form them. The inheritance of the fertilized egg includes the genetic potentials of the organism.
5. The preformed nuclear instructions include the ability to respond to environmental stimuli in specific ways.
6. The ectoderm gives rise to the epidermis, nervous system, and pigment cells.
7. The mesoderm generates the kidneys, gonads, bones, heart, and blood cells.
8. The endoderm forms the lining of the digestive tube and the respiratory system.
9. Karl von Baer's principles state that the general features of a large group of animals appear earlier in the embryo than do the specialized features of a smaller group. As each embryo of a given species develops, it diverges from the adult forms of other species. The early embryo of a "higher" animal species is not like the adult of a "lower" animal.
10. Labeling cells with dyes shows that some cells differentiate where they form, while others migrate from their original sites and differentiate in their new locations. Migratory cells include neural crest cells and the precursors of germ cells and blood cells.
11. "Community of embryonic structure reveals community of descent" (Charles Darwin).
12. Homologous structures in different species are those organs whose similarity is due to their sharing a common ancestral structure. Analogous structures are those organs whose similarity comes from their serving a similar function (but which are not derived from a common ancestral structure).
13. Congenital anomalies can be caused by genetic factors (mutations, aneuploidies, translocations) or by environmental agents (certain chemicals, certain viruses, radiation).
14. Syndromes consists of sets of developmental abnormalities that "run together."
15. Organs that are linked in developmental syndromes share either a common origin or a common mechanism of formation.
16. If growth is isometric, a twofold change in weight will cause a 1.26-fold expansion in length.
17. Allometric growth can create dramatic changes in the structure of organisms.
18. Complex patterns may be self-generated by reaction-diffusion events, wherein the activator of a local phenomenon stimulates the production of more of itself as well as the production of a more diffusible inhibitor.

B. LIFE CYCLES AND DEVELOPMENTAL PATTERNS

1. The life cycle can be considered a central unit in biology. The adult form need not be paramount. In a sense, the life cycle is the organism.
2. The basic life cycle consists of fertilization, cleavage, gastrulation, germ layer formation, organogenesis, metamorphosis, adulthood, and senescence.
3. Reproduction need not be sexual. Some organisms, such as *Volvox* and *Dictyostelium,* exhibit both asexual reproduction and sexual reproduction.
4. Cleavage divides the zygote into numerous cells called blastomeres.
5. In animal development, gastrulation rearranges the blastomeres and forms the three germ layers.
6. Organogenesis often involves interactions between germ layers to produce distinct organs.
7. Germ cells are the precursors of the gametes. Gametogenesis forms the sperm and the eggs.
8. There are three main ways to provide nutrition to the developing embryo: (1) supply the embryo with yolk; (2) form a larval feeding stage between the embryo and the adult; or (3) create a placenta between the mother and the embryo.
9. Life cycles must be adapted to the nonliving environment and interwoven with other life cycles.
10. Don't regress your tail until you've formed your hindlimbs.
11. There are several types of evidence. Correlation between phenomenon A and phenomenon B does not imply that A causes B or that B causes A. Loss-of-function data (if A is experimentally removed, B does not occur) suggests that A causes B, but other explanations are possible. Gainof- function data (if A happens where or when it does not usually occur, then B also happens in this new time or place) is most convincing.
12. Protostomes and deuterostomes represent two different sets of variations on development. Protostomes form the mouth first, while deuterostomes form the anus first.

C. EXPERIMENTAL EMBRYOLOGY

1. There are norms of reaction that describe an embryo's inherited ability to develop a range of phenotypes. The environment can play a role in selecting which phenotype is expressed. (Examples include temperature-dependent sex determination and seasonal phenotypic changes in caterpillars and butterflies.)
2. Developing organisms are adapted to the ecological niches in which they develop. (Examples include the ability of frog eggs exposed to sunlight to repair DNA damage.)

3. Before cells overtly differentiate into the many cell types of the body, they undergo a "covert" commitment to a certain fate. This commitment is first labile (the specification step) but later becomes irreversible (the determination step).
4. In autonomous specification, removal of a blastomere from an embryo causes the absence in the embryo of those tissues formed by that blastomere. This mechanism of specification produces a mosaic pattern of development. (Examples include early snail and tunicate embryos.)
5. In autonomous specification, morphogenetic determinants are apportioned to different blastomeres during cell cleavage. (An example is the yellow crescent cytoplasm that is found in the muscle-forming cells of tunicate embryos.)
for by the other cells' changing their fates. Each cell can potentially give rise to more cell types than it normally does. This produces a regulative pattern of development wherein cell fates are determined relatively late. (Examples include frog and mammalian embryos.)
7. In conditional specification, the fate of a cell often depends upon its neighbors ("whom it meets").
8. In conditional specification, groups of cells can have their fates determined according to a concentration gradient of morphogen. The cells specified by such a morphogen can constitute a field.
9. In syncytial specification, the fates of cells can be determined by gradients of morphogens within the egg cytoplasm.
10. Different cell types can sort themselves into regions by means of cell surface molecules such as cadherins. These molecules can be critical in patterning cells into tissues and organs.

D. GENES AND DEVELOPMENT

1. Development connects genotype and phenotype.
2. Nuclear genes are not lost or mutated during development. The genome of every cell is equivalent.
3. The exceptions to the rule of genomic equivalence are the lymphocytes. During differentiation, these cells rearrange their DNA to create new immunoglobulin and antigen receptor genes.
4. Genomic equivalence is implied by metaplasia, in which one differentiated cell type becomes another differentiated cell type. An example is the transdifferentiation of the salamander dorsal iris into a lens when the lens is removed.
5. The ability of nuclei from differentiated cells to direct the development of complete adult organisms has recently confirmed the principle of genomic equivalence.
6. The cloning of human beings, as well as regenerating damaged organs or enhancing physical abilities, may soon be possible through cloning technology and the use of embryonic stem cells.
7. Only a small percentage of the genome is expressed in any particular cell.
8. Polytene chromosomes, in which the DNA has replicated without separating (as in larval *Drosophila* salivary glands), show regions where DNA is being transcribed. Different cell types show different regions of DNA being transcribed.
9. Northern blots, in situ hybridization, and the polymerase chain reaction can show which cells are transcribing particular genes.
10. The functions of a gene often can be ascertained by antisense mRNA, transgenic expression, or (in the case of mammals) gene knockouts.
11. Knowledge of gene activity in humans can be obtained by candidate gene mapping or positional cloning

E. DEVELOPMENTAL GENETICS

1. Differential gene expression from genetically identical nuclei creates different cell types. Differential gene expression can occur at the levels of gene transcription, nuclear RNA processing, mRNA translation, and protein modification.
2. Genes are usually repressed. Activation of a gene often means inhibiting its repressor. This leads to thinking in double and triple negatives: Activation is often the inhibition of the inhibitor; repression is the inhibition of the inhibitor of the inhibitor.
3. Eukaryotic genes contain promoter sequences to which RNA polymerase can bind to initiate transcription. The eukaryotic RNA polymerases are bound by a series of proteins called basal transcription factors.
4. Eukaryotic genes expressed in specific cell types contain enhancer sequences that regulate their transcription in time and space.
5. Specific transcription factors can recognize specific sequences of DNA in the promoter and enhancer regions. They activate or repress transcription from the genes to which they have bound.
6. Enhancers work in a combinatorial fashion. The binding of several transcription factors can act to promote or inhibit transcription from a certain promoter. In some cases transcription is activated only if *both* factor A *and* factor B are present, while in other cases, transcription is activated if *either* factor A *or* factor B is present.
7. A gene encoding a transcription factor can keep itself activated if the transcription factor it encodes also activates its own promoter. Thus, a transcription factor gene can have one set of enhancer sites to initiate its activation and a second set of enhancer sites (that bind the encoded transcription factor) to maintain its activation.
8. Often, the same transcription factors that are used during the differentiation of a particular cell type are also used to activate the genes for that cell type's specific products. For instance, Pax6 is needed both for the differentiation of the lens and for the transcription of the lens crystallin genes, and Mitf is needed for pigment cell differentiation and for the transcription of the genes whose products catalyze the synthesis of melanin.
9. Enhancers can act as silencers to suppress the transcription of a gene in inappropriate cell types.
10. Locus control regions may function by making relatively large portions of a chromosome accessible to transcription factors.
11. Transcription factors act in different ways to regulate RNA synthesis. Some transcription factors stabilize RNA polymerase binding to the DNA, some disrupt nucleosomes, and some increase the efficiency of transcription.
12. Transcription correlates with a lack of methylation on the promoter and enhancer regions of genes. Methylation differences can account for examples of genomic imprinting, wherein a gene transmitted through the sperm

is expressed differently from the same gene transmitted through the egg.
13. Dosage compensation enables the X chromosome-derived products of males (which have one X chromosome per cell in fruit flies and mammals) to equal the X chromosome-derived products of females (which have two X chromosomes per cell). This compensation is accomplished at the level of transcription, either by accelerating transcription from the lone X chromosome in males (*Drosophila*) or by inactivating a large portion of one of the two X chromosomes in females (mammals).
14. X chromosome inactivation in placental mammals is generally random and involves the activation of the *Xist* gene on the chromosome that will be inactivated.
15. Differential RNA selection can allow certain transcripts to enter the cytoplasm while preventing other transcripts from leaving the nucleus.
16. Differential RNA splicing can create a family of related proteins by causing different regions of the nRNA to be read as exons and introns. What is an exon in one set of circumstances may be an intron in another.
17. Some messages are translated only at certain times. The oocyte, in particular, uses translational regulation to set aside certain messages that it transcribes during egg development but uses only after the egg is fertilized. This activation is often accomplished either by the removal of inhibitory proteins or by the polyadenylation of the message.
18. Many messenger RNAs are localized to particular regions of the oocyte or other cells. This localization appears to be regulated by the 3´ untranslated region of the mRNA.

F. CELL-CELL COMMUNICATION

1. Inductive tissue interaction involves inducer and responding tissues.
2. The ability to respond to inductive signals depends upon the competence of the responding cells.
3. Reciprocal induction occurs when the two interacting tissues are both inducers and are competent to respond to each other's signals.
4. Cascades of inductive events are responsible for organ formation.
5. Regionally specific inductions can generate different structures from the same tissue.
6. The ability to respond to inducers is determined by the genetic state of the responding tissue.
7. Juxtacrine interactions are inductions that occur between the cell membranes of adjacent cells or between a cell membrane and an extracellular matrix secreted by another cell.
8. Paracrine interactions occur when a cell or tissue secretes proteins that induce changes in neighboring cells.
9. Paracrine factors are inducing proteins that bind to cell membrane receptors in competent responding cells.
10. Competent cells respond to paracrine factors through signal transduction pathways. Competence is the ability to bind and to respond to the inducers, and it is often the result of a prior induction.
11. Signal transduction pathways begin with the paracrine or juxtacrine factor causing a conformational change in its cell membrane receptor. The new shape results in enzymatic activity in the cytoplasmic domains of the receptor protein. This allows the receptor to phosphorylate other cytoplasmic proteins, thereby activating a dormant kinase activity. Eventually, a transcription factor (or set of factors) is activated that activates or represses specific gene activity.
12. Pleiotropy is the phenomenon of many phenotypic changes being caused by one mutation. Mosaic pleiotropy results when the mutant gene is used in different parts of the body and each part is separately altered. Relational pleiotropy occurs when a particular defect caused by the mutant gene affects other parts of the body that do not express the gene.
13. Genetic heterogeneity results when multiple genes are needed to create a particular phenotype. Often mutant genes for a paracrine factor cause syndromes similar to those generated by mutant genes for the factor's receptor.
14. Phenotypic heterogeneity results when the same mutation produces different phenotypic effects in different individuals. It is caused by the interactions between gene products.
15. Dominant mutations (in which only one mutant gene of the diploid pair is necessary to produce an abnormal phenotype) can be caused by haploinsufficiency, gain-of-function mutations, or dominant negative alleles.
16. Programmed cell death is one possible response to inductive stimuli. Apoptosis is a critical part of life.
17. There is cross-talk between signal transduction pathways, which allows the cell to respond to multiple inputs simultaneously.

G. FERTILIZATION

1. Fertilization accomplishes two separate activities: sex (the combining of genes derived from two parents) and reproduction (the creation of a new organism).
2. The events of conception usually include: (1) contact and recognition between sperm and egg; (2) regulation of sperm entry into the egg; (3) fusion of genetic material from the two gametes; and (4) activation of egg metabolism to start development.
3. The sperm head consists of a haploid nucleus and an acrosome. The acrosome is derived from the Golgi apparatus and contains enzymes needed to digest extracellular coats surrounding the egg. The neck of the sperm contains the mitochondria and the centriole that generates the microtubules of the flagellum. Energy for flagellar motion comes from mitochondrial ATP and a dynein ATPase in the flagellum.
4. The egg contains a haploid nucleus, and an enlarged cytoplasm storing ribosomes, mRNAs, and nutritive proteins. Other mRNAs and proteins, used as morphogenetic factors, are also stored in the egg. Cortical granules lie beneath the egg's plasma membrane. Many eggs also contain protective agents needed for survival in their particular environment.
5. Surrounding the egg plasma membrane is an extracellular layer often used in sperm recognition. In most animals, this extracellular layer is the vitelline envelope. In mammals, it is the much thicker zona pellucida.
6. In many species, eggs secrete diffusible molecules that attract and activate the sperm.
7. In sea urchins, the acrosome reaction is initiated by compounds in the egg jelly. The acrosomal vesicle undergoes exocytosis to release its enzymes. Globular actin polymerizes to extend the acrosomal process. Bindin on the acrosomal process is recognized by a protein complex on the sea urchin egg surface.

8. In mammals, sperm must be capacitated in the female reproductive tract before they are capable of fertilizing the egg.
9. Mammalian sperm bind to the zona pellucida before undergoing the acrosome reaction. In the mouse, this binding is mediated by ZP3 (zona protein 3) and one or many sperm proteins that recognize it. The mammalian acrosome reaction is initiated on the zona pellucida, and the acrosomal enzymes are concentrated there.
10. Fusion between sperm and egg is mediated by protein molecules whose hydrophobic groups can merge the sperm and egg plasma membranes. In sea urchins, bindin may mediate gamete fusion. In mammals, fertilin proteins in the sperm bind to integrins in the egg and allow the membranes to fuse.
11. Polyspermy results when two sperm fertilize the egg. It is usually lethal, since it results in three sets of chromosomes divided among four cells.
12. There are often two blocks to polyspermy. The fast block is electrical and is mediated by sodium ions: the egg membrane resting potential rises, and sperm can no longer fuse with the egg. The slow block is physical and is mediated by calcium ions. A wave of calcium ions propagates from the point of sperm entry, causing the cortical granules to fuse with the egg cell membrane. The released contents of the granules cause the vitelline membrane to rise and to harden into the fertilization envelope.
13. In mammals, blocks to polyspermy include the modification of the zona proteins by the contents of the cortical granules. Sperm can no longer bind to the zona.
14. Inositol 1,4,5-triphosphate (IP3) is believed to be responsible for releasing calcium ions from storage in the endoplasmic reticulum. DAG (diacylglycerol) is thought to initiate the rise in egg pH. The free calcium ions, supported by the alkalization of the egg, activate egg metabolism, protein synthesis, and DNA synthesis.
15. The male pronucleus and the female pronucleus migrate toward each other, replicating DNA as they move.
16. In sea urchins, the two pronuclei merge and a diploid zygote nucleus is formed. In mammals, the pronuclei disintegrate as they approach each other, and their chromosomes gather around a common metaphase plate.
17. Some genes are transmitted differently depending on whether they are from the egg or the sperm. Methylation differences determine if these genes are to be expressed in the early embryo.
18. Microtubular changes cause cytoplasmic movements. These rearrangements of cytoplasm can be critical in specifying which portions of the egg are going to develop into which organs.

H. EARLY INVERTEBRATE DEVELOPMENT

1. During cleavage, most cells do not grow. Rather, the volume of the oocyte is cleaved into numerous cells. The major exceptions to this rule are mammals.
2. The blastomere cell cycle is governed by the synthesis and degradation of cyclin. Cyclin synthesis promotes the formation of MPF, and MPF promotes mitosis. Degradation of cyclin brings the cell back to the S phase. The G phases are added at the midblastula transition.
3. "Blast" vocabulary: A blastomere is a cell derived from cleavage in an early embryo. A blastula is an embryonic structure composed of blastomeres. The cavity in the blastula is the blastocoel. If the blastula lacks a blastocoel, it is a stereo blastula. A mammalian blastula is called a blastocyst (in Chapter 11), and the invagination where gastrulation begins is the blastopore.
4. The movements of gastrulation include invagination, involution, ingression, delamination, and epiboly.
5. Three axes are the foundations of the body: the anterior-posterior axis (head to tail or mouth to anus), the dorsal-ventral axis (back to belly), and the right-left axis (between the two lateral sides of the body).
6. In all four invertebrates described here, cleavage is holoblastic. In the sea urchin, cleavage is radial; in the snail, spiral; in the tunicate, bilateral; and in the nematode, rotational.
7. In the tunicate, snail, and nematode, gastrulation occurs when there are relatively few cells, and the blastopore becomes the mouth. This is the protostome mode of gastrulation.
8. Body axes in these species are established in different ways. In some, such as the sea urchin and tunicate, the axes are established at fertilization through determinants in the egg cytoplasm. In other species, such as the nematode and snail, the axes are established by cell interactions later in development.
9. In the sea urchin, gastrulation occurs only after thousands of cells have formed, and the blastopore becomes the anus. This is the deuterostome mode of gastrulation, and is characteristic only of echinoderms and chordates.
10. In sea urchins, cell fates are determined by signaling. The micromeres constitute a major signaling center. β-catenin is important for the inducing capacity of the micromeres.
11. Differential cell adhesion is important in regulating sea urchin gastrulation. The micromeres delaminate first from the vegetal plate. They form the primary mesenchyme which becomes the skeletal rods of the pluteus larva. The vegetal plate invaginates to form the endodermal archenteron, with a tip of secondary mesenchyme cells. The archenteron elongates by convergent extension and is guided to the future mouth region by the secondary mesenchyme.
12. Snails exhibit spiral cleavage and form stereoblastulae, having no blastocoels. The direction of the spiral cleavage is regulated by a factor encoded by the mother and placed into the oocyte. Spiral cleavage can be modified by evolution, and adaptations of spiral cleavage have allowed some molluscs to survive in otherwise harsh conditions.
13. The polar lobe of certain molluscs contains the determinants for mesoderm and endoderm. These will enter the D blastomere.
14. The tunicate fate map is identical on its right and left sides. The yellow cytoplasm contains muscle-forming determinants; these act autonomously. The nervous system of tunicates is formed conditionally, by interactions between blastomeres.
15. The soil nematode *Caenorhabditis elegans* was chosen as a model organism because it has a small number of cells, a small genome, is easily bred and maintained, has a short lifespan, can be genetically manipulated, and has a cuticle through which one can see cell movements.
16. In the early divisions of the *C. elegans* zygote, one daughter cell becomes a founder cell (producing differentiated descendants) and the other becomes a stem cell (producing other founder cells and the germ line).

17. Blastomere identity in *C. elegans* is regulated by both autonomous and conditional specification.

I. DROSOPHILA DEVELOPMENT AND AXIS SPECIFICATION

1. *Drosophila* cleavage is superficial. The nuclei divide 13 times before forming cells. Before cell formation, the nuclei reside in a syncytial blastoderm. Each nucleus is surrounded by an actinfilled cytoplasm.
2. When the cells form, the *Drosophila* embryo undergoes a midblastula transition, wherein the cleavages become asynchronous and new mRNA is made. The amount of chromatin determines the timing of this transition.
3. Gastrulation begins with the invagination of the most ventral region, the presumptive mesoderm. This causes the formation of a ventral furrow. The germ band expands such that the future posterior segments curl just behind the presumptive head.
4. Maternal effect genes are responsible for the initiation of anterior-posterior polarity. *Bicoid* mRNA is sequestered by its 3′ UTR in the future anterior by the cytoskeleton; *nanos* mRNA is sequestered by its 3′ UTR in the future posterior pole. *Hunchback* and *caudal* messages are seen throughout the embryo.
5. At fertilization, *bicoid* and *nanos* messages are translated. A gradient of Bicoid protein activates more hunchback transcription in the anterior. Moreover, Bicoid inhibits the translation of caudal mRNA. A gradient of Nanos in the posterior inhibits the translation of hunchback mRNA. Caudal protein is made in the posterior.
6. Bicoid and Hunchback proteins activate the genes responsible for the anterior portion of the fly; Caudal activates genes responsible for posterior development.
7. The unsegmented anterior and posterior are regulated by the activation of the Torso protein at the anterior and posterior poles of the egg.
8. The gap genes respond to concentrations of the maternal effect gene proteins. Their protein products interact with each other such that each gap gene protein defines specific regions of the embryo.
9. The gap gene proteins activate and repress the pair-rule genes. The pair-rule genes have modular promoters such that they become activated in the seven "stripes." Their boundaries are defined by the gap genes. These genes form seven bands of transcription along the anterior-posterior axis, each one comprising two parasegments.
10. The pair-rule gene products activate *engrailed* and *wingless* expression in adjacent cells. The *engrailed*expressing cells form the anterior boundary of each parasegment. These cells form a signaling center that organizes the cuticle formation and segmental structure of the embryo.
11. The homeotic selector genes are found in two complexes on chromosome 3 of *Drosophila.* Together these are called Hom-C, the homeotic gene complex. The genes are arranged in the same order as their transcriptional expression. These genes specify each segment, and mutations in these genes are capable of transforming one segment into another.
12. The expression of each homeotic selector gene is regulated by the gap and pair-rule genes. Their expression is refined and maintained by interactions whereby the protein products interact with genes, preventing the transcription of neighboring Hom-C genes.
13. In Ultrabithorax mutations, the third thoracic segment becomes specifed as the second thoracic segment. This converts the halteres into wings. When Antennapedia is expressed in the head as well as in the thorax, it represses antenna formation, allowing legs to form where the antenna should be.
14. The targets of the Hom-C proteins are the realisator genes. These include *Distal-less* and *Wingless* genes (in the thoracic segments).
15. Dorsal-ventral polarity is regulated by the entry of the Dorsal protein into the nucleus. Dorsal-ventral polarity is initiated by the nucleus being positioned in the dorsal-anterior of the oocyte and transcribing the *gurken* message. This message is transported to the region above the nucleus and adjacent to the follicle cells.
16. The *gurken* mRNA is translated into the Gurken protein, which is secreted from the oocyte and binds to its receptor, Torpedo, on the follicle cells. This dorsalizes the follicle cells, preventing them from synthesizing Pipe.
17. The Pipe protein in the ventral follicle cells modifies an as yet unknown factor that modifies the Nudel protein. This allows the Nudel protein to activate a cascade of proteolysis in the space between the ventral follicle cells and the ventral cells of the embryo.
18. As a result of the cascade, the Spätzle protein is activated and binds to the Toll protein on the ventral embryonic cells.
19. The activated Toll protein activates Pelle and Tube to phosphorylate the Cactus protein, which has been bound to the Dorsal protein. Phosphorylated Cactus protein is degraded, allowing Dorsal protein to enter the nucleus.
20. Once in the nucleus, Dorsal protein activates the genes responsible for the ventral cell fates and represses those genes whose proteins would specify dorsal cell fates. Since a gradient of Dorsal protein enters the various nuclei, those at the most ventral surface become mesoderm, those more lateral become neurogenic ectoderm.
21. Organs form at the intersection of dorsal-ventral and anterior-posterior regions of gene expression.

J. EARLY DEVELOPMENT AND AXIS FORMATION IN AMPHIBIANS

1. Amphibian cleavage is holoblastic, but unequal due to the presence of yolk in the vegetal hemisphere.
2. Amphibian gastrulation begins with the invagination of the bottle cells, followed by the coordinated involution of the mesoderm and the epiboly of the ectoderm. Vegetal rotation plays a significant role in directing the involution.
3. The driving forces for ectodermal epiboly and the convergent extension of the mesoderm are the intercalation events in which several tissue layers merge. Fibronectin plays a critical role in enabling the mesodermal cells to migrate into the embryo.
4. The dorsal lip of the blastopore forms the organizer tissue of the amphibian gastrula. This tissue dorsalizes the ectoderm, transforming it into neural tissue, and it transforms ventral mesoderm into lateral mesoderm.
5. The organizer consists of pharyngeal endoderm, head mesoderm, notochord, and dorsal blastopore lip. The organizer functions by secreting proteins (Noggin, chordin, and follistatin) that block the BMP signal that

would otherwise ventralize the mesoderm and activate the epidermal genes in the ectoderm.
6. In the head region, an addition set of proteins (Cerberus, Frzb, Dickkopf) block the Wnt signal from the ventral and lateral mesoderm.
7. The organizer is itself induced by the Nieuwkoop center, located in the dorsalmost vegetal cells. This center is formed by cortical rotation during fertilization, which translocates the Dishevelled protein to the dorsal side of the egg.
8. The Dishevelled protein stabilizes β-catenin in the dorsal cells of the embryo. Thus, the Nieuwkoop center is formed by the accumulation of β-catenin, which can complex with Tcf3 to form a transcription factor complex that can activate the transcription of the *siamois* gene.
9. The *siamois* product and a TGF-β signal (perhaps from Vg1) can activate the *goosecoid* gene in the organizer. The *goosecoid* gene can activate other genes that cause the organizer to function.
10. Other posteriorizing signals (Wnt3a, retinoic acid, eFGF) can influence the anterior-posterior specification of the neural tube.
11. The left-right axis appears to be initiated at fertilization through the Vg1 protein. In a still unknown fashion, this protein activates a Nodal protein solely on the left side of the body. As in other vertebrates, the Nodal protein activates expression of Pitx2, which is critical in distinguishing left-sidedness from right sidedness
in the heart and gut tubes.

K. THE EARLY DEVELOPMENT OF VERTEBRATES

1. Fishes, reptiles, and birds undergo discoidal meroblastic cleavage, wherein the early cell divisions do not cut through the yolk of the egg. These cells form a blastoderm.
2. In fishes, the deep cells form between the yolk syncytial layer and the enveloping layer. These cells migrate over the top of the yolk, forming the hypoblast and epiblast layers. On the future dorsal side, these layers intercalate to form the embryonic shield, a structure homologous to the amphibian organizer. Transplantation of the embryonic shield into the ventral side of another embryo will cause the formation of a second embryonic axis.
3. There appear to be two signaling centers supplying anterior-posterior information in fishes, one located at the border between the neural and surface ectoderm, the other in the lateral mesoderm.
4. In chick embryos, early cleavage forms an area opaca and an area pellucida. The region between them is the marginal zone. Gastrulation begins at the posterior marginal zone, as the hypoblast and primitive streak both start there.
5. The primitive streak is derived from anterior epiblast cells and the central cells of the posterior marginal zone. As the primitive streak extends rostrally, Hensen's node is formed. Cells migrating through Hensen's node become chordamesoderm (notochord) cells. These extend up to the presumptive midbrain, where they meet the prechordal plate.
6. The prechordal plate induces the formation of the forebrain; the chordamesoderm induces the formation of the midbrain, hindbrain, and spinal cord. The first cells migrating laterally through the primitive streak become endoderm, displacing the hypoblast. The mesoderm cells then migrate through. Meanwhile, the surface ectoderm undergoes epiboly around the entire yolk.
7. In birds, gravity is critical in determining the anterior-posterior axis, while pH differences appear crucial for distinguishing dorsal from ventral. The left-right axis is formed by the expression of *nodal* on the left side of the embryo, which signals *pitx2* expression on the left side of developing organs.
8. Mammals undergo holoblastic rotational cleavage, characterized by a slow rate of division, a unique cleavage orientation, lack of divisional synchrony, and the formation of a blastocyst.
9. The blastocyst forms after the blastomeres undergo compaction. It contains outer cells the trophoblast cells that become the chorion, and an inner cell mass that becomes the amnion and the embryo.
10. The chorion forms the fetal portion of the placenta, which functions to provide oxygen and nutrition to the embryo, to provide hormones for the maintenance of pregnancy, and to provide barriers to the mother's immune system.
11. Mammalian gastrulation is not unlike that of birds. There appear to be two signaling centers one in the node and one in the anterior visceral endoderm. The latter is critical for generating the forebrain, while the former is critical in inducing the axial structures caudally from the midbrain.
12. Hox genes pattern the anterior-posterior axis and help to specify positions along that axis. If Hox genes are knocked out, segment-specific malformations can arise. Similarly, causing the ectopic expression of Hox genes can alter the body axis.
13. The homology of gene structure and the similarity of expression patterns between *Drosophila* and mammalian Hox genes suggests that this patterning mechanism is extremely ancient.
14. The mammalian left-right axis is specified similarly to that of the chick.

L. CENTRAL NERVOUS SYSTEM AND EPIDERMIS

1. The neural tube forms from the shaping and folding of the neural plate. In primary neurulation, the surface ectoderm folds into a tube that separates from the surface. In secondary neurulation, the ectoderm forms a cord and then forms a cavity within it.
2. Primary neurulation is regulated by both intrinsic and extrinsic forces. Intrinsic wedging occurs within cells of the hinge regions to bend the neural plate. Extrinsic forces include the migration of the surface ectoderm towards the center of the embryo.
3. Neural tube closure is also a mixture of extrinsic and intrinsic forces. In humans, if the neural tube fails to close various diseases can result.
4. The neural crest cells arise at the lateral borders of the neural tube and surface ectoderm. They become located between the neural tube and surface ectoderm, and they migrate away from this region to become peripheral neural, glial, and pigment cells.
5. There is a gradient of maturity in many embryos, especially those of amniotes. The anterior develops earlier than the posterior.
6. The dorsal-ventral patterning of the neural tube is accomplished by proteins of the TGF-β family secreted

from the surface ectoderm and roof of the neural tube, and from Sonic hedgehog protein secreted by the notochord and floor plate cells. Both types of protein appear to work through gradients.

7. The brain forms three primary vesicles: prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). The prosencephalon and rhombencephalon will become subdivided.

8. The brain expands through fluid secretion putting positive pressure on the vesicles.

9. The neurons of the brain are organized into cortices (layers) and nuclei (clusters).

10. New neurons are formed by mitosis in the neural tube. The neural precursors can migrate away from the neural tube and form a new layer. Neurons forming later have to migrate through the existing layers. This forms the cortical layers. The germinal zone at the lumen of the neural tube is called the ventricular zone. The new layer is called the mantle zone (gray matter).

11. In the cerebellum, a second germinal zone the external granule layer is formed. Other neurons migrate out of the ventricular zone on the processes of glial cells.

12. The cerebral cortex in humans has six layers, and the mantle zone is called the neocortex. Cell fates are often fixed as they undergo their last division. Neurons derived from the same stem cell may end up in different functional regions of the brain.

13. Neural stem cells have been observed in the adult human brain. We now believe humans can continue making neurons throughout life, although at nowhere near the fetal rate.

14. Dendrites receive signals from other neurons, while axons transmit them. The place where the signaling takes place (through the release of neurotransmitters) is called a synapse.

15. Axons grow from the nerve cell body, or soma. They are led by the growth cone.

16. The chordate and arthropod systems, though structurally very different, appear to be specified through the same set of genetic instructions.

17. The retina forms from the optic vesicle that extends from the brain. Pax6 plays a major role in eye formation, and the downregulation of Pax6 by Sonic hedgehog in the center of the brain splits the eye-forming region of the brain in half. If Sonic hedeghog is not expressed there, a single medial eye results.

18. The photoreceptor cells gather the light and transmit the impulse through interneurons to the retinal ganglion cells. The axons of the retinal ganglion cells form the optic nerve.

19. The lens and cornea form from the surface ectoderm. Both must become transparent.

20. The basal layer of the surface ectoderm becomes the stratum germinativum, or germinal layer of the skin. These cells divide to produce a stem cell and a cell committed to become an epidermal cell (keratinocyte). Stem cells appear to be able to make hair.

21. Paracrine factors such as TGF-_ and FGF7 are important in normal skin development.

22. Cutaneous appendages hair, feathers, and scales are formed by epithelial-mesenchymal interactions between the epidermis and the dermal mesoderm.

M. NEURAL CREST CELLS AND AXONAL SPECIFICITY

1. The neural crest is a transitory structure. Its cells migrate to become numerous different cell types.

2. Trunk neural crest cells can migrate dorsolaterally into the ectoderm, where they become melanocytes. They can also migrate ventrally, to become sympathetic and parasympathetic neurons and adrenal medulla cells.

3. A portion of the anterior trunk neural crest enters the heart and forms the separation between the pulmonary artery and aorta.

4. The cranial neural crest cells enter the pharyngeal arches to become the cartilage of the jaw and the bones of the middle ear. They also form the bones of the frontonasal process, the papillae of the teeth, and the cranial nerves.

5. The formation of the neural crest depends on interactions between the prospective epidermis and the neural plate. Paracrine factors from these regions induce the formation of transcription factors that enable neural crest cells to emigrate.

6. The path a neural crest cell takes depends on the extracellular matrix it meets.

7. Trunk neural crest cells will migrate through the anterior portion of each somite, but not through the posterior portion of a somite. Ephrin proteins are expressed in the posterior portion of each somite and appear to prevent neural crest cell migration.

8. Some neural crest cells appear to be capable of forming large repertoire of cell types. Other neural crest cells may be committed to a fate even before migrating. The final destination of the neural crest cell can sometimes change the specification of the neural crest cell.

9. The fates of the cranial neural crest cells are to a great extent controlled by the Hox genes.

10. Teeth develop through an elaborate dialogue between the neural crest-derived mesenchyme and the jaw epithelium. The mesenchyme becomes the odontoblasts, while the epithelium generates the ameloblasts.

11. The major signaling center of the tooth is the enamel knot. It secretes several paracrine factors that regulate cell proliferation and differentiation in both the mesencyme and epithelium.

12. The specification of the motor neurons is done according to their place in the neural tube. The LIM family of transcription factors plays an important role in this specification.

13. Targets of the motor neurons are specified before the motor neurons extend into the periphery.

14. The growth cone is the locomotor organelle of the neuron, and it senses the environmental cues. (It has been called a "neural crest cell on a leash" because the growth cone and the neural crest cell both are migratory and sense the environment.)

15. Axons can find their targets without neuronal activity.

16. Some proteins are generally permissive to neuron adhesion and provide substrates on which axons can migrate. Other substances prohibit migration.

17. Some growth cones recognize molecules which are present in very specific areas and therefore will be guided by these molecules to their respective targets.

18. Some neurons are "kept in line" by repulsive molecules. If they wander off the path to their target, these molecules bring them back. Some molecules, such

as the semaphorins, are selectively repulsive to a particular set of neurons.
19. Some neurons sense gradients of a protein and are brought to their target by following these gradients. The netrins may work in this fashion.
20. Target selection can be brought about by neurotrophins, proteins that are made by the target tissue that stimulate the particular set of axons that can innervate it. In some cases, the target makes only enough of these factors to support a single axon.
21. Address selection is activity-dependent. An active neuron can suppress synapse formation by other neurons on the same target.
22. Retinal ganglial axons in frogs and chick send axons that bind to specific regions of the optic tectum. This process is mediated by numerous interactions, and the target selection appears to be mediated through ephrins.
23. In some instances, fetal neurons can integrate into adult brains and re-establish damaged synapses.
24. Some behaviors appear to be innate ("hard-wired") while others are learned. Experience can strengthen certain neural connections.

N. PARAXIAL AND INTERMEDIATE MESODERM

1. The paraxial mesoderm forms blocks of tissue called somites. Somites give rise to three major divisions: the dermatome, the myotome, and the sclerotome.
2. The dermatome of the somite forms the back dermis. The sclerotome of the somite forms the vertebral cartilage. In thoracic vertebrae, the sclerotome cells also form the proximal portions of the ribs.
3. The epaxial myotome forms the back musculature. The hypaxial myotome forms the muscles of the body wall, limb, and tongue.
4. Somites are formed from the segmental plate (unsegmented mesoderm) by a combination of proteins. The Hairy protein, a transcription factor, appears to specify the somite boundaries. Notch and Eph receptor systems may be involved in the separation of the somites from the unsegmented paraxial mesoderm. N-cadherin and fibronectin appear to be important in causing these cells to become epithelial.
5. The somite regions are specified by paracrine factors secreted by neighboring tissue. The sclerotome is specified to a large degree by the Sonic hedgehog protein, secreted by the notochord and floor plate cells. The dermatome is specified by neurotrophin-3, secreted by the roof plate cells of the neural tube.
6. The two myotome regions are specified by different factors. The epaxial myotome is specified by Wnt proteins from the dorsal neural tube. The hypaxial myotome is specified by BMP4 (and perhaps other proteins) secreted by the lateral plate mesoderm. In both instances, myogenic bHLH transcription factors are induced in the cells that will become muscles the hypaxial and epaxial myoblasts.
7. Muscle formation involves the myoblasts ceasing to divide, aligning themselves, and fusing.
8. The major lineages that form the skeleton are the somites (axial skeleton), lateral plate mesoderm (appendages), and neural crest (skull and face).
9. There are two major types of ossification. Intramembranous ossification occurs in the skull bones and in turtle shells. Here, mesenchyme is directly converted into bone. In endochondral ossification, the mesenchyme cells become cartilage. These cartilagenous models are later become replaced by bone cells.
10. The replacement of cartilage by bone during endochondral ossification depends upon the mineralization of the cartilage matrix.
11. In the long bones of humans and other mammals, the ends contain cartilagenous regions called epiphyseal growth plates. The cartilage in these regions proliferate to make the bone bigger. Eventually, the cartilage is replaced by bone and growth stops.
12. The hollowing out of bone for the bone marrow is accomplished by osteoclasts. Osteoclasts continually remodel bone throughout a person's lifetime.
13. The intermediate mesoderm generates the kidneys and gonads.
14. The metanephric kidney of mammals is formed by the reciprocal interactions of the metanephrogenic mesenchyme and a branch of the nephric duct called the ureter bud.
15. The metanephrogenic mesenchyme becomes competent to become kidney tubules by expressing WT1. WT1 is also thought to enable that mesenchyme to secrete GDNF and HGF. These two factors are secreted by the mesoderm and induce the formation of the ureteric bud.
16. The ureteric bud secretes FGF2 and BMP7 to prevent apoptosis in the kidney mesenchyme. Without these factors, the kidney mesenchyme dies. FGF2 also makes the mesenchyme competent to respond to LIF.
17. The ureter bud also secretes LIF, and this protein induces the competent kidney mesenchyme to become epithelial tubules. As they form the kidney tubules, the cells secrete Wnt4, which promotes and maintains their epithelialization.
18. The condensing mesenchyme secretes paracrine factors (most of them members of the TGF-β superfamily) that mediate the branching of the ureter bud. The branching depends upon the extracellular matrix of the epithelium.

O. LATERAL MESODERM AND ENDODERM

1. The lateral plate mesoderm splits into two layers. The dorsal layer is the somatic (parietal) mesoderm, which underlies the ectoderm and forms the somatopleure. The ventral layer is the splanchnic (visceral) mesoderm, which overlies the endoderm and forms the splanchnopleure.
2. The space between these two layers is the body cavity, the coelom.
3. The heart arise from splanchnic mesoderm on both sides of the body. This region of cells is called the cardiogenic mesoderm.
4. The Nkx2-5 transcription factor is important in specifying cells to become cardiogenic mesoderm. These cells migrate from the sides to the midline of the embryo, in the neck region.
5. Cardiogenic mesoderm forms the endocardium (which is continuous with the blood vessels) and the myocardium (the muscular component of the heart).
6. The endocardial tubes form separately and then fuse. The looping of the heart transforms the original anterior-posterior polarity of the heart tube into a right-left polarity.
7. In mammals, fetal circulation differs dramatically from adult circulation. When the infant takes its first breath,

changes in air pressure close the foramen ovale through which blood had passed from the right to the left atrium. At that time, the lungs, rather than the placenta, become the source of oxygen.

8. Blood vessel formation is constrained by physiological, evolutionary, and physical parameters. The subdividing of a large vessel into numerous smaller ones allows rapid transport of the blood to regions of gas and nutrient diffusion.

9. Blood vessels are constructed by two processes, vaculogenesis and angiogenesis. Vasculogenesis involves the condensing of visceral mesoderm cells to form blood islands. The outer cells of these islands become endothelial (blood vessel) cells. Angiogenesis involves remodeling existing blood vessels.

10. Numerous paracrine factors are essential in blood vessel formation. FGF2 is needed for specifying the angioblasts. VEGF is essential for the differentiation of angioblasts. Angiopoietin- 1 allows the smooth muscle cells (and smooth muscle-like pericytes) to cover the vessels. Ephrin ligands and Eph receptor tyrosine kinases are critical for capillary bed formation.

11. The pluripotential hematopoietic stem cell generates other pluripotential stem cells, as well as lineage-restricted stem cells. It gives rise to both blood cells and lymphocytes.

12. The CFU-S is a blood stem cell that can generate the more committed stem cells for the different blood lineages. Hematopoietic inductive microenvironments determine the direction of the blood cell differentiation.

13. In mammals, embryonic blood stem cells are provided by the blood islands near the yolk. The definitive adult blood stem cells come from the aorta-gonad-mesonephros region within the embryo.

14. The endoderm constructs the digestive tube and the respiratory tube.

15. Four pairs of pharyngeal pouches become the endodermal lining of the eustacian tube, tonsils, thymus, and parathyroid glands. The thyroid also forms in this region of endoderm.

16. The gut tissue forms by reciprocal interactions between the endoderm and the mesoderm. Sonic hedgehog from the endoderm appears to play a role in inducing a nested pattern of Hox gene expression in the mesoderm surrounding the gut. The regionalized mesoderm then instructs the endodermal tube to become the different organs of the digestive tube.

17. The pancreas forms in a region of endoderm that lacks Sonic hedgehog expression. The Pdx1 transcription factor is expressed in this region.

18. The respiratory tube is derived as an outpocketing of the digestive tube. The regional specificity of the mesenchyme it meets determines whether the tube remains straight (as in the tracheae) or branches (as in the alveoli).

19. The yolk sac and allantois are derived from the splanchnopleure. The yolk sac (in birds and reptiles) allows yolk nutrients to pass into the blood. The allantois collects nitrogenous wastes.

20. The chorion and amnion are made by the somatopleure. In birds and reptiles, the chorion abuts the shell and allows for gas exchange. The amnion in birds, reptiles, and mammals bathes the embryo in amnionic fluid.

P. THE TETRAPOD LIMB

1. The places where limbs emerge from the body axis depend upon Hox gene expression.

2. The specification of the limb field into a hindlimb or forelimb bud is determined by *Tbx4* and *Tbx5* expression.

3. The proximal-distal axis of the developing limb is determined by the induction of the ectoderm at the dorsal-ventral boundary to form the apical ectodermal ridge (AER). This induction is caused by an FGF, probably FGF10. The AER secretes FGF8, which keeps the underlying mesenchyme proliferative and undifferentiated. This mesenchyme is called the progress zone.

4. As the limb grows outward, the stylopod forms first, then the zeugopod, and the autopod is formed last. Each of these phases involves the expression of Hox genes, and the formation of the autopod involves a reversal of Hox gene expression that distinguishes fish fins from tetrapod limbs.

5. The anterior-posterior axis is defined by the expression of Sonic hedgehog in the posterior mesoderm of the limb bud. This region is called the zone of polarizing activity (ZPA). If the ZPA or Sonic hedgehog-secreting cells or beads are placed in the anterior margin, they establish a second, mirror-image pattern of Hox gene expression and a corresponding mirror-image duplication of the digits.

6. The ZPA is established by the interaction of FGF8 from the AER and mesenchyme made competent to express Sonic hedgehog by its expression of particular Hox genes. Sonic hedgehog acts, probably in an indirect manner, to change the expression of the Hox genes in the limb bud.

7. The dorsal-ventral axis is formed, in part, by the expression of Wnt7a in the dorsal portion of the limb ectoderm. Wnt7a also maintains the expression of Sonic hedgehog in the ZPA and FGF4 in the posterior AER. FGF4 and Sonic hedgehog reciprocally maintain each other's expression.

8. Cell death in the limb is necessary for the formation of digits and joints. It is mediated by BMPs. The effects of BMPs can be regulated by the Noggin protein, and the BMPs can be involved both in inducing apoptosis and in differentiating the mesenchymal cells into cartilage.

Q. SEX DETERMINATION

1. In mammals, primary sex determination (the determination of gonadal sex) is a function of the sex chromosomes. XX individuals are females, XY individuals are males.

2. The Y chromosome plays a key role in male sex determination. XY and XX mammals both have a bipotential gonad that makes the primary sex cords. In XY animals, these cords continue to be formed within the gonad, and eventually differentiate into the Sertoli cells of the testes. The interstitial mesenchyme becomes the Leydig cells.

3. In XX individuals, the internal sex cords degenerate, and a second set of cortical sex cords emerges. These remain on the periphery of the gonad. Germ cells enter the sex cords, but will not be released from the gonad until puberty. The epithelium of the sex cords becomes the granulosa cells; the mesenchyme becomes the thecal cells.

4. In humans, the *SRY* gene is the testis-determining factor on the Y chromosome. It synthesizes a DNA-binding protein that is thought to compete with the *DAX1* protein. It is thought that if SRY is produced at a high enough level, it activates (either directly or indirectly) the *SF1* gene and inhibits the *WNT4* gene.
5. The *SF1* product is believed to activate the *SOX9* gene, as well as several other genes involved in synthesizing steroid hormones and anti-Müllerian duct hormone (AMH). *SOX9* may organize the genital ridge epithelium to form testes, but the corresponding ovary-forming genes have not yet been found, although the *WNT-4* gene may be important in this regard.
6. Secondary sex determination in mammals involves the hormones produced by the developing gonads. Under estrogenic stimulation, the Müllerian duct differentiates into the oviducts, uterus, cervix, and upper portion of the vagina. In male mammals, the Müllerian duct is destroyed by the AMH produced by the Sertoli cells, while the testosterone produced by the Leydig cells enables the Wolffian duct to differentiate into the vas deferens and seminal vesicle. In female mammals, the Wolffian duct degenerates because of the lack of testosterone.
7. The conversion of testosterone to dihydrotestosterone in the genital rudiment and prostate gland precursor enables the differentiation of the penis, scrotum, and prostate gland.
8. Individuals with mutations of these hormones or their receptors may have a distinction between their primary and secondary sex characteristics.
9. In *Drosophila,* sex is determined by the ratio of X chromosomes to autosomes, and the Y chromosome does not play a role in sex determination. There are no sex hormones, so each cell makes a sex determination decision.
10. The *Drosophila Sxl* gene is activated in females (by proteins encoded on the X chromosomes) and is repressed in males (by factors encoded on the autosomes). Sxl protein acts as an RNA splicing factor to splice an inhibitory exon from the *tra* transcript. Therefore, female flies have an active Tra protein, while males do not.
11. The Tra protein also acts as an RNA splicing factor to splice exons of the *doublesex* transcript. The *doublesex* gene is transcribed in both XX and XY cells, but its pre-mRNA is processed to form different mRNAs, depending on whether Tra is present. The proteins translated from both messages are active, and they activate or inhibit transcription of a set of genes involved in producing the sexually dimorphic traits of the fly.
12. In turtles and alligators, sex is often determined by the temperature during the time of gonad determination. Since estrogen is necessary for ovary development, it is possible that differing levels of aromatase (the enzyme that can convert testosterone into estrogen) distinguish male from female patterns of gonadal differentiation.
13. In some species, such as *Bonellia* and *Crepidula,* sex determination is brought about by the position of the individual with regard to other individuals of the same species.

R. METAMORPHOSIS, REGENERATION, AND AGING

1. Amphibian metamorphosis includes both morphological and biochemical changes. Some structures are remodeled, some are replaced, and some new structures are formed.
2. Many changes during amphibian metamorphosis are regionally specific. The tail epidermis dies, the head epidermis does not. An eye will persist even if transplanted into a degenerating tail.
3. The hormones responsible for amphibian metamorphosis are the thyroid hormones thyroxine (T4) and triiodothyronine (T3). The coordination of metamorphic changes appears to be due to early changes that occur at low concentrations of the thyroid hormones. This is called the threshold concept. The molecular basis for the autoinduction of thyroid hormones may be the ability of thyroid hormones to induce production of more thyroid hormone receptor protein. Thyroid hormones act predominantly at the transcriptional level.
4. Heterochrony involves changing the relative rate of development in different parts of the animal. In animals with direct development, the tadpole stage has been lost. Some frogs, for instance, form limbs while in the egg.
5. In neoteny, the juvenile (larval) form is slowed down, while the gonads and germ cells mature at their normal rate. In progenesis, the gonads and germ cells mature rapidly, while the rest of the body matures normally. In both instances, the animal can mate while in its larval form.
6. In ametabolous insects, there is direct development. In hemimetabolous insects, there is a nymph stage wherein the immature organism is usually a smaller version of the adult. In holometabolous insects, there is a dramatic metamorphosis from larva to pupa to sexually mature adult.
7. In the period between larval molts, the larva is called an instar. After the last instar stage, the larva undergoes a metamorphic molt to become a pupa. The pupa will undergo an instar molt to become an adult.
8. During the pupal stage, the imaginal discs and histoblasts grow and differentiate to produce the structures of the adult body.
9. The anterior-posterior, dorsal-ventral, and proximal-distal axes are sequentially specified and involve interactions between different compartments in the imaginal discs.
10. Molting is caused by the hormone hydroxyecdysone. In the presence of high titres of juvenile hormone, the molt is an instar molt. In low concentrations of juvenile hormone, the molt produces a pupa; and if no juvenile hormone is present, the molt is an imaginal molt.
11. The ecdysone receptor gene can produce nRNA that can form at least three different proteins. The types of ecdysone receptors in a cell may influence the response of that cell to hydroxyecdysone. The ecdysone receptors bind to DNA to activate or repress transcription.
12. There are three major types of regeneration. In epimorphosis (such as regenerating limbs), tissues dedifferentiate into a blastema, divide, and re-differentiate into the new structure. In morphallaxis (characteristic of hydra), there is a repatterning of existing tissue with little or no growth. In compensatory regeneration (such as in the liver), cells divide but retain their differentiated state.

13. In the regenerating salamander limb, the epidermis forms an apical ectodermal cap. The cells beneath it dedifferentiate to form a blastema. The differentiated cells lose their adhesions and reenter the cell cycle. This does not happen in mammals.
14. In hydras, there appear to be head activation gradients, head inhibition gradients, foot activation gradients, and foot inhibition gradients. Hydra budding occurs where these gradients are minimal.
15. In mammals, medical researchers are testing whether paracrine factors may permit local regeneration. Bone and neural cells are being returned to embryonic conditions in the hopes that they will regrow. Natural inhibitors of neural regeneration have recently been discovered, and their circumvention may allow spinal cord regeneration.
16. The maximum life span of a species is how long its longest observed member has lived. It is largely characteristic of a given species. Life expectancy is the time at which approximately 50 percent of the members of a given population of a species still survive.
17. There are several levels at which we can study aging, including cellular, biochemical, and genetic studies. Reactive oxygen species (ROS) can damage cell membranes, inactivate proteins, and mutate DNA. Mutations that alter the ability to make or degrade ROS can change the life span of the mutants.
18. Mitochondria may be a target for proteins that regulate aging.
19. Aging is the time-related deterioration of the physiological functions necessary for survival and reproduction. The phenotypic changes of senescence (which affect all members of the species) are not to be confused with diseases of senescence, such as cancer and heart disease (which affect individuals).

S. THE GERM LINE

1. The precursors of the gametes the sperm and eggs are the primordial germ cells. They form outside the gonads and migrate into the gonads during development.
2. In many species, a distinctive germ plasm exists. It often contains the Oskar, Vasa, and Nanos proteins or the mRNAs encoding them.
3. In *Drosophila,* the germ plasm becomes localized in the posterior of the embryo and forms pole cells, the precursors of the gametes. In frogs, the germ plasm originates in the vegetal portion of the oocyte.
the gonads. In mammals, a similar migration is seen, and fibronectin pathways may also be used. Stem cell factor is critical in this migration, and the germ cells proliferate as they travel.
5. In birds, the germ plasm is first seen in the germinal crescent. The germ cells migrate through the blood, then leave the blood vessels and migrate into the genital ridges.
6. Germ cell migration in *Drosophila* occurs in several steps involving passive translocation, repulsion from the endoderm, and attraction to the gonads.
7. Before meiosis, the DNA is replicated and remains bound at the kinetochore. Homologous chromosomes are connected through the synaptonemal complex. The configuration of the four chromatids is called a tetrad.
8. The first division of meiosis separates the homologous chromosomes. The second division of meiosis splits the kinetochore and separates the chromatids.
9. The meiosis/mitosis decision in nematodes is regulated by the Delta proteins, which bind to the Notch proteins on the PGCs. The decision for a germ cell to become either a sperm or an egg is regulated at the level of translation of the *fem-3* message.
10. Spermatogenic meiosis in mammals is characterized by the production of four gametes per meiosis and by the absence of meiotic arrest. Oogenic meiosis is characterized by the production of one gamete per meiosis and by an arrest at first meiotic prophase to allow the egg to grow.
11. In some species, meiosis is modified such that a diploid egg is formed. These species can produce a new generation parthenogenetically, without fertilization.
12. The egg not only synthesizes numerous compounds, but also absorbs material produced by other cells. Moreover, it localizes many proteins and messages to specific regions of the cytoplasm, often tethering them to the cytoskeleton.
13. The *Xenopus* oocyte transcribes actively from lampbrush chromosomes during the first meiotic prophase.
14. In *Drosophila,* the oocyte is relatively dormant in terms of transcription. Rather, nurse cells make mRNAs that enter the developing oocyte. Which of the cells derived from the primordial germ cell becomes the oocyte and which become nurse cells is determined by the fusome and the pattern of divisions.
15. In mammals, the hormones of the menstrual cycle integrate the ovarian cycle, the uterine cycle, and the cervical cycle. This integration allows the uterus to be ready to receive an embryo shortly after ovulation occurs.

T. PLANT DEVELOPMENT

1. Plants are characterized by alternation of generations.
2. A multicellular diploid sporophyte produces haploid spores via meiosis. These spores divide mitotically to produce a haploid gametophyte. Mitotic divisions within the gametophyte produce the gametes. The diploid sporophyte results from the fusion of two gametes.
3. The male gamete, pollen, arrives at the style of the female gametophyte and effects fertilization through the pollen tube. Two sperm cells move through the pollen tube; one joins with the ovum to form the zygote, and the other is involved in the formation of the endosperm.
4. Plant embryos develop deeply embedded in parental tissue. The parent tissue provides nutrients but only minimal patterning information.
5. Early embryogenesis is characterized by the establishment of the shoot-root axis and by radial patterning yielding three tissue systems. Pattern emerges by regulation of planes of cell division and the directions of cell expansion, since plant cells do not move during development.
6. As the embryo matures, a food reserve is established.
7. Pattern is elaborated during postembryonic development, when meristems construct the reiterative structures of the plant.
8. The germ line is not reserved early in development. Coordination of signaling among leaves, roots, and shoot meristems regulates the transition to the reproductive state. Reproduction may be followed by genetically programmed senescence of the parent plant.

U. THE ENVIRONMENTAL REGULATION OF DEVELOPMENT

1. The environment can affect development in several ways. Development is sometimes cued to normal circumstances that the organism can expect to find in its environment. The larvae of many species will not begin metamorphosis until they find a suitable substrate. In other instances, symbiotic relationships between two or more species are necessary for the complete development of one or more of the species.
2. Developmental plasticity makes it possible for environmental circumstances to elicit different phenotypes from the same genotype. Many species have a broad reaction norm, wherein the genotype can respond in a graded way to environmental conditions.
3. Some species exhibit polyphenisms, in which distinctly different phenotypes are evoked by different environmental cues.
4. Seasonal cues such as photoperiod, temperature, or type of food can alter development in ways that make the organism more fit. Changes in temperature also are responsible for determining sex in several organisms, including many types of reptiles and insects.
5. Predator-induced polyphenisms have evolved such that the prey species can respond morphologically to the presence of a specific predator. In some instances, this induced adaptation can be transmitted to the progeny of the prey.
6. The differentiation of immunocompetent cells and the formation of synapses in the visual system are examples where experience influences the phenotype.
7. Compounds found in the environment (teratogens) can disrupt normal development. Teratogens can be naturally occurring substances or synthetic ones.
8. Alcohol and retinoic acid are two of the most intensively studied human teratogens. They may produce their teratogenic effects through more than one pathway.
9. It is possible that numerous compounds may be acting as hormone mimics or antagonists disrupt normal development by interfering with the endocrine system.
10. Genetic differences can predispose individuals to being affected by teratogens.

V. EVOLUTIONARY DEVELOPMENTAL BIOLOGY

1. Evolution is caused by the inheritance of changes in development. Modifications of embryonic or larval development can create new phenotypes that can then be selected.
2. Darwin's concept of "descent with modification" explained both homologies and adaptations. The similarities of structure were due to common ancestry (homology), while the modifications were due to natural selection (adaptation to the environmental circumstances).
3. The Urbilaterian ancestor can be extrapolated by looking at the developmental genes common to invertebrates and vertebrates and which perform similar functions. These include the Hox genes that specify body segments, the *tinman* gene that regulates heart development, the *Pax6* gene that specifies those regions able to form eyes, and the genes that instruct head and tail formation.
4. Changes in the targets of Hox genes can alter what the Hox genes specify. The Ubx protein, for instance, specifies halteres in flies and hindwings in butterflies.
5. Changes of Hox gene expression within a region can alter the structures formed by that region.
For instance, changes in the expression of Ubx and abdA in insects regulate the production of prolegs in the abdominal segments of the larvae.
6. Changes in Hox gene expression between body regions can alter the structures formed by that region. In crustaceans, different Hox expression patterns enable the body to have or to lack maxillipeds on its thoracic segments.
7. Changes in Hox gene expression are correlated with the limbless phenotypes in snakes.
8. Changes in Hox gene number may allow Hox genes to take on new functions. Large changes the numbers of Hox genes correlate with major transitions in evolution.
9. Duplications of genes may also enable these genes to become expressed in new places. The formation of new cell types may result from duplicated genes whose regulation has diverged.
10. In addition to structures being homologous, developmental pathways can be homologous. Here, one has homologous proteins organized in homologous ways. These pathways can be used for different developmental phenomena in different organisms and within the same organism.
11. Deep homology results when the homologous pathway is utilized for the same function in greatly diverged organisms. The instructions for forming the central nervous system and for forming limbs are possible examples of deep homology.
12. Modularity allows for parts of the embryo to change without affecting other parts.
13. The dissociation of one module from another is shown by heterochrony (changing in the timing of the development of one region with respect to another) and by allometry (when different parts of the organism grow at different rates).
14. Allometry can create new structures (such as the pocket gopher cheek pouch) by crossing a threshold.
15. Duplication and divergence are important mechanisms of evolution. On the gene level, the Hox genes, the Distal-less genes, the MyoD genes, and many other gene families started as single genes. The diverged members can assume different functions.
16. Co-option (recruitment) of existing genes and pathways for new functions is a fundamental mechanism for creating new phenotypes. One such recruitment is the limb development pathway being used to form eyespots in butterfly wings.
17. Developmental modules can include several tissue types such that correlated progression occurs. here, a change in one portion of the module causes changes in the other portions. When skeletal bones change, the nerves and muscles serving them also change.
18. Tissue interactions have to be conserved, and if one component changes, the other must. If a ligand changes, its receptor must change. Reproductive isolation may result from changes in sperm or egg proteins.
19. Developmental constraints prevent certain phenotypes from occurring. Such restraints may be physical (no rotating limbs), morphogenetic (no middle finger smaller than its neighbors), or phyletic (no neural tube without a notochord).

D. Plant Developmental Biology

An overview of plant development

The fundamental questions in developmental biology are similar for plants* and animals. Their developmental strategies, which have evolved over millions of years, have many commonalities; however, some of the challenges and solutions found in plants are sufficiently unique to warrant a separate discussion in this chapter.

Land plants have their origins in the freshwater green algae, and the transition to land correlates with the evolution of an increasingly protected embryo. Mosses, ferns, gymnosperms (conifers, cycads, and ginkgos), and angiosperms (flowering plants) all develop from protected embryos. Two examples of embryo protection are the seed coat that first appeared in the gymnosperms and the fruit that characterizes the angiosperms. As we have seen, embryo protection is also a theme in animal development. What are the differences?

1. Plants do not gastrulate. Plant cells are trapped within rigid cellulose walls that generally prevent cell and tissue migration. Plants, like animals, develop three basic tissue systems (dermal, ground, and vascular), but do not rely on gastrulation to establish this layered system of tissues. Plant development is highly regulated by the environment, a strategy that is adaptive for a stationary organism.

2. Plants have sporic meiosis rather than gametic meiosis. That is, spores, not gametes, are produced by meiosis. Gametes are produced by mitotic divisions following meiosis.

3. The life cycle of land plants (as well as many other plants) includes both diploid and haploid multicellular stages. This type of life cycle is referred to as alternation of generations. The evolutionary trend has been toward a reduction in the size of the haploid generation.

4. Germ cells are not set aside early in development. This is also the case in several animal phyla, but it is true for all plants.

5. Plants undergo extended morphogenesis. Clusters of actively dividing cells called **meristems**, which are similar to stem cells in animals, persist long after maturity. Meristems allow for reiterative development and the formation of new structures throughout the life of the plant.

6. Plants have tremendous developmental plasticity. Many plant cells are highly plastic. While cloning in animals also illustrates plasticity, plants depend more heavily on this developmental strategy. For example, if a shoot is grazed by herbivores, meristems in the leaf often grow out to replace the lost part. (This strategy has similarities to the regeneration seen in some animals.) Whole plants can even be regenerated from some single cells. In addition, a plant's form (including branching, height, and relative amounts of vegetative and reproductive structures) is greatly influenced by environmental factors such as light and temperature, and a wide range of morphologies can result from the same genotype. This amazing level of plasticity may help compensate for the plant's lack of mobility.

7. Plants may tolerate higher genetic loads than animals. Plant genomes can carry a much greater load of mutations than animals before the phenotype is affected. For example, half of the maize (corn) genome appears to be made up of foreign DNA. Most of it is in the form of retroelements that resemble retroviruses. The maize plant appears to function quite well with all of this "hitchhiking" DNA. Animals also have a significant amount of foreign DNA, but aneuploidy and polyploidy can be developmentally harmful to them. When plants are aneuploid or polyploid, the consequences can be adaptive. Many flowers found in the florist shop and the wheat used for bread flour are examples of successful polyploids. Despite these major differences among many plants and animals, developmental genetic studies are revealing some commonalities between them in the regulation of basic molecular mechanisms of patterning, along with evolutionarily distinct solutions to the problem of creating three-dimensional form from a single cell.

Plant Life Cycles

The plant life cycle alternates between haploid and diploid generations. Embryonic development is seen only in the diploid generation. The embryo, however, is produced by the fusion of gametes, which are formed only by the haploid generation. So understanding the relationship between the two generations is important in the study of plant development.

Unlike animals, plants have multicellular haploid and multicellular diploid stages in their life cycle. Gametes develop in the multicellular haploid **gametophyte** (from the Greek *phyton,* "plant"). Fertilization gives rise to a multicellular diploid **sporophyte**, which produces haploid spores via meiosis. This type of life cycle is called a **haplodiplontic** life cycle. It differs from our own **diplontic** life cycle, in which only the gametes are in the haploid state. In haplodiplontic life cycles, gametes are not the direct result of a meiotic division. Diploid sporophyte cells undergo meiosis to produce haploid **spores**. Each spore goes through mitotic divisions to yield a multicellular, haploid gametophyte. Mitotic divisions within the gametophyte are required to produce the gametes. The diploid sporophyte results from the fusion of two gametes. Among the Plantae, the gametophytes and sporophytes of a species have distinct morphologies (in some algae they look alike). How a single genome can be used to create two unique morphologies is an intriguing puzzle.

All plants alternate generations. There is an evolutionary trend from sporophytes that are nutritionally dependent on autotrophic (self-feeding) gametophytes to the opposite –gametophytes that are dependent on autotrophic sporophytes. This trend is exemplified by comparing the life cycles of a moss, a fern, and an angiosperm. (Gymnosperm life cycles bear many similarities to those of angiosperms; the distinctions will be explored in the context of angiosperm development.)

The "leafy" moss you walk on in the woods is the gametophyte generation of that plant. Mosses are

heterosporous, which means they make two distinct types of spores; these develop into male and female gametophytes. Male gametophytes develop reproductive structures called **antheridia** (singular, antheridium) that produce sperm by mitosis. Female gametophytes develop **archegonia** (singular, archegonium) that produce eggs by mitosis. Sperm travel to a neighboring plant via a water droplet, are chemically attracted to the entrance of the archegonium, and fertilization results. The embryonic sporophyte develops within the archegonium, and the mature sporophyte stays attached to the gametophyte. The sporophyte is not photosynthetic. Thus both the embryo and the mature sporophyte are nourished by the gametophyte. Meiosis within the capsule of the sporophyte yields haploid spores that are released and eventually germinate to form a male or female gametophyte.

Ferns follow a pattern of development similar to that of mosses, although most (but not all) ferns are **homosporous**. That is, the sporophyte produces only one type of spore within a structure called the **sporangium**. One gametophyte can produce both male and female sex organs. The greatest contrast between the mosses and the ferns is that both the gametophyte and the sporophyte of the fern photosynthesize and are thus autotrophic; the shift to a dominant sporophyte generation is taking place.

At first glance, angiosperms may appear to have a diplontic life cycle because the gametophyte generation has been reduced to just a few cells. However, mitotic division still follows meiosis in the sporophyte, resulting in a multicellular gametophyte, which produces eggs or sperm. All of this takes place in the the organ that characterizes the angiosperms: the flower. Male and female gametophytes have distinct morphologies (i.e., angiosperms are heterosporous), but the gametes they produce no longer rely on water for fertilization. Rather, wind or members of the animal kingdom deliver the male gametophyte **pollen** to the female gametophyte.

Another evolutionary innovation is the production of a seed coat, which adds an extra layer of protection around the embryo. The seed coat is also found in the gymnosperms. A further protective layer, the fruit, is unique to the angiosperms and aids in the dispersal of the enclosed embryos by wind or animals.

The remainder of this chapter provides a detailed exploration of angiosperm development from fertilization to senescence. Keep in mind that the basic haplodiplontic life cycle seen in the mosses and ferns is also found in the angiosperms, continuing the trend toward increased nourishment and protection of the embryo.

Have you ever wondered why there are no moss trees? Aside from the fact that the gametophytes of mosses (and other plants) do not have the necessary structural support and transport systems to attain tree height, it would be very difficult for a sperm to swim up a tree!

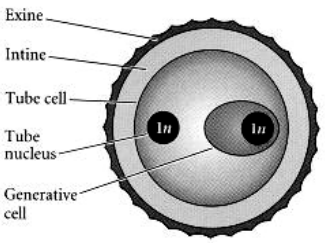
It *is* possible to have tree ferns, for two reasons. First, the gametophyte develops on the ground, where water can facilitate fertilization. Secondly, unlike mosses, the fern sporophyte has vascular tissue, which provides the support and transport system necessary to achieve substantial height.

Gamete Production in Angiosperms

Like those of mosses and ferns, angiosperm gametes are produced by the gametophyte generation. Angiosperm gametophytes are associated with flowers. The gametes they produce join to form the sporophyte. The study of embryonic development in plants is therefore the study of early sporophyte development. In angiosperms, the sporophyte is what is commonly seen as the plant body. The shoot meristem of the sporophyte produces a series of vegetative structures.

At a certain point in development, internal and external signals trigger a shift from vegetative to reproductive (flower-producing) development. Once the meristem becomes floral, it initiates the development of floral parts sequentially in whorls of organs modified from leaves. The first and second whorls become **sepals** and **petals**, respectively; these organs are sterile. The pollen-producing **stamens** are initiated in the third whorl of the flower. The **carpel** in the fourth whorl contains the female gametophyte. The stamens contain four groups of cells, called the **microsporangia** (pollen sacs), within an **anther**. The microsporangia undergo meiosis to produce **microspores**. Unlike most ferns, angiosperms are heterosporous, so the prefix *micro* is used to identify the spores that mitotically yield the male gametophytes pollen grains. The inner wall of the pollen sac, the **tapetum**, provides nourishment for the developing pollen.

Pollen



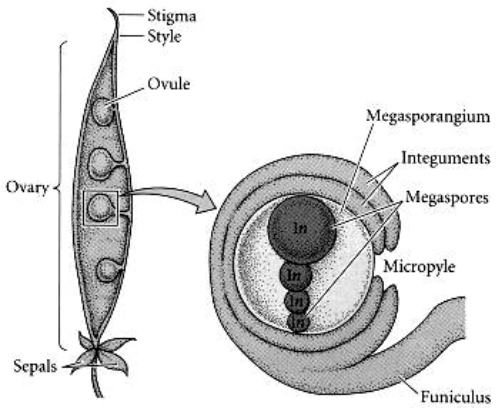
The pollen grain is an extremely simple multicellular structure. The outer wall of the pollen grain, the **exine**, is composed of resistant material provided by both the tapetum (sporophyte generation) and the microspore (gametophyte generation). The inner wall, the **intine**, is produced by the microspore. A mature pollen grain consists of two cells, one within the other. The **tube cell** contains a **generative cell** within it. The generative cell divides to produce two sperm. The tube cell nucleus guides pollen germination and the growth of the pollen tube after the pollen lands on the stigma of a female gametophyte. One of the two sperm will fuse with the egg cell to produce the next sporophyte generation. The second sperm will participate in the formation of the endosperm, a structure that provides nourishment for the embryo.

The ovary

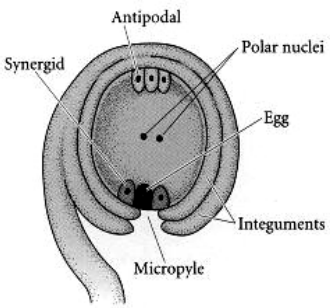
The fourth whorl of organs within the flower forms the **carpel**, which gives rise to the female gametophyte.

The carpel consists of the **stigma** (where the pollen lands), the **style**, and the **ovary**. Following fertilization, the ovary wall will develop into the **fruit**. This unique angiosperm structure provides further protection for the developing embryo and also enhances seed dispersal by

frugivores (fruit-eating animals). Within the ovary are one or more **ovules** attached by a **placenta** to the ovary wall. Fully developed ovules are called **seeds**. The ovule has one or two outer layers of cells called the **integuments**. These enclose the **megasporangium**, which contains sporophyte cells that undergo meiosis to produce **megaspores**.



There is a small opening in the integuments, called the **micropyle**, through which the pollen tube will grow. The integuments an innovation first appearing in the gymnosperms develop into the **seed coat**, which protects the embryo by providing a waterproof physical barrier. When the mature embryo disperses from the parent plant, diploid sporophyte tissue accompanies the embryo in the form of the seed coat and the fruit. Within the ovule, meiosis and unequal cytokinesis yield four megaspores. The largest of these megaspores undergoes three mitotic divisions to produce a seven-celled **embryo sac** with eight nuclei (Figure).



One of these cells is the egg. The two **synergid cells** surrounding the egg may be evolutionary remnants of the archegonium (the female sex organ seen in mosses and ferns). The **central cell** contains two or more polar nuclei, which will fuse with the second sperm nucleus and develop into the polyploid endosperm. Three **antipodal cells** form at the opposite end of the embryo sac from the synergids and degenerate before or during embryonic development. There is no known function for the antipodals. Genetic analyses of female gametophyte development in maize and *Arabidopsis* are providing insight into the regulation of the specific steps in this process.

Pollination

Pollination refers to the landing and subsequent germination of the pollen on the stigma. Hence it involves an interaction between the gametophytic generation of the male (the pollen) and the sporophytic generation of the female (the stigmatic surface of the carpel). Pollination can occur within a single flower (self-fertilization), or pollen can land on a different flower on the same or a different plant. About 96% of flowering plant species produce male and female gametophytes on the same plant. However, about 25% of these produce two different types of flowers on the same plant, rather than **perfect flowers** containing both male and female gametophytes. **Staminate** flowers lack carpels, while **carpellate** flowers lack stamens. Maize plants, for example, have staminate (tassel) and carpellate (ear) flowers on the same plant. Such species are considered to be **monoecious** (Greek *mono,* "one"; *oecos,* "house"). The remaining 4% of species (e.g., willows) produce staminate and carpellate flowers on separate plants. These species are considered to be **dioecious** ("two houses"). Only a few plant species have true sex chromosomes.

The terms "male" and "female" are most correctly applied only to the gametophyte generation of heterosporous plants, not to the sporophyte. The arrival of a viable pollen grain on a receptive stigma does not guarantee fertilization.

Interspecific incompatibility refers to the failure of pollen from one species to germinate and/or grow on the stigma of another species. **Intraspecific incompatibility** is incompatibility that occurs within a species. **Self-incompatibility** incompatibility between the pollen and the stigmas of the same individual is an example of intraspecific incompatibility. Self-incompatibility blocks fertilization between two genetically similar gametes, increasing the probability of new gene combinations by promoting outcrossing (pollination by a different individual of the same species). Groups of closely related plants can contain a mix of self-compatible and self-incompatible species.

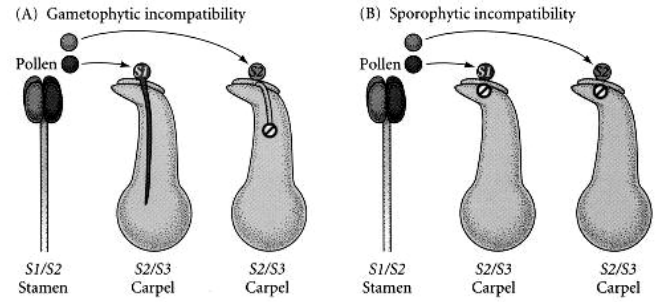


Figure. Self-incompatibility. S1,S2, and S3 are different alleles of the self-incompatibility (*S*) locus. (A) Plants with gametophytic self-incompatibility reject pollen only when the genotype of the pollen matches one of the carpel's two alleles. (B) In sporophytic self-incompatibility, the genotype of the pollen parent, not just of the haploid pollen grain itself, can trigger an incompatibility response.

Several different self-incompatibility systems have evolved. Recognition of self depends on the multiallelic self-incompatibility (*S*) locus. Gametophytic self-incompatibility occurs when the *S* allele of the pollen grain matches either of the *S* alleles of the stigma (remember that the stigma is part of the diploid sporophyte generation, which has two *S* alleles). In this case, the pollen tube begins developing, but stops before reaching the micropyle. Sporophytic self-incompatibility occurs when one of the two *S* alleles of the pollen-producing sporophyte (not the gametophyte) matches one of the *S* alleles of the stigma. Most likely, sporophyte contributions to the exine are responsible.

The *S* locus consists of several physically linked genes that regulate recognition and rejection of pollen. An *S*

gene has been cloned that codes for an RNase called S RNase, which is sufficient, in the gametophytically self-incompatible petunia pistil, to recognize and reject self-pollen. The pollen component recognized is most likely a different gene in the *S* locus, but has not yet been identified in either gametophytically or sporophytically self-incompatible plants. In sporophytic self-incompatibility, a ligand on the pollen is thought to bind to a membrane-bound kinase receptor in the stigma that starts a signaling process leading to pollen rejection. The mechanism of pollen degradation is unclear, but appears to be highly specific.

If the pollen and the stigma are compatible, the pollen takes up water (hydrates) and the pollen tube emerges. The pollen tube grows down the style of the carpel toward the micropyle. The tube nucleus and the sperm cells are kept at the growing tip by bands of callose (a complex carbohydrate). It is possible that this may be an exception to the "plant cells do not move" rule, as the generative cell(s) appear to move ahead via adhesive molecules. Pollen tube growth is quite slow in gymnosperms (up to a year), while in some angiosperms the tube can grow as rapidly as 1 cm/hour.

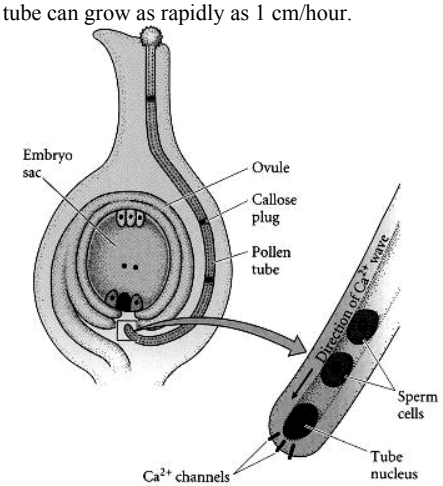


Figure. Calcium and pollen tube tip growth. After compatible pollen germinates, the pollen tube grows toward the micropyle. Calcium plays a key role in the growth of the tube.

Calcium has long been known to play an essential role in pollen tube growth. Calcium accumulates in the tip of the pollen tubes, where open calcium channels are concentrated. There is direct evidence that pollen tube growth in the field poppy is regulated by a slow-moving calcium wave controlled by the phosphoinositide signaling pathway (Figure). Cytoskeletal investigations show that organelle positioning during pollen tube growth depends on interactions with cytoskeletal components. This must link to signaling, but the specifics are still unknown.

Genetic approaches have been useful in investigating how the growing pollen tube is guided toward unfertilized ovules. In *Arabidopsis,* the pollen tube appears to be guided by a long distance signal from the ovule. Analysis of pollen tube growth in ovule mutants of *Arabidopsis* indicates that the haploid embryo sac is particularly important in the long-range guidance of pollen tube growth. Mutants with defective sporophyte tissue in the ovule but a normal haploid embryo sac appear to stimulate normal pollen tube development.

While the evidence points primarily to the role of the gametophyte generation in pollen tube guidance, diploid cells may make some contribution. Two *Arabidopsis* genes, *POP2* and *POP3,* have been identified that specifically guide pollen tubes to the ovule with no other apparent effect on the plant. These genes function in both the pollen and the pistil, thus implicating the sporophyte generation in the guidance system

Fertilization

The growing pollen tube enters the embryo sac through the micropyle and grows through one of the synergids. The two sperm cells are released, and a **double fertilization** event occurs. One sperm cell fuses with the egg, producing the zygote that will develop into the sporophyte. The second sperm cell fuses with the bi- or multinucleate central cell, giving rise to the **endosperm**, which nourishes the developing embryo. This second event is not true fertilization in the sense of male and female gametes undergoing syngamy (fusion). That is, it does not result in a zygote, but in nutritionally supportive tissue. (When you eat popcorn, you are eating "popped" endosperm.) The other accessory cells in the embryo sac degenerate after fertilization.

The zygote of the angiosperm produces only a single embryo; the zygote of the gymnosperm, on the other hand, produces two or more embryos after cell division begins, by a process known as cleavage embryogenesis. Double fertilization, first identified a century ago, is generally restricted to the angiosperms, but it has also been found in the gymnosperms *Ephedra* and *Gnetum,* although no endosperm forms. Friedman (1998) has suggested that endosperm may have evolved from a second zygote "sacrificed" as a food supply in a gymnosperm with double fertilization. Investigations of the most closely related extant relative of the basal angiosperm, *Amborella,* should provide information on the evolutionary origin of the endosperm.

Fertilization is not an absolute prerequisite for angiosperm embryonic development. Embryos can form within embryo sacs from haploid eggs and from cells that did not divide meiotically. This phenomenon is called **apomixis** (Greek, "without mixing"), and results in viable seeds. The viability of the resulting haploid sporophytes indicates that ploidy alone does not account for the morphological distinctions between the gametophyte and the sporophyte. Embryos can also develop from cultured sporophytic tissue. These embryos develop with no associated endosperm, and they lack a seed coat.

Embryonic Development

Experimental studies

The angiosperm zygote is embedded within the ovule and ovary and thus is not readily accessible for experimental manipulation. The following approaches, however, can yield information on the formation of the plant embryo:

- **Histological studies** of embryos at different stages show how carefully regulated cell division results in the construction of an organism, even without the ability to move cells and tissues to shape the embryo.
- **Culture experiments** using embryos isolated from ovules and embryos developing de novo from cultured sporophytic tissue provide information on the interactions between the embryo and surrounding sporophytic and endosperm tissue.
- **In vitro fertilization experiments** provide information on gamete interactions. **Biochemical analyses** of embryos at different stages of development provides information on such things as the stage-specific gene products necessary for patterning and establishing food reserves.

Genetic and molecular analyses of developmental mutants characterized using the above approaches have greatly enhanced our understanding of embryonic development. **Clonal analysis** involves marking individual cells and following their fate in development. For example, seeds heterozygous for a pigmentation gene may be irradiated so that a certain cell loses the ability to produce pigment. Its descendants will form a colorless sector that can be identified and related to the overall body pattern.

Embryogenesis

In plants, the term **embryogenesis** covers development from the time of fertilization until dormancy occurs. The basic body plan of the sporophyte is established during embryogenesis; however, this plan is reiterated and elaborated after dormancy is broken. The major challenges of embryogenesis are

1. To establish the basic body plan. **Radial patterning** produces three tissue systems, and **axial patterning** establishes the apical-basal (shoot-root) axis.

2. To set aside meristematic tissue for postembryonic elaboration of the body structure (leaves, roots, flowers, etc.).

3. To establish an accessible food reserve for the germinating embryo until it becomes autotrophic.

Embryogenesis is similar in all angiosperms in terms of the establishment of the basic body plan (see Figure). There are differences in pattern elaboration, however, including differences in the precision of cell division patterns, the extent of endosperm development, cotyledon development, and the extent of shoot meristem development.

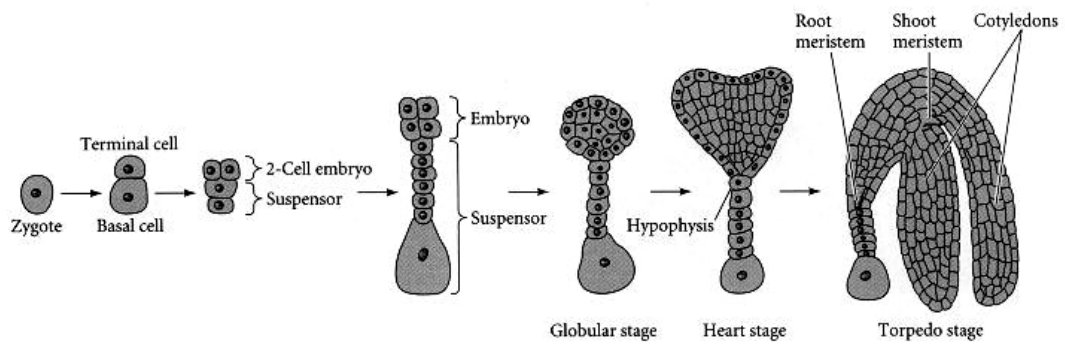


Figure. Angiosperm embryogenesis. A representative dicot is shown; a monocot would develop only a single cotyledon. While there are basic patterns of embryogenesis in angiosperms, there is tremendous morphological variation among species

Polarity is established in the first cell division following fertilization. The establishment of polarity has been investigated using brown algae as a model system. The zygotes of these plants are independent of other tissues and amenable to manipulation.

The initial cell division results in one smaller cell, which will form the rhizoid (root homologue) and anchor the rest of the plant, and one larger cell, which gives rise to the thallus (main body of the sporophyte). The point of sperm entry fixes the position of the rhizoid end of the apical-basal axis. This axis is perpendicular to the plane of the first cell division. F-actin accumulates at the rhizoid pole. However, light or gravity can override this fixing of the axis and establish a new position for cell division. Once the apical basal axis is established, secretory vesicles are targeted to the rhizoid pole of the zygote. These vesicles contain material for rhizoid outgrowth, with a cell wall of distinct macromolecular composition. Targeted secretion may also help orient the first plane of cell division. Maintenance of rhizoid versus thallus fate early in development depends on information in the cell walls. Cell wall information also appears to be important in angiosperms.

The basic body plan of the angiosperm laid down during embryogenesis also begins with an asymmetrical cell division, giving rise to a **terminal cell** and a **basal cell** (Figure). The terminal cell gives rise to the **embryo proper**. The basal cell forms closest to the micropyle and gives rise to the **suspensor**. The **hypophysis** is found at the interface between the suspensor and the embryo proper. In many species it gives rise to some of the root cells. (The suspensor cells divide to form a filamentous or spherical organ that degenerates later in embryogenesis.) In both gymnosperms and angiosperms, the suspensor orients the absorptive surface of the embryo toward its food source; in angiosperms, it also appears to serve as a nutrient conduit for the developing embryo. Culturing isolated embryos of scarlet runner beans with and without the suspensor has demonstrated the need for a suspensor through the heart stage in dicots. Embryos cultured with a suspensor are twice as likely to

survive as embryos cultured without an attached suspensor at this stage. The suspensor may be a source of hormones. In scarlet runner beans, younger embryos without a suspensor can survive in culture if they are supplemented with the growth hormone gibberellic acid.

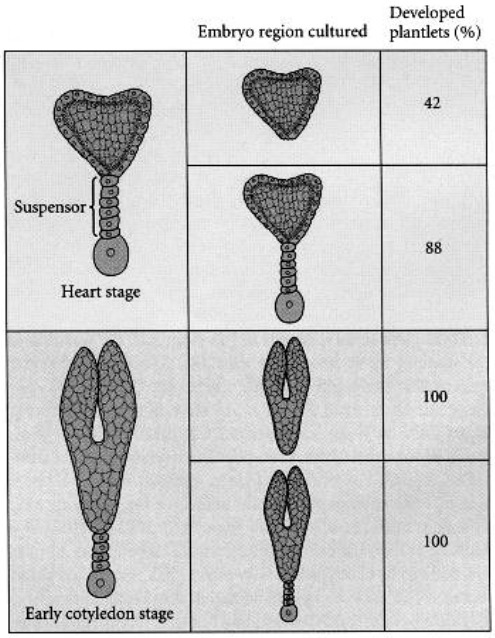
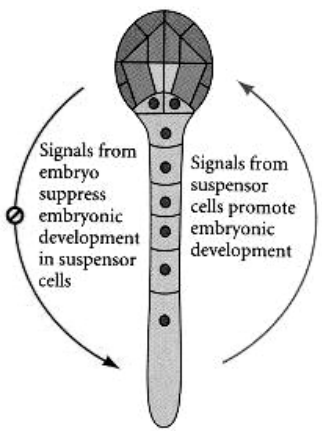


Figure. Role of the suspensor in dicot embryogenesis. Culturing scarlet runner bean embryos with and without their suspensors has demonstrated that the suspensor is essential at the heart-shaped stage, but not later.

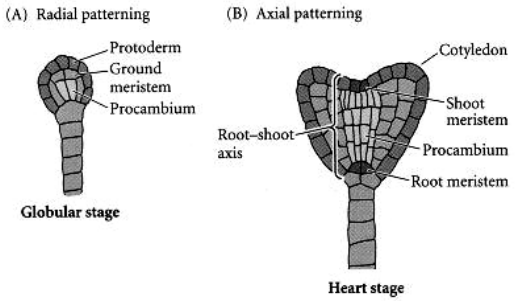


As the establishment of apical-basal polarity is one of the key achievements of embryogenesis, it is useful to consider why the suspensor and embryo proper develop unique morphologies. Here the study of embryo mutants in maize and *Arabidopsis* has been particularly helpful. Investigations of suspensor mutants (*sus1*,*sus2*, and *raspberry1*) of *Arabidopsis* have provided genetic evidence that the suspensor has the capacity to develop embryo-like structures (Figure).

In these mutants, abnormalities in the embryo proper appear prior to suspensor abnormalities. Earlier experiments in which the embryo proper was removed also demonstrated that suspensors could develop like embryos. A signal from the embryo proper to the suspensor may be important in maintaining suspensor identity and blocking the development of the suspensor as an embryo. Molecular analyses of these and other genes are providing insight into the mechanisms of communication between the suspensor and the embryo proper.

Maternal effect genes play a key role in establishing embryonic pattern in animals. The role of extrazygotic genes in plant embryogenesis is less clear, and the question is complicated by at least three potential sources of influence: sporophytic tissue, gametophytic tissue, and the polyploid endosperm. All of these tissues are in close association with the egg/zygote. Endosperm development could also be affected by maternal genes. Sporophytic and gametophytic maternal effect genes have been identified in *Arabidopsis,* and it is probable that the endosperm genome influences the zygote as well. The first maternal effect gene identified, *SHORT INTEGUMENTS 1* (*SIN1*), must be expressed in the sporophyte for normal embryonic development. Two transcription factors (FBP7 and FBP11) are needed in the petunia sporophyte for normal endosperm development. A female gametophytic maternal effect gene, *MEDEA* (after Euripides' Medea, who killed her own children), has protein domains similar to those of a *Drosophila* maternal effect gene. Curiously, *MEDEA* is in the Polycomb gene group, whose products alter chromatin, directly or indirectly, and affect transcription. *MEDEA* affects an imprinted gene that is expressed by the female gametophyte and by maternally inherited alleles in the zygote, but not by paternally inherited alleles. How significant maternal effect genes are in establishing the sporophyte body plan is still an unanswered question.

Radial and axial patterns develop as cell division and differentiation continue (Figure). The cells of the embryo proper divide in transverse and longitudinal planes to form a **globular stage** embryo with several tiers of cells. Superficially, this stage bears some resemblance to cleavage in animals, but the nuclear/cytoplasmic ratio does not necessarily increase. The emerging shape of the embryo depends on regulation of the planes of cell division and expansion, since the cells are not able to move and reshape the embryo. Cell division planes in the outer layer of cells become restricted, and this layer, called the **protoderm**, becomes distinct. Radial patterning emerges at the globular stage as the three tissue systems (dermal, ground, and vascular) of the plant are initiated. The **dermal tissue** (epidermis) will form from the protoderm and contribute to the outer protective layers of the plant. **Ground tissue** (cortex and pith) forms from the ground meristem, which lies beneath the protoderm. The **procambium**, which forms at the core of the embryo will give rise to the **vascular tissue** (xylem and phloem), which will function in support and transport. The differentiation of each tissue system is at least partially independent. For example, in the *keule* mutant of *Arabidopsis,* the dermal system is defective while the inner tissue systems develop normally.



The globular shape of the embryo is lost as **cotyledons** ("first leaves") begin to form. Dicots have two cotyledons, which give the embryo a heart-shaped appearance as they form. The axial body plan is evident

by this **heart stage** of development. Hormones (specifically, auxins) may mediate the transition from radial to bilateral symmetry. In monocots, such as maize, only a single cotyledon emerges.

In many plants, the cotyledons aid in nourishing the plant by becoming photosynthetic after germination (although those of some species never emerge from the ground). In some cases peas, for example the food reserve in the endosperm is used up before germination, and the cotyledons serve as the nutrient source for the germinating seedling. Even in the presence of a persistent endosperm (as in maize), the cotyledons store food reserves such as starch, lipids, and proteins. In many monocots, the cotyledon grows into a large organ pressed against the endosperm and aids in nutrient transfer to the seedling. Upright cotyledons can give the embryo a torpedo shape. In some plants, the cotyledons grow sufficiently long that they must bend to fit within the confines of the seed coat. The embryo then looks like a walking stick. By this point, the suspensor is degenerating.

The **shoot apical meristem** and **root apical meristem** are clusters of stem cells that will persist in the postembryonic plant and give rise to most of the sporophyte body. The root meristem is partially derived from the hypophysis in some species. All other parts of the sporophyte body are derived from the embryo proper. Genetic evidence indicates that the formation of the shoot and root meristems is regulated independently. This independence is demonstrated by the *dek23* maize mutant and the *shootmeristemless* (*STM*) mutant of *Arabidopsis,* both of which form a root meristem but fail to initiate a shoot meristem. The *STM* gene, which has been cloned, is expressed in the late globular stage, before cotyledons form. Genes have also been identified that specifically affect the development of the root axis during embryogenesis. Mutations of the *HOBBIT* gene in *Arabidopsis*, for example, affect the hypophysis derivatives and eliminate root meristem function.

The shoot apical meristem will initiate leaves after germination and, ultimately, the transition to reproductive development. In *Arabidopsis,* the cotyledons are produced from general embryonic tissue, not from the shoot meristem. In many angiosperms, a few leaves are initiated during embryogenesis. In the case of *Arabidopsis,* clonal analysis points to the presence of leaves in the mature embryo, even though they are not morphologically well developed. Clonal analysis has demonstrated that the cotyledons and the first two true leaves of cotton are derived from embryonic tissue rather than an organized meristem.

Clonal analysis experiments provide information on cell fates, but do not necessarily indicate whether or not cells are determined for a particular fate. Cells, tissues, and organs are shown to be determined when they have the same fate in situ, in isolation, and at a new position in the organism. Clonal analysis has demonstrated that cells that divide in the wrong plane and "move" to a different tissue layer often differentiate according to their new position. Position, rather than clonal origin, appears to be the critical factor in embryo pattern formation, suggesting some type of cell-cell communication. Microsurgery experiments on somatic carrot embryos demonstrate that isolated pieces of embryo can often replace the missing complement of parts. A cotyledon removed from the shoot apex will be replaced. Isolated embryonic shoots can regenerate a new root; isolated root tissue regenerates cotyledons, but is less likely to regenerate the shoot axis. Although most embryonic cells are pluripotent and can generate organs such as cotyledons and leaves, only meristems retain this capacity in the postembryonic plant body.

*Asymmetrical cell division is also important in later angiosperm development, including the formation of guard cells of leaf stomata and of different cell types in the ground and vascular tissue systems. Another intriguing characteristic of these mutants is that cell differentiation occurs in the absence of morphogenesis. Thus, cell differentiation and morphogenesis can be uncoupled in plant development. Mendel's famous wrinkled-seed mutant (the *rugosus* or *r* allele) has a defect in a starch branching enzyme that affects starch, lipid, and protein biosynthesis in the seed and leads to defective cotyledons.

Dormancy

From the earliest stages of embryogenesis, there is a high level of zygotic gene expression. As the embryo reaches maturity, there is a shift from constructing the basic body plan to creating a food reserve by accumulating storage carbohydrates, proteins, and lipids. Genes coding for seed storage proteins were among the first to be characterized by plant molecular biologists because of the high levels of specific storage protein mRNAs that are present at different times in embryonic development. The high level of metabolic activity in the developing embryo is fueled by continuous input from the parent plant into the ovule. Eventually metabolism slows, and the connection of the seed to the ovary is severed by the degeneration of the adjacent supporting sporophyte cells. The seed dessicates (loses water), and the integuments harden to form a tough seed coat. The seed has entered **dormancy**, officially ending embryogenesis. The embryo can persist in a dormant state for weeks or years, a fact that affords tremendous survival value. There have even been examples of seeds found stored in ancient archaeological sites that germinated after thousands of years of dormancy!

Maturation leading to dormancy is the result of a precisely regulated program. The *viviparous* mutation in maize, for example, produces genetic lesions that block dormancy. The apical meristems of *viviparous* mutants behave like those of ferns, with no pause before producing postembryonic structures. The embryo continues to develop, and seedlings emerge from the kernels on the ear attached to the parent plant. Recently a group of plant genes have been identified that belong to the Polycomb group, which regulates early development in mammals, nematodes, and insects. These genes encode chromatin silencing factors, which may play an important role in seed formation.

Plant hormones are critical in dormancy, and linking them to genetic mechanisms is an active area of research. The hormone **abscisic acid** is important in maintaining dormancy in many species. **Gibberellins**, another class of hormones, are important in breaking dormancy.

Germination

The postembryonic phase of plant development begins with **germination**. Some dormant seeds require a period of **after-ripening** during which low-level metabolic activities continue to prepare the embryo for germination. Highly evolved interactions between the seed and its environment increase the odds that the germinating seedling will survive to produce another generation. Temperature, water, light, and oxygen are all key in determining the success of germination.

Stratification is the requirement for chilling (5°C) to break dormancy in some seeds. In temperate climates, this adaptation ensures germination only after the winter months have passed. In addition, seeds have maximum germination rates at moderate temperatures of 25°-30°C and often will not germinate at extreme temperatures. Seeds such as lettuce require light (specifically, the red wavelengths) for germination; thus seeds will not germinate so far below ground that they use up their food reserves before photosynthesis is possible.

Desiccated seeds may be only 5 20% water. **Imbibition** is the process by which the seed rehydrates, soaking up large volumes of water and swelling to many times its original size. The **radicle** (primary embryonic root) emerges from the seed first to enhance water uptake; it is protected by a root cap produced by the root apical meristem. Water is essential for metabolic activity, but so is oxygen. A seed sitting in a glass of water will not survive. Some species have such hard protective seed coats that they must be **scarified** (scratched or etched) before water and oxygen can cross the barrier. Scarification can occur by the seed being exposed to the weather and other natural elements over time, or by its exposure to acid as the seed passes through the gut of a frugivore. The frugivore thus prepares the seed for germination, as well as dispersing it to a site where germination can take place.

During germination, the plant draws on the nutrient reserves in the endosperm or cotyledons. Interactions between the embryo and endosperm in monocots use gibberellin as a signal to trigger the breakdown of starch into sugar. As the shoot reaches the surface, the differentiation of chloroplasts is triggered by light. Seedlings that germinate in the dark have long, spindly stems and do not produce chlorophyll. This environmental response allows plants to use their limited resources to reach the soil surface, where photosynthesis will be productive. The delicate shoot tip must be protected as the shoot pushes through the soil. Three strategies for protecting the shoot tip have evolved (Figure):

1. Cotyledons protect the shoot tip.
2. The **epicotyl** (the stem above the cotyledons) bends so that stem tissue, rather than the shoot tip, pushes through the soil.
3. In monocots, a special leaflike structure, the **coleoptile**, forms a protective sheath around the shoot tip.

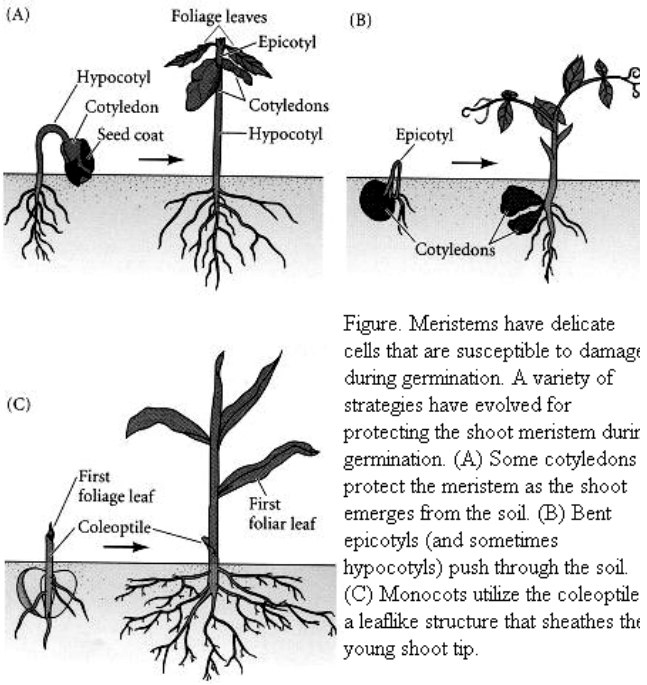


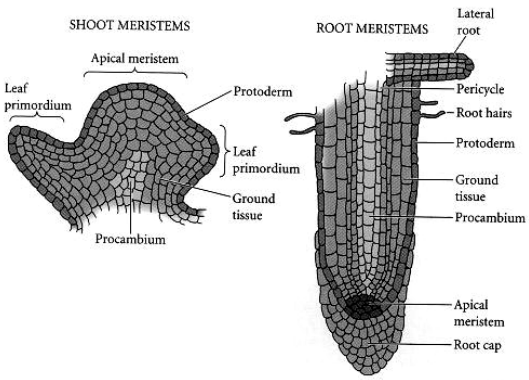
Figure. Meristems have delicate cells that are susceptible to damage during germination. A variety of strategies have evolved for protecting the shoot meristem during germination. (A) Some cotyledons protect the meristem as the shoot emerges from the soil. (B) Bent epicotyls (and sometimes hypocotyls) push through the soil. (C) Monocots utilize the coleoptile, a leaflike structure that sheathes the young shoot tip.

Vegetative Growth

When the shoot emerges from the soil, most of the sporophyte body plan remains to be elaborated. Figure shows the basic parts of the mature sporophyte plant, which will emerge from meristems.

Meristems

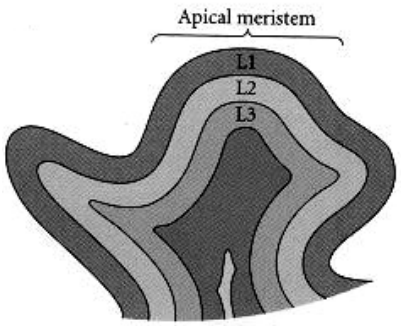
As has been mentioned, **meristems** are clusters of cells that allow the basic body pattern established during embryogenesis to be reiterated and extended after germination. Meristematic cells are similar to stem cells in animals. They divide to give rise to one daughter cell that continues to be meristematic and another that differentiates. Meristems fall into three categories: apical, lateral, and intercalary.



Apical meristems occur at the growing shoot and root tips. Root apical meristems produce the root cap, which consists of lubricated cells that are sloughed off as the meristem is pushed through the soil by cell division and elongation in more proximal cells. The root apical meristem also gives rise to daughter cells that produce the three tissue systems of the root. New root apical meristems are initiated from tissue within the core of the root and emerge through the ground tissue and dermal tissue. Root meristems can also be derived secondarily

from the stem of the plant; in the case of maize, this is the major source of root mass.

The shoot apical meristem produces stems, leaves, and reproductive structures. In addition to the shoot apical meristem initiated during embryogenesis, axillary shoot apical meristems (axillary buds) derived from the original one form in the axils (the angles between leaf and stem). Unlike new root meristems, these arise from the surface layers of the meristem.



Angiosperm apical meristems are composed of up to three layers of cells (labeled L1, L2, and L3) on the plant surface (Figure). One way of investigating the contributions of different layers to plant structure is by constructing chimeras. Plant chimeras are composed of layers having distinct genotypes with discernible markers. When L2, for example, has a different genotype than L1 or L3, all pollen will have the L2 genotype, indicating that pollen is derived from L2.

Chimeras have also been used to demonstrate classical induction in plants, in which, as in animal development, one layer influences the developmental pathway of an adjacent layer. The size of the shoot apical meristem is precisely controlled by intercellular signals, most likely between layers of the meristem. Mutations in the *Arabidopsis CLAVATA* genes, for example, lead to increased meristem size and the production of extra organs. *STM* has the opposite effect, and double mutant phenotypes are consistent with the hypothesis that the two work together to maintain meristem size. Perhaps they balance the rate of cell division (which enlarges the meristem) and the rate of cell differentiation in the periphery of the meristem (which decreases meristem size).

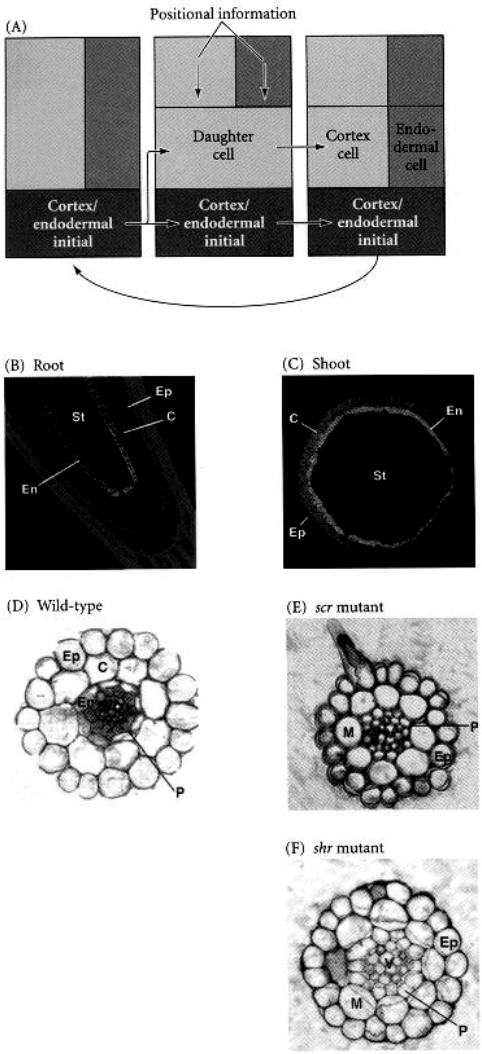
Lateral meristems are cylindrical meristems found in shoots and roots that result in secondary growth (an increase in stem and root girth by the production of vascular tissues). Monocot stems do not have lateral meristems, but often have **intercalary meristems** inserted in the stems between mature tissues. The popping sound you can hear in a cornfield on a summer night is actually caused by the rapid increase in stem length due to intercalary meristems.

Root development

Radial and axial patterning in roots begins during embryogenesis and continues throughout development as the primary root grows and lateral roots emerge from the pericycle cells deep within the root. Laser ablation experiments eliminating single cells and clonal analyses have demonstrated that cells are plastic and that position is the primary determinant of fate in early root development. Analyses of root radial organization mutants have revealed genes with layer specific activity. We will illustrate these findings by looking at two *Arabidopsis* genes that regulate ground tissue fate.

In wild-type *Arabidopsis,* there are two layers of root ground tissue. The outer layer becomes the cortex, and the inner layer becomes the endodermis, which forms a tube around the vascular tissue core. The *SCARECROW* (*SCR*) and *SHORT-ROOT* (*SHR*) genes have mutant phenotypes with one, instead of two, layers of root ground tissue. The *SCR* gene is necessary for an asymmetrical cell division in the initial layer of cells, yielding a smaller endodermal cell and a larger cortex cell. The *scr* mutant expresses markers for both cortex and endodermal cells, indicating that differentiation progresses in the absence of cell division. *SHR* is responsible for endodermal cell specification. Cells in the *shr* mutant do not develop endodermal features.

Axial patterning in roots may be morphogen-dependent, paralleling some aspects of animal development. A variety of experiments have established that the distribution of the plant hormone **auxin** organizes the axial pattern. A peak in auxin concentration at the root tip must be perceived for normal axial patterning.



As discussed earlier, distinct genes specifying root and shoot meristem formation have been identified; however,

root and shoot development may share common groups of genes that regulate cell fate and patterning. This appears to be the case for the *SCR* and *SHR* genes. In the shoot, these genes are necessary for the normal gravitropic response, which is dependent on normal endodermis formation (a defect in mutants of both genes; see figure C). It's important to keep in mind that there are a number of steps between establishment of the basic pattern and elaboration of that pattern into anatomical and morphological structure. Uncovering the underlying control mechanisms is likely to be the most productive strategy in understanding how roots and shoots develop.

Shoot development

The unique aboveground architectures of different plant species have their origins in shoot meristems. Shoot architecture is affected by the amount of axillary bud outgrowth. Branching patterns are regulated by the shoot tip a phenomenon called apical dominance and plant hormones appear to be the factors responsible. Auxin is produced by young leaves and transported toward the base of the leaf. It can suppress the outgrowth of axillary buds. Grazing and flowering often release buds from apical dominance, at which time branching occurs.

Cytokinins can also release buds from apical dominance. Axillary buds can initiate their own axillary buds, so branching patterns can get quite complex. Branching patterns can be regulated by environmental signals so that an expansive canopy in an open area maximizes light capture. Asymmetrical tree crowns form when two trees grow very close to each other. In addition to its environmental plasticity, shoot architecture is genetically regulated. In several species, genes have now been identified that regulate branching patterns.

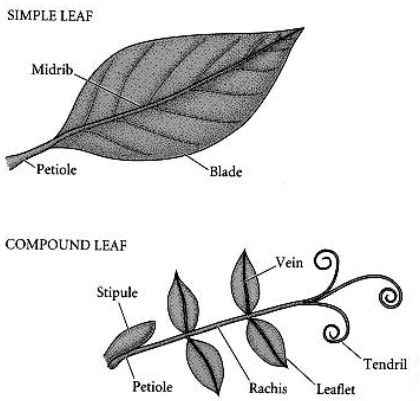
Leaf primordia (clusters of cells that will form leaves) are initiated at the periphery of the shoot meristem. The union of a leaf and the stem is called a **node**, and stem tissue between nodes is called an **internode**. In a simplistic sense, the mature sporophyte is created by stacking node/internode units together. **Phyllotaxy**, the positioning of leaves on the stem, involves communication among existing and newly forming leaf primordia. Leaves may be arranged in various patterns, including a spiral, 180-degree alternation of single leaves, pairs, and whorls of three or more leaves at a node.

Experimentation has revealed a number of mechanisms for maintaining geometrically regular spacing of leaves on a plant, including chemical and physical interactions of new leaf primordial with the shoot apex and with existing primordia.

It is not clear how a specific pattern of phyllotaxy gets started. Descriptive mathematical models can replicate the observed patterns, but reveal nothing about the mechanism. Biophysical models (e.g., of the effects of stress/strain on deposition of cell wall material, which affects cell division and elongation) attempt to bridge this gap. Developmental genetics approaches are promising, but few phyllotactic mutants have been identified. One candidate is the *terminal ear* mutant in maize, which has irregular phyllotaxy. The wild-type gene is expressed in a horseshoe-shaped region, with a gap where the leaf will be initiated. The plane of the horseshoe is perpendicular to the axis of the stem.

Leaf development

Leaf development includes commitment to become a leaf, establishment of leaf axes, andmorphogenesis, giving rise to a tremendous diversity of leaf shapes. Culture experiments have assessed when leaf primordia become determined for leaf development. Research on ferns and angiosperms indicates that the youngest visible leaf primordia are not determined to make a leaf; rather, these young primordia can develop as shoots in culture. The programming for leaf development occurs later. The radial symmetry of the leaf primordium becomes dorsal-ventral, or flattened, in all leaves. Two other axes, the proximal-distal and lateral, are also established. The unique shapes of leaves result from regulation of cell division and cell expansion as the leaf blade develops. There are some cases in which selective cell death (apoptosis) is involved in the shaping of a leaf, but differential cell growth appears to be a more common mechanism.



Leaves fall into two categories, simple and compound. There is much variety in simple leaf shape, from smooth-edged leaves to deeply lobed oak leaves. Compound leaves are composed of individual leaflets (and sometimes tendrils) rather than a single leaf blade. Whether simple and compound leaves develop by the same mechanism is an open question. One perspective is that compound leaves are highly lobed simple leaves. An alternative perspective is that compound leaves are modified shoots. The ancestral state for seed plants is believed to be compound, but for angiosperms it is simple. Compound leaves have arisen multiple times in the angiosperms, and it is not clear if these are reversions to the ancestral state. Developmental genetic approaches are being applied to leaf morphogenesis. The Class I *KNOX* genes are homeobox genes that include *STM* and the *KNOTTED 1* (*KN1*) gene in maize. Gain-offunction mutations of *KN1* cause meristem-like bumps to form on maize leaves. In wild-type plants, this gene is expressed in meristems. When *KN1,* or the tomato homologue *LeT6,* has its promoter replaced with a promoter from cauliflower mosaic virus and is inserted into the genome of tomato, the gene is expressed at high levels throughout the plant, and the leaves become "super compound". Simple leaves become more lobed (but not compound) in response to overexpression of *KN1,* consistent with the hypothesis that compound leaves may be an extreme case of lobing in simple leaves. The role of *KN1* in shoot

meristem and leaf development, however, is consistent with the hypothesis that compound leaves are modified shoots.
A second gene, *LEAFY,* that is essential for the transition from vegetative to reproductive development also appears to play a role in compound leaf development. It was identified in *Arabidopsis* and snapdragon (in which it is called *FLORICAULA*), and has homologues in other angiosperms. The pea homologue (*UNIFOLIATA*) has a mutant phenotype in which compound leaves are reduced to simple leaves. This finding is also indicative of a regulatory relationship between shoots and compound leaves.

In some compound leaves, developmental decisions about leaf versus tendril formation are also made. Mutations of two leaf-shape genes can individually and in sum dramatically alter the morphology of the compound pea leaf. The *acacia* mutant (*tl*) converts tendrils to leaflets; *afilia* (*af*) converts leaflet to tendrils. The *af tl* double mutant has a complex architecture and resembles a parsley leaf.

At a more microscopic level, the patterning of stomata (openings for gas and water exchange) and trichomes (hairs) across the leaf is also being investigated. In monocots, the stomata form in parallel files, while in dicots the distribution appears more random. In both cases, the patterns appear to maximize the evenness of stomata distribution . Genetic analysis is providing insight into the mechanisms regulating this distribution. A common gene group appears to be working in both shoots and roots, affecting the distribution pattern of both trichomes and root hairs.

*The similarities between plant meristem cells and animal stem cells may extend to the molecular level, indicating that stem cells existed before plants and animals pursued separate phylogenetic pathways. Homology has been found between genes required for plant meristems to persist and genes expressed in *Drosophila* germ line stem cells.

This phenomenon, called *fasciation,* is found in many species, including peas and tomatoes.

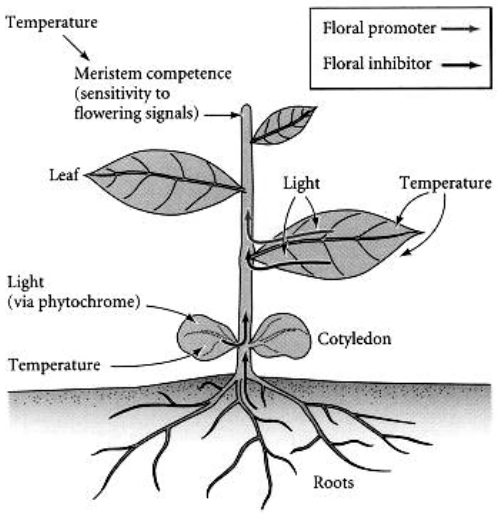
The Vegetative-to-Reproductive Transition

Unlike some animal systems in which the germ line is set aside during early embryogenesis, the germ line in plants is established only after the transition from vegetative to reproductive development that is, flowering. The vegetative and reproductive structures of the shoot are all derived from the shoot meristem formed during embryogenesis. Clonal analysis indicates that no cells are set aside in the shoot meristem of the embryo to be used solely in the creation of reproductive structures. In maize, irradiating seeds causes changes in the pigmentation of some cells. These seeds give rise to plants that have visually distinguishable sectors descended from the mutant cells. Such sectors may extend from the vegetative portion of the plant into the reproductive regions, indicating that maize embryos do not have distinct reproductive compartments.

Maximal reproductive success depends on the timing of flowering and on balancing the number of seeds produced with resources allocated to individual seeds. As in animals, different strategies work best for different organisms in different environments. There is a great diversity of flowering patterns among the over 300,000 angiosperm species, yet there appears to be an underlying evolutionary conservation of flowering genes and common patterns of flowering regulation.

A simplistic explanation of the flowering process is that a signal from the leaves moves to the shoot apex and induces flowering. In some species, this flowering signal is a response to environmental conditions. The developmental pathways leading to flowering are regulated at numerous control points in different plant organs (roots, cotyledons, leaves, and shoot apices) in various species, resulting in a diversity of flowering times and reproductive architectures. The nature of the flowering signal, however, remains unknown.

Some plants, especially woody perennials, go through a **juvenile phase**, during which the plant cannot produce reproductive structures even if all the appropriate environmental signals are present. The transition from the juvenile to the adult stage may require the acquisition of competence by the leaves or meristem to respond to an internal or external signal. Grafting and organ culture experiments, mutant analyses, and molecular analyses give us a framework for describing the reproductive transition in plants (Figure). Grafting experiments have identified the sources of signals that promote or inhibit flowering and have provided information on the developmental acquisition of meristem competence to respond to these signals.



Analyses of mutants and molecular characterization of genes are yielding information on the mechanics of these signal-response mechanisms. Leaves produce a graft-transmissible substance that induces flowering. In some species, this signal is produced only under specific **photoperiods** (day lengths), while other species are day neutral and will flower under any photoperiod. Not all leaves may be competent to perceive or pass on photoperiodic signals. The **phytochrome** pigments transduce these signals from the external environment. The structure of phytochrome is modified by red and far-red light, and these changes can initiate a cascade of events leading to the production of either floral promoter

or floral inhibitor. Leaves, cotyledons, and roots have been identified as sources of floral inhibitors in some species.

A critical balance between inhibitor and promoter is needed for the reproductive transition. In some species, meristems change in their competence to respond to flowering signals during development. **Vernalization**, a period of chilling, can enhance the competence of shoots and leaves to perceive or produce a flowering signal. The reproductive transition depends on both meristem competence and signal strength (Figure).

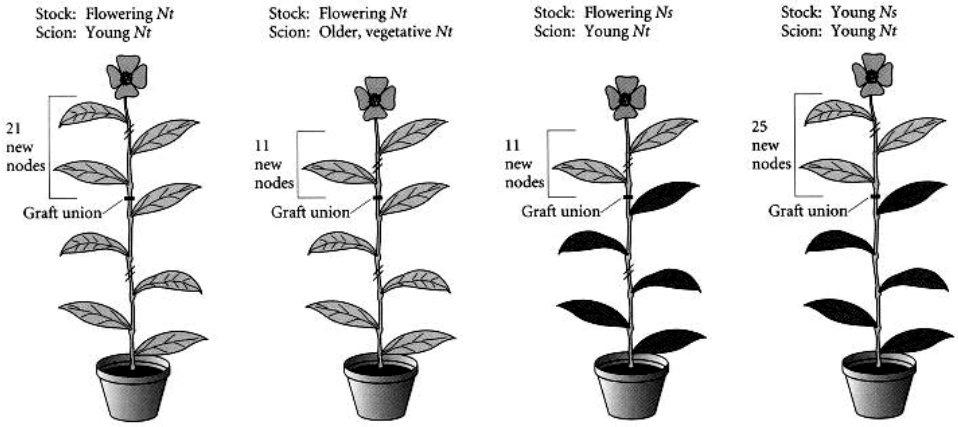
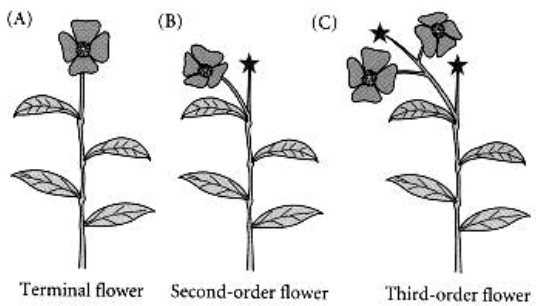


Figure. The interaction of competence and signal strength. Nicotiana tabacum (Nt) is a day-neutral plant that flowers when the meristem gains competence to respond to internal signals. N. silvestris (Ns) is a long-day plant that flowers when the floral signal(s) reach a critical level. These grafting experiments illustrate that a young Nt shoot is less competent to respond to the Nt flowering signal than an older Nt shoot. Young Nt shoots respond quickly to the flowering signal from flowering Ns plants, but flower later when grafted to young Ns plants with a lower level of signal. The scion is the shoot that is grafted on to the stock; the stock is the rooted portion of the plant from which the shoot has been excised.

Shoot tip culture experiments in several species (including tobacco, sunflower, and peas) have demonstrated that determination for reproductive function can occur before reproductive morphogenesis. That is, isolated shoot tips that are determined for reproductive development but are morphologically vegetative will produce the same number of nodes before flowering in situ and in culture.

The "black box" between environmental signals and the production of a flower is vanishing rapidly, especially in the model plant *Arabidopsis.* The signaling pathways from light via different phytochromes to key flowering genes are being elucidated. Molecular explanations are revealing redundant pathways that ensure that flowering will occur. Light-dependent, gibberellins dependent, vernalization-dependent, and autonomous pathways that regulate the floral transition have been genetically dissected.

The ancestral angiosperm is believed to have formed a terminal flower directly from the terminal shoot apex. In modern angiosperms, a variety of flowering patterns exist in which the terminal shoot apex is indeterminate, but axillary buds produce flowers. This observation introduces an intermediate step into the reproductive process: the transition of a vegetative meristem to an **inflorescence meristem**, which initiates axillary meristems that can produce floral organs, but does not directly produce floral parts itself. The inflorescence is the reproductive backbone (stem) that displays the flowers. The inflorescence meristem probably arises through the action of a gene that suppresses terminal flower formation. The *CENTRORADIALUS* (*CEN*) gene in snapdragons suppresses terminal flower formation. It suppresses expression of *FLORICAULA* (*FLO*), which specifies floral meristem identity. Curiously, the expression of *FLO* is necessary for *CEN* to be turned on. The *Arabidopsis* homologue of *CEN* (*TERMINAL FLOWER 1* or *TFL1*) is expressed during the vegetative phase of development as well, and has the additional function of delaying the commitment to inflorescence development. Overexpression of *TFL1* in transgenic *Arabidopsis* extends the time before a terminal flower forms. *TFL1* must delay the reproductive transition. Garden peas branch one more time than snapdragons do before forming a flower. That is, the axillary meristem does not directly produce a flower, but acts as an inflorescence meristem that initiates floral meristems. Two genes, *DET* and *VEG1,* are responsible for this more complex inflorescence, and only when both are nonfunctional is a terminal flower formed (Figure).



The next step in the reproductive process is the specification of floral meristems those meristems that will actually produce flowers. In *Arabidopsis, LEAFY* (*LFY*), *APETALA 1* (*AP1*), and *CAULIFLOWER* (*CAL*) are **floral meristem identity genes**. *LFY* is the homologue of *FLO* in snapdragons, and its upregulation during development is key to the transition to reproductive development. Expression of these genes is necessary for the transition from an inflorescence

meristem to a floral meristem. Mutants (*lfy*) tend to form leafy shoots in the axils where flowers form in wild-type plants; they are unable to make the transition to floral development. If *LFY* is overexpressed, flowering occurs early. For example, when aspen was transformed with an *LFY* gene that was expressed throughout the plant, the time to flowering was dramatically shortened from years to month. *AP1* and *CAL* are closely related and redundant genes. The *cal* mutant looks like the wildtype plant, but *ap1 cal* double mutants produce inflorescences that look like cauliflower heads.

Floral meristem identity genes initiate a cascade of gene expression that turns on region specifying (**cadastral**) genes, which further specify pattern by initiating transcription of **floral organ identity genes** (Weigel 1995). *SUPERMAN* (*SUP*) is an example of a cadastral gene in *Arabidopsis* that plays a role in specifying boundaries for organ identity gene expression. Three classes (A, B, and C) of organ identity genes are necessary to specify the four whorls of floral organs (Figures).

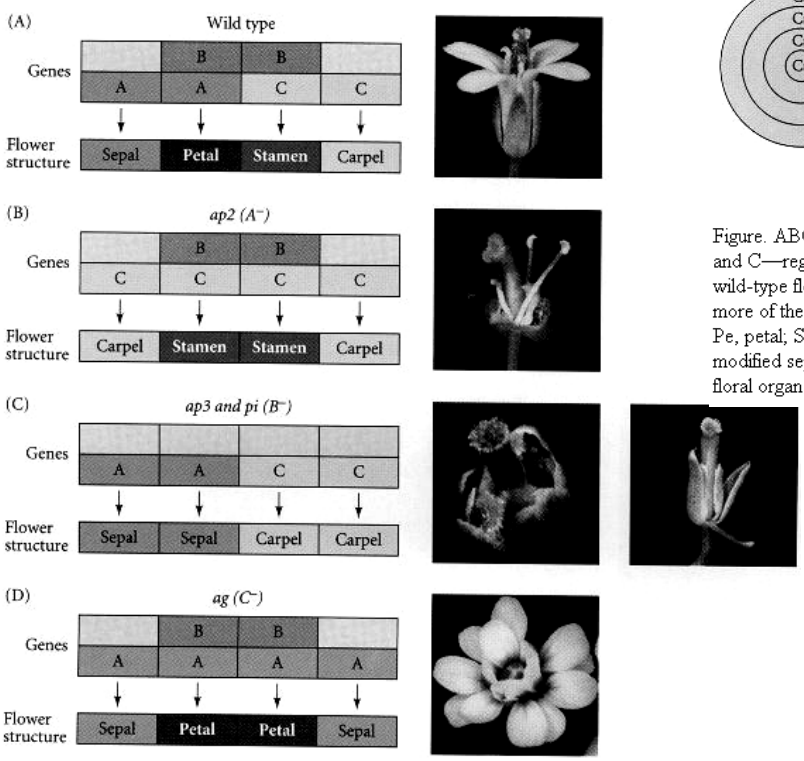


Figure. Wild-type and mutant phenotypes of the Arabidopsis class A (ap2), class B (ap3, pi), and class C (ag) floral organ identity genes.

They are homeotic genes (but not Hox genes) and include *AP2*,*AGAMOUS* (*AG*), *AP3*, and *PISTILLATA* (*PI*) in *Arabidopsis*. Class A genes (*AP2*) alone specify sepal development. Class A genes and class B genes (*AP3* and *PI*) together specify petals. Class B and class C (*AG*) genes are necessary for stamen formation; class C genes alone specify carpel formation. When all of these homeotic genes are not expressed in a developing flower, floral parts become leaflike. The ABC genes code for transcription factors that initiate a cascade of events leading to the actual production of floral parts. In addition to the ABC genes, class D genes are now being investigated that specifically regulate ovule development. The ovule evolved long before the other angiosperm floral parts, and while its development is coordinated with that of the carpel, one would expect more ancient, independent pathways to exist.

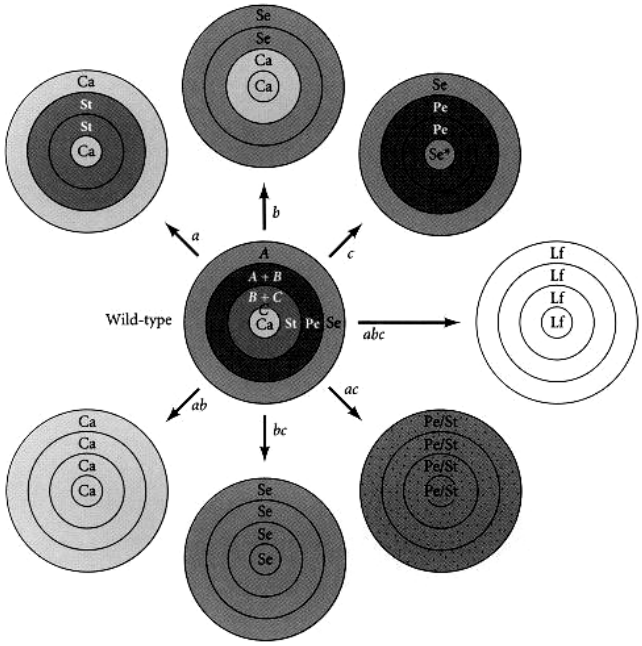


Figure. ABC model for floral organ specification. Three classes of genes—A,B, and C—regulate organ identity in flowers. The central diagram represents the wild-type flower; surrounding diagrams represent mutants that are missing one or more of these gene functions (indicated by the lowercase a,b, or c). Se, sepal; Pe, petal; St, stamen; Ca, carpel; Pe/St, a hybrid petal/stamen; Lf, leaf; se*, a modified sepal indicating that other genes (possibly ovule genes) also regulate floral organ specification.

Senescence

Flowering and **senescence** (a developmental program leading to death) are closely linked in many angiosperms. Individual flower petals in some species senesce following pollination. Orchids, which stay fresh for long periods of time if they are not pollinated, are a good example. Fruit ripening (and ultimately over-ripening) is an example of organ senescence. Whole-plant senescence leads to the death of the entire sporophyte generation. **Monocarpic** plants flower once and then senesce. **Polycarpic** plants, such as the bristlecone pine, can live thousands of years (4900 years is the current record) and flower repeatedly. In polycarpic plants, death is by accident; in monocarpic plants, it appears to be genetically programmed. Flowers and fruits play a key role in the process, and their removal can sometimes delay senescence. In some legumes, senescence can be delayed by removing the developing seed in other words, the embryo may trigger senescence in the parent plant. During flowering and fruit development, nutrients are reallocated from other parts of the plant to support the development of the next generation. The reproductive structures become a nutrient sink, and this can lead to whole-plant senescence.

E1. Programmed Cell Death

Programmed cell-death (PCD) is death of a cell in any form, mediated by an intracellular program. In contrast to necrosis, which is a form of cell-death that results from acute tissue injury and provokes an inflammatory response, PCD is carried out in a regulated process which generally confers advantage during an organism's life-cycle. PCD serves fundamental functions during both plant and metazoa (multicellular animals) tissue development.

1. Types

- Apoptosis or Type I cell-death
- Autophagic or Type II cell-death (cytoplasmic: characterized by the formation of large vacuoles which eat away organelles in a specific sequence prior to the nucleus being destroyed.)

Besides these two types of PCD, other pathways have been discovered. Called "non-apoptotic programmed cell-death" (or "caspase-independent programmed cell-death" or "necrosis-like programmed cell-death") these alternative routes to death are as efficient as apoptosis and can function as either backup mechanisms or the main type of PCD.

Other forms of programmed cell death include anoikis, almost identical to apoptosis except in its induction; cornification, a form of cell death exlusive to the eyes; excitotoxicity and Wallerian degeneration.Plant cells undergo particular processes of PCD which are similar to autophagic cell death. However, some common features of PCD are highly conserved in both plants and metazoa.

2. Apoptosis

Apoptosis is a form of programmed cell death in multicellular organisms. It is one of the main types of programmed cell death (PCD) and involves a series of biochemical events leading to a characteristic cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Processes of disposal of cellular debris whose results do not damage the organism differentiate apoptosis from necrosis.

In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose; the result is that the digits are separate. Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight.

Research on apoptosis has increased substantially since the early 1990s. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer.

3. Functions

3.1 Cell termination: Apoptosis can occur when a cell is damaged beyond repair, infected with a virus, or undergoing stress conditions such as starvation. DNA damage from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumour-suppressing gene p53. The "decision" for apoptosis can come from the cell itself, from the surrounding tissue, or from a cell that is part of the immune system. In these cases apoptosis functions to remove the damaged cell, preventing it from sapping further nutrients from the organism, or to prevent the spread of viral infection.

Apoptosis also plays a role in preventing cancer; if a cell is unable to undergo apoptosis, due to mutation or biochemical inhibition, it can continue dividing and develop into a tumour. For example, infection by papillomaviruses causes a viral gene to interfere with the cell's p53 protein, an important member of the apoptotic pathway. This interference in the apoptotic capability of the cell plays a critical role in the development of cervical cancer.

3.2 Homeostasis: In the adult organism, the number of cells is kept relatively constant through cell death and division. Cells must be replaced when they become diseased or malfunctioning; but proliferation must be compensated by cell death. This balancing process is part of the homeostasis required by living organisms to maintain their internal states within certain limits. Some scientists have suggested homeodynamics as a more accurate term. The related term allostasis reflects a balance of a more complex nature by the body.

Homeostasis is achieved when the rate of mitosis (cell division) in the tissue is balanced by cell death. If this equilibrium is disturbed, one of two potentially fatal disorders occurs:

- The cells are dividing faster than they die, effectively developing a tumor.
- The cells are dividing slower than they die, which results in a disorder of cell loss.
- The organism must orchestrate a complex series of controls to keep homeostasis tightly controlled, a process that is ongoing for the life of the organism and involves many different types of cell signaling. Impairment of any one of these controls can lead to a diseased state; for example, dysregulation of signaling pathway has been implicated in several forms of cancer. The pathway, which conveys an anti-apoptotic signal, has been found to be activated in pancreatic adenocarcinoma tissues.

3.3. Development: Programmed cell death is an integral part of both plant and animal tissue development. Development of an organ or tissue is often preceded by the extensive division and differentiation of a particular cell, the resultant mass is then "pruned" into the correct form by apoptosis. Unlike necrosis, cellular death caused by injury, apoptosis results in cell shrinkage and fragmentation. This allows the cells to be efficiently phagocytosed and their components reused without releasing potentially harmful intracellular substances (such as hydrolytic enzymes, for example) into the surrounding tissue.

Research on chick embryos has suggested how selective cell proliferation, combined with selective apoptosis, sculpts developing tissues in vertebrates. During vertebrate embryo development, structures called the notochord and the floor plate secrete a gradient of the signaling molecule (Shh), and it is this gradient that directs cells to form patterns in the embryonic neural tube: cells that receive Shh in a receptor in their membranes called Patched1 (Ptc1) survive and proliferate; but, in the absence of Shh, one of the ends of this same Ptc1 receptor (the carboxyl-terminal, inside the membrane) is cleaved by caspase-3, an action that exposes an apoptosis-producing domain.

During development, apoptosis is tightly regulated and different tissues use different signals for inducing apoptosis. In birds, bone morphogenetic proteins (BMP) signaling is used to induce apoptosis in the interdigital tissue. In Drosophila flies, steroid hormones regulate cell death. Developmental cues can also induce apoptosis, such as the sex-specific cell death of hermaphrodite specific neurons in *C. elegans* males through low TRA-1 transcription factor activity (TRA-1 helps prevent cell death).

3.4. Lymphocyte interactions: The development of B lymphocytes and the development of T lymphocytes in the human body is a complex process that effectively creates a large pool of diverse cells to begin with, then weeds out those potentially damaging to the body. Apoptosis is the mechanism by which the body removes both the ineffective and the potentially-damaging immature cells, and in T-cells is initiated by the withdrawal of survival signals.

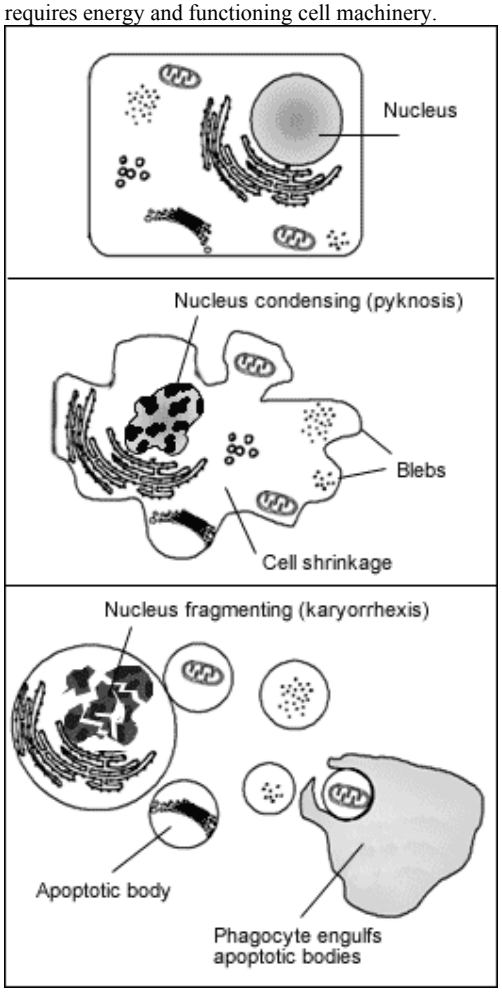
Cytotoxic T-cells are able to directly induce apoptosis in cells by opening up pores in the target's membrane and releasing chemicals that bypass the normal apoptotic pathway. The pores are created by the action of secreted perforin, and the granules contain granzyme B, a serine protease that activates a variety of caspases by cleaving aspartate residues.

4. Process

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines, and therefore must either cross the plasma membrane or transduce to effect a response. These signals may positively or negatively induce apoptosis; in this context the binding and subsequent initiation of apoptosis by a molecule is termed positive, whereas the active repression of apoptosis by a molecule is termed negative.

Intracellular apoptotic signalling is a response initiated by a cell in response to stress, and may ultimately result in cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration (e.g. by membrane damage) are all factors that can lead to the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis.

Before the actual process of cell death is carried out by enzymes, apoptotic signals must be connected to the actual death pathway by way of regulatory proteins. This step allows apoptotic signals to either culminate in cell death, or be aborted should the cell no longer need to die. Several proteins are involved, however two main methods of achieving regulation have been identified; targeting mitochondria functionality, or directly transducing the signal via adapter proteins to the apoptotic mechanisms. The whole preparation process requires energy and functioning cell machinery.



4.1 Mitochondrial regulation: The mitochondria are essential to multicellular life. Without them, a cell ceases to respire aerobically and quickly dies - a fact exploited by some apoptotic pathways. Apoptotic proteins that

target mitochondria affect them in different ways; they may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out. There is also a growing body of evidence that indicates that nitric oxide (NO) is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable.

Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cytosol following an increase in permeability. SMAC binds to inhibitor of apoptosis proteins (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of a group of cysteine proteases called caspases, which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability.

Cytochrome c is also released from mitochondria due to formation of a channel, MAC, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis. Once cytochrome c is released it binds with Apaf-1 and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3.

MAC is itself subject to regulation by various proteins, such as those encoded by the mammalian Bcl-2 family of anti-apoptopic genes, the homologs of the ced-9 gene found in C. elegans. Bcl-2 proteins are able to promote or inhibit apoptosis either by direct action on MAC or indirectly through other proteins. It is important to note that the actions of some Bcl-2 proteins are able to halt apoptosis even if cytochrome c has been released by the mitochondria.

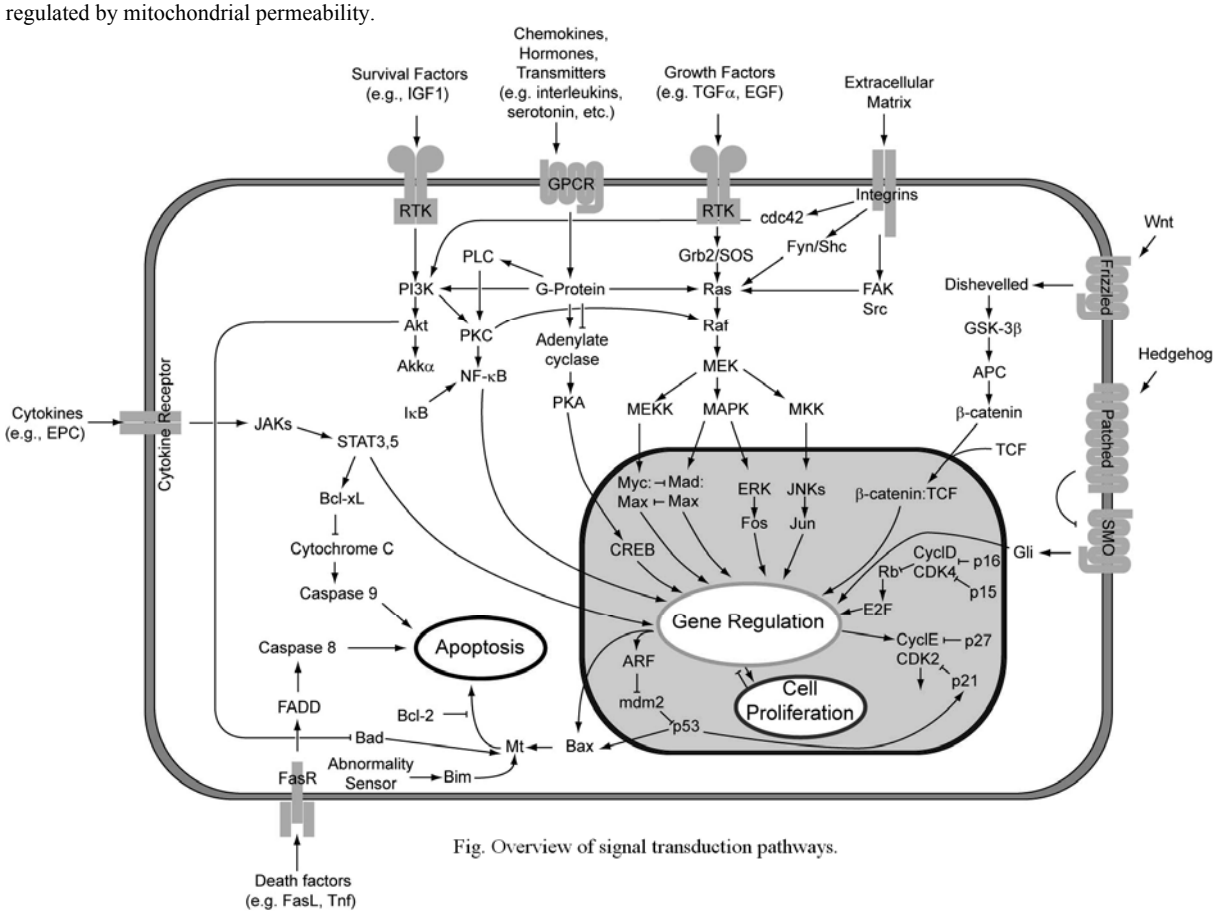


Fig. Overview of signal transduction pathways.

4.2 Direct signal transduction: Two important examples of the direct initiation of apoptotic mechanisms in mammals include the TNF-induced (tumour necrosis factor) model and the Fas-Fas ligand-mediated model, both involving receptors of the TNF receptor (TNFR) family coupled to extrinsic signals.

TNF is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses. The link between TNF and apoptosis shows why an abnormal production of TNF plays a fundamental role in several human diseases, especially in autoimmune diseases.

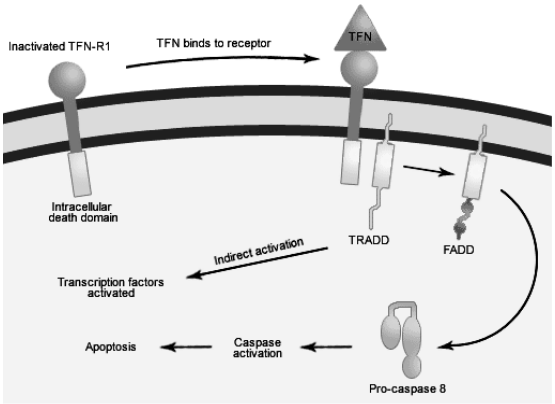


Fig. Overview of TNF signalling in apoptosis, an example of direct signal transduction

The Fas receptor (also known as Apo-1 or CD95) binds the Fas ligand (FasL), a transmembrane protein part of the TNF family. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10. In some types of cells (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis. In other types of cells (type II), the Fas-DISC starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8.

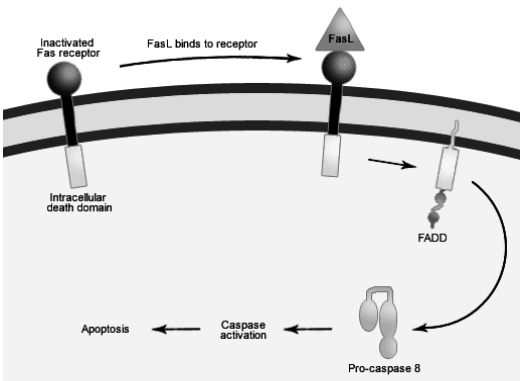


Fig. Overview of Fas signalling in apoptosis, an example of direct signal transduction

Following TNF-R1 and Fas activation in mammalian cells a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-Xl and Bcl-2) members of the Bcl-2 family is established. This balance is the proportion of pro-apoptotic homodimers that form in the outer-membrane of the mitochondrion. The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC. Control of pro-apoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood, but it has been found that a mitochondrial outer-membrane protein, VDAC2, interacts with BAK to keep this potentially-lethal apoptotic effector under control. When the death signal is received, products of the activation cascade displace VDAC2 and BAK is able to be activated.

There also exists a caspase-independent apoptotic pathway that is mediated by AIF (apoptosis-inducing factor).

4.3 Execution: Although many pathways and signals lead to apoptosis, there is only one mechanism that actually causes the death of the cell in this process; after the appropriate stimulus has been received by the cell and the necessary controls exerted, a cell will undergo the organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology that can be observed with a microscope:

- Cell shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton by caspases.
- The cytoplasm appears dense, and the organelles appear tightly packed.
- Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis.
- The nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA.
- The cell membrane shows irregular buds known as blebs.
- The cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed.

Apoptosis progresses quickly and its products are quickly removed, making it difficult to detect or visualize. During karyorrhexis, endonuclease activation leaves short DNA fragments, regularly spaced in size. These give a characteristic "laddered" appearance on agar gel after electrophoresis. Tests for DNA laddering differentiate apoptosis from ischemic or toxic cell death.

4.4 Removal of dead cells: Dying cells that undergo the final stages of apoptosis display phagocytotic molecules, such as phosphatidylserine, on their cell surface. Phosphatidylserine is normally found on the cytosolic surface of the plasma membrane, but is redistributed during apoptosis to the extracellular surface by a hypothetical protein known as scramblase. These molecules mark the cell for phagocytosis by cells possessing the appropriate receptors, such as macrophages. Upon recognition, the phagocyte reorganizes its cytoskeleton for engulfment of the cell. The removal of dying cells by phagocytes occurs in an orderly manner without eliciting an inflammatory response.

5. Implication in disease

5.1 Defective apoptotic pathways: The many different types of apoptotic pathways contain a multitude of different biochemical components, many of them not yet understood. As a pathway is more or less sequential in nature it is a victim of causality; removing or modifying one component leads to an effect in another. In a living organism this can have disastrous effects, often in the form of disease or disorder. A discussion of every

disease caused by modification of the various apoptotic pathways would be impractical, but the concept overlying each one is the same: the normal functioning of the pathway has been disrupted in such a way as to impair the ability of the cell to undergo normal apoptosis. This results in a cell that lives past its "use-by-date" and is able to replicate and pass on any faulty machinery to its progeny, increasing the likelihood of the cell becoming cancerous or diseased.

A recently-described example of this concept in action can be seen in the development of a lung cancer called NCI-H460. The X-linked inhibitor of apoptosis protein (XIAP) is overexpressed in cells of the H460 cell line. XIAPs bind to the processed form of caspase-9, and suppress the activity of apoptotic activator cytochrome c, therefore overexpression leads to a decrease in the amount of pro-apoptotic agonists. As a consequence, the balance of anti-apoptotic and pro-apoptotic effectors is upset in favour of the former, and the damaged cells continue to replicate despite being directed to die.

5.2 p53 disregulation: The tumor-suppressor protein p53 accumulates when DNA is damaged due to a chain of biochemical reactions. Part of this pathway includes alpha-interferon and beta-interferon, which induce transcription of the p53 gene and result in the increase of p53 protein level and enhancement of cancer cell-apoptosis. p53 prevents the cell from replicating by stopping the cell cycle at G1, or interphase, to give the cell time to repair, however it will induce apoptosis if damage is extensive and repair efforts fail. Any disruption to the regulation of the p53 or interferon genes will result in impaired apoptosis and the possible formation of tumors.

5.3 HIV progression: The progression of the human immunodeficiency virus (HIV) to AIDS is primarily due to the depletion of CD4+ T-helper lymphocytes, which leads to a compromised immune system. One of the mechanisms by which T-helper cells are depleted is apoptosis, which can be the end-product of multiple biochemical pathways:

- HIV enzymes inactivate anti-apoptotic Bcl-2 and simultaneously activate pro-apoptotic procaspase-8. This does not directly cause cell death but primes the cell for apoptosis should the appropriate signal be received.
- HIV products may increase levels of cellular proteins which have a promotive effect on Fas-mediated apoptosis.
- HIV proteins decrease the amount of CD4 glycoprotein marker present on the cell membrane.
- Released viral particles and proteins present in extracellular fluid are able to induce apoptosis in nearby "bystander" T-helper cells.
- HIV decreases the production of molecules involved in marking the cell for apoptosis, giving the virus time to replicate and continue releasing apoptotic agents and virions into the surrounding tissue.
- The infected CD4+ cell may also receive the death signal from a cytotoxic T cell, leading to apoptosis.

In addition to apoptosis, infected cells may also die as a direct consequence of the viral infection.

5.4 Viral infection: Viruses can trigger apoptosis of infected cells via a range of mechanisms including:

- Receptor binding.
- Activation of protein kinase R (PKR).
- Interaction with p53.

Expression of viral proteins coupled to MHC proteins on the surface of the infected cell, allowing recognition by cells of the immune system (such as Natural Killer and cytotoxic T cells) that then induce the infected cell to undergo apoptosis.

Most viruses encode proteins that can inhibit apoptosis. Several viruses encode viral homologs of Bcl-2. These homologs can inhibit pro-apoptotic proteins such as BAX and BAK, which are essential for the activation of apoptosis. Examples of viral Bcl-2 proteins include the Epstein-Barr virus BHRF1 protein and the adenovirus E1B 19K protein. Some viruses express caspase inhibitors that inhibit caspase activity and an example is the CrmA protein of cowpox viruses. Whilst a number of viruses can block the effects of TNF and Fas. For example the M-T2 protein of myxoma viruses can bind TNF preventing it from binding the TNF receptor and inducing a response. Furthermore, many viruses express p53 inhibitors that can bind p53 and inhibit its transcriptional transactivation activity. Consequently p53 cannot induce apoptosis since it cannot induce the expression of pro-apoptotic proteins. The adenovirus E1B-55K protein and the hepatitis B virus HBx protein are examples of viral proteins that can perform such a function.

Interestingly, viruses can remain intact from apoptosis particularly in the latter stages of infection. They can be exported in the apoptotic bodies that pinch off from the surface of the dying cell and the fact that they are engulfed by phagocytes prevents the initiation of a host response. This favours the spread of the virus.

6. Programmed cell-death in plant tissue

In "APL regulates vascular tissue identity in Arabidopsis", Bonke and colleagues state that one of the two long-distance transport systems in vascular plants, xylem, consists of several cell-types "the differentiation of which involves deposition of elaborate cell-wall thickenings and programmed cell-death." The authors emphasize that the products of plant PCD play an important structural role.

Basic morphological and biochemical features of PCD have been conserved in both plant and animal kingdoms. It should be noted, however, that specific types of plant cells carry out unique cell-death programs. These have common features with animal apoptosis -- for instance, nuclear DNA degradation -- but they also have their own peculiarities, such as nuclear degradation being triggered by the collapse of the vacuole in tracheary elements of the xylem.

Janneke Balk and Christopher J. Leaver, of the Department of Plant Sciences, University of Oxford, carried out research on mutations in the mitochondrial genome of sun-flower cells. Results of this research suggest that mitochondria play the same key role in vascular plant PCD as in other eukaryotic cells.

7. PCD in pollen prevents inbreeding

During pollination, plants enforce self-incompatibility (SI) as an important means to prevent self-fertilization. Research on the corn poppy (Papaver rhoeas) has revealed that proteins in the pistil on which the pollen lands, interact with pollen and trigger PCD in incompatible (ie. self) pollen. The researchers, Steven G. Thomas and Veronica E. Franklin-Tong, also found that the response involves rapid inhibition of pollen-tube growth, followed by PCD.

8. Programmed cell death in slime molds

The social slime mold Dictyostelium discoideum has the peculiarity of adopting either a predatory amoeba-like behavior in its unicellular form, or coalescing into a mobile slug-like form when dispersing the spores which will give birth to the next generation.

The stalk is composed of dead cells which have undergone a type of PCD that shares many features of an autophagic cell-death: massive vacuoles forming inside cells, a degree of chromatin condensation, but no DNA-fragmentation. The structural role of the residues left by the dead cells is reminiscent of the products of PCD in plant tissue.

D. discoideum is a slime mold, part of a branch which may have emerged from eukaryotic ancestors about a billion years before the present. They apparently emerged after the ancestors of green-plants and the ancestors of fungi and animals had differentiated. But in addition to their place in the evolutionary tree, the fact that PCD has been observed in the humble, simple, six-chromosome *D. discoideum* has additional significance: it permits the study of a developmental PCD path which does not depend on the caspases which are characteristic of apoptosis.

9. Evolutionary origin of PCD

Biologists had long suspected that mitochondria originated from bacteria which had been incorporated as endosymbionts ("living together inside") of larger eukaryotic cells. It was Lynn Margulis who from 1967 on championed this theory, which has since become widely accepted. The most convincing evidence for this theory is the fact that mitochondria possess their own DNA and are equipped with genes and replication apparatus.

This evolutionary step would have been more than risky for the primitive eukaryotic cells which began to engulf the energy-producing bacteria and conversely, a perilous step for the ancestors of mitochondria which began to invade their proto-eukaryotic hosts. This process is still evident today, between human white blood-cells and bacteria. Most of the time, invading bacteria are destroyed by the white blood-cells; however, it is not uncommon for the chemical warfare waged by prokaryotes to succeed, with the consequence known as infection by its resulting damage.

One of these rare evolutionary events, about two billion years before the present, made it possible for certain eukaryotes and energy-producing prokaryotes not only to coexist, but to mutually benefit from their symbiosis.

Mitochondriate eukaryotic cells live poised between life and death, because mitochondria still retain their repertoire of molecules which can trigger cell suicide. This process has now been evolved to happen only when programmed. Given certain signals to cells (such as feedback from neighbors, stress or DNA-damage), mitochondria release caspase activators which trigger the cell-death inducing biochemical cascade. As such, the cell-suicide mechanism is now crucial to all of our lives.

E2. Senescence and Ageing

Senescence refers to the biological processes of a living organism approaching an advanced age (i.e., the combination of processes of deterioration which follow the period of development of an organism). The word senescence is derived from the Latin word senex, meaning "old man" or "old age" or "advanced in age".

1. Cellular senescence

Cellular senescence is the phenomenon where normal diploid differentiated cells lose the ability to divide, normally after about 50 cell divisions in vitro, some cells become senescent before because of DNA double strand breaks, toxins etc. This phenomenon is also known as "replicative senescence", the "Hayflick phenomenon", or the Hayflick limit in honour of Dr. Leonard Hayflick who was the first to publish this information in 1965. In response to DNA damage (including shortened telomeres) cells either age or self-destruct (apoptosis, programmed cell death) if the damage cannot be repaired. In this 'cellular suicide', the death of one, or more, cells may benefit the organism as a whole. For example, in plants the death of the water-conducting xylem cells (tracheids and vessel elements) allows the cells to function more efficiently and so deliver water to the upper parts of a plant.

2. Ageing of the whole organism

Organismal senescence is the aging of whole organisms. The term ageing has become so commonly equated with senescence that the terms will be used interchangeably in this article. Ageing is generally characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased risk of ageing-associated diseases. Because of this, death is the ultimate consequence of ageing. Differences in maximum life span among species correspond to different "rates of ageing". For example, inherited differences in the rate of ageing make a mouse elderly at 3 years and a human elderly at 90 years. These genetic differences affect a variety of physiological processes, including the efficiency of DNA repair, antioxidant enzymes, and rates of free radical production.

Senescence of the organism gives rise to the Gompertz-Makeham law of mortality, which says that mortality rate rises rapidly with age.

Some animals, such as some reptiles and fish, age slowly. Some even exhibit "negative senescence", in which mortality falls with age, in disagreement with the Gompertz-Makeham "law".

Whether cell senescence (Hayflick limit) plays a causative role in organismal aging is at present and is an on-going area of active investigation.

3. Theories of aging

The process of senescence is complex, and may derive from a variety of different mechanisms and exist for a variety of different reasons. However, senescence is not universal, and scientific evidence suggests that cellular senescence evolved in certain species as a mechanism to prevent the onset of cancer. In a few simple species, senescence is negligible and cannot be detected. All such species have no "post-mitotic" cells; they reduce the effect of damaging free radicals by cell division and dilution. Such species are not immortal, however, as they will eventually fall prey to trauma or disease. Moreover, average lifespans can vary greatly within and between species. This suggests that both genetic and environmental factors contribute to ageing.

Traditionally, theories that explain senescence have generally been divided between the programmed and stochastic theories of ageing. Programmed theories imply that ageing is regulated by biological clocks operating throughout the life span. This regulation would depend on changes in gene expression that affect the systems responsible for maintenance, repair and defense responses. Stochastic theories blame environmental impacts on living organisms that induce cumulative damage at various levels as the cause of ageing, examples which range from damage to DNA, damage to tissues and cells by oxygen radicals (widely known as free radicals countered by the even more well known antioxidants), and cross-linking.

Conversely, ageing is seen as a progressive failure of homeodynamics (homeostasis) involving genes for the maintenance and repair, stochastic events leading to molecular damage and molecular heterogeneity, and chance events determining the probability of death. Since complex and interacting systems of maintenance and repair comprise the homeodynamic (old term, homeostasis) space of a biological system, ageing is considered to be a progressive shrinkage of homeodynamic space mainly due to increased molecular heterogeneity.

3.1. Evolutionary theories: Ageing is believed to have evolved because of the increasingly smaller probability of an organism still being alive at older age, due to predation and accidents, both of which may be random and age-invariant. It is thought that strategies which result in a higher reproductive rate at a young age, but shorter overall lifespan, result in a higher lifetime reproductive success and are therefore favoured by natural selection. Essentially, ageing is therefore the result of investing resources in reproduction, rather than maintenance of the body (the "Disposable Soma" theory), in light of the fact that accidents, predation and disease will eventually kill the organism no matter how much energy is devoted to repair of the body. Various other, or more specific, theories of ageing exist, and are not necessarily mutually exclusive.

The geneticist J. B. S. Haldane wondered why the dominant mutation which causes Huntington's disease remained in the population, why natural selection had not eliminated it. The onset of this neurological disease is (on average) at age 45 and is invariably fatal within 10-20 years. Haldane assumed, probably reasonably, that in human prehistory, few survived until age 45. Since few were alive at older ages and their contribution to the next generation was therefore small relative to the large cohorts of younger age groups, the force of selection against such late-acting deleterious mutations was correspondingly small. However, if a mutation affected younger individuals, selection against it would be strong. Therefore, late-acting deleterious mutations could accumulate in populations over evolutionary time through genetic drift. This principle has been demonstrated experimentally. And it is these later-acting deleterious mutations which are believed to cause, or perhaps more correctly allow, age-related mortality.

Peter Medawar formalised this observation in his mutation accumulation theory of ageing. "The force of natural selection weakens with increasing age — even in a theoretically immortal population, provided only that it is exposed to real hazards of mortality. If a genetic disaster... happens late enough in individual life, its consequences may be completely unimportant". The 'real hazards of mortality' are typically predation, disease and accidents. So, even an immortal population, whose fertility does not decline with time, will have fewer individuals alive in older age groups. This is called 'extrinsic mortality.' Young cohorts, not depleted in numbers yet by extrinsic mortality, contribute far more to the next generation than the few remaining older cohorts, so the force of selection against late-acting deleterious mutations, which only affect these few older individuals, is very weak. The mutations may not be selected against, therefore, and may spread over evolutionary time into the population.

The major testable prediction made by this model is that species which have high extrinsic mortality in nature will age more quickly and have shorter intrinsic lifespans. This is borne out among mammals, the most well studied in terms of life history. There is a correlation among mammals between body size and lifespan, such that larger species live longer than smaller species in controlled/optimum conditions, but there are notable exceptions. For instance, many bats and rodents are similarly sized, yet bats live much, much longer. For instance, the little brown bat, half the size of a mouse, can live 30 years in the wild. A mouse will live 2–3 years even with optimum conditions. The explanation is that bats have fewer predators, so therefore low extrinsic mortality. Thus more individuals survive to later ages so the force of selection against late-acting deleterious mutations is stronger. Fewer late-acting deleterious mutations = slower ageing = longer lifespan. Birds are also warm-blooded and similarly sized to many small mammals, yet live often 5–10 times as long. They clearly

have fewer predation pressures compared with ground-dwelling mammals. And seabirds, which generally have the fewest predators of all birds, live longest.

Also, when examining the body-size vs. lifespan relationship, predator mammals tend to have longer lifespans than prey animals in a controlled environment such as a zoo or nature reserve. The explanation for the long lifespans of primates (such as humans, monkeys and apes) relative to body size is that their intelligence and often sociality helps them avoid becoming prey. Being a predator, being smart and working together all reduce extrinsic mortality.

Another evolutionary theory of ageing was proposed by George C. Williams and involves antagonistic pleiotropy. A single gene may affect multiple traits. Some traits that increase fitness early in life may also have negative effects later in life. But because many more individuals are alive at young ages than at old ages, even small positive effects early can be strongly selected for, and large negative effects later may be very weakly selected against. Williams suggested the following example: perhaps a gene codes for calcium deposition in bones which promotes juvenile survival and will therefore be favored by natural selection; however this same gene promotes calcium deposition in the arteries, causing negative effects in old age. Therefore negative effects in old age may reflect the result of natural selection for pleiotropic genes which are beneficial early in life. In this case, fitness is relatively high when Fisher's reproductive value is high and relatively low when Fisher's reproductive value is low.

3.2. Gene regulation: A number of genetic components of ageing have been identified using model organisms, ranging from the simple budding yeast Saccharomyces cerevisiae to worms such as Caenorhabditis elegans and fruit flies (Drosophila melanogaster). Study of these organisms has revealed the presence of at least two conserved ageing pathways.

One of these pathways involves the gene Sir2, a NAD^+-dependent histone deacetylase. In yeast, Sir2 is required for genomic silencing at three loci: the yeast mating loci, the telomeres and the ribosomal DNA (rDNA). In some species of yeast replicative ageing may be partially caused by homologous recombination between rDNA repeats; excision of rDNA repeats results in the formation of extrachromosomal rDNA circles (ERCs). These ERCs replicate and preferentially segregate to the mother cell during cell division, and are believed to result in cellular senescence by titrating away (competing for) essential nuclear factors. ERCs have not been observed in other species (nor even all strains of the same yeast species) of yeast (which also display replicative senescence), and ERCs are not believed to contribute to ageing in higher organisms such as humans (they have not been shown to accumulate in mammals in a similar manner to yeast). Extrachromosomal circular DNA (eccDNA) has been found in worms, flies and humans. The origin and role of eccDNA in ageing, if any, is unknown.

Despite the lack of a connection between circular DNA and ageing in higher organisms, extra copies of Sir2 are capable of extending the lifespan of both worms and flies (though in flies, this finding has not been replicated by other investigators, and the activator of Sir2, resveratrol, does not reproducibly increase lifespan in either species). Whether the Sir2 homologues in higher organisms have any role in lifespan is unclear, but the human SIRT1 protein has been demonstrated to deacetylate p53, Ku70, and the forkhead family of transcription factors. SIRT1 can also regulate acetylates such as CBP/p300, and has been shown to deacetylate specific histone residues.

RAS1 and RAS2 also affect ageing in yeast and have a human homologue. RAS2 overexpression has been shown to extend lifespan in yeast.

Other genes regulate ageing in yeast by increasing the resistance to oxidative stress. Superoxide dismutase, a protein that protects against the effects of mitochondrial free radicals, can extend yeast lifespan in stationary phase when overexpressed.

In higher organisms, ageing is likely to be regulated in part through the insulin/IGF-1 pathway. Mutations that affect insulin-like signaling in worms, flies and the growth hormone/IGF1 axis in mice are associated with extended lifespan. In yeast, Sir2 activity is regulated by the nicotinamidase PNC1. PNC1 is transcriptionally upregulated under stressful conditions such as caloric restriction, heat shock, and osmotic shock. By converting nicotinamide to niacin, it removes nicotinamide, which inhibits the activity of Sir2. A nicotinamidase found in humans, known as PBEF, may serve a similar function, and a secreted form of PBEF known as visfatin may help to regulate serum insulin levels. It is not known, however, whether these mechanisms also exist in humans since there are obvious differences in biology between humans and model organisms.

Sir2 activity has been shown to increase under calorie restriction. Due to the lack of available glucose in the cells more NAD^+ is available and can activate Sir2. Resveratrol, a polyphenol found in the skin of red grapes, was reported to extend the lifespan of yeast, worms, and flies (the lifespan extension in flies and worms have proved irreproducible by independent investigators). It has been shown to activate Sir2 and therefore mimics the effects of calorie restriction, if one accepts that caloric restriction is indeed depedent on Sir2.

Gene expression is imperfectly controlled, and it is possible that random fluctuations in the expression levels of many genes contribute to the ageing process as suggested by a study of such genes in yeast. Individual cells, which are genetically identical, none-the-less can have substantially different responses to outside stimuli, and markedly different lifespans, indicating the epigenetic factors play an important role in gene expression and ageing as well as genetic factors.

3.3. Cellular senescence: As noted above, senescence is not universal, and senescence is not observed in single-celled organisms that reproduce through the process of cellular mitosis. Moreover, cellular senescence is not observed in many organisms, including perennial plants, sponges, corals, and lobsters. In those species where cellular senescence is observed, cells eventually become post-mitotic when they can no longer replicate themselves through the process of cellular mitosis -- i.e.,

cells experience replicative senescence. How and why some cells become post-mitotic in some species has been the subject of much research and speculation, but (as noted above) it is widely believed that cellular senescence evolved as a way to prevent the onset and spread of cancer. Somatic cells that have divided many times will have accumulated DNA mutations and would therefore be in danger of becoming cancerous if cell division continued.

Lately the role of telomeres in cellular senescence has aroused general interest, especially with a view to the possible genetically adverse effects of cloning. The successive shortening of the chromosomal telomeres with each cell cycle is also believed to limit the number of divisions of the cell, thus contributing to ageing. There have, on the other hand, also been reports that cloning could alter the shortening of telomeres. Some cells do not age and are therefore described as being "biologically immortal." It is theorized by some that when it is discovered exactly what allows these cells, whether it be the result of telomere lengthening or not, to divide without limit that it will be possible to genetically alter other cells to have the same capability. It is further theorized that it will eventually be possible to genetically engineer all cells in the human body to have this capability by employing gene therapy and thereby stop or reverse ageing, effectively making the entire organism potentially immortal.

Cancer cells are usually immortal. This evasion of cellular senescence is the result, in about 85% of tumors, of up-activation of their telomerase genes. This simple observation suggests that reactivation of telomerases in healthy individuals could greatly increase their cancer risk.

Whether cell senecence plays any role in organismal aging is at present unknown, and is an active area of investigation. Mice lacking telomerase do not immediately show accelerated aging.

3.4 Chemical damage: Earliest ageing theory was the Rate of Living Hypothesis described by Raymond Pearl in 1928, based on the idea that fast basal metabolic rate corresponds to short maximum life span (much as a rapidly running machine will experience more damage from wear). (The idea had been posited earlier by Max Rubner).

While there is likely some validity to this theory, in the form of various types of specific damage detailed below which, all other things being equal may reduce lifespan, in general this theory does not adequately explain the differences in lifespan either within, or between, species. Calorically-restricted animals process as much, or more, calories per gram of body mass, as their ad libitum fed counterparts, yet exhibit substantially longer lifespans. Similarly, metabolic rate is a poor predictor of lifespan for birds, bats and other species which presumably have reduced mortality from predation, and therefore have evolved long lifespans even in the presence of very high metabolic rates.

With respect to specific types of chemical damage caused by metabolism, it is suggested that damage to long-lived biopolymers, such as structural proteins or DNA, caused by ubiquitous chemical agents in the body such as oxygen and sugars, are in part responsible for ageing. The damage can include breakage of biopolymer chains, cross-linking of biopolymers, or chemical attachment of unnatural substituents (haptens) to biopolymers.

Under normal aerobic conditions, approximately 4% of the oxygen metabolized by mitochondria is converted to superoxide ion which can subsequently be converted to hydrogen peroxide, hydroxyl radical and eventually other reactive species including other peroxides and singlet oxygen, which can in turn generate free radicals capable of damaging structural proteins and DNA. Certain metal ions found in the body, such as copper and iron, may participate in the process. (In Wilson's disease, a hereditary defect which causes the body to retain copper, some of the symptoms resemble accelerated senescence.) These processes are termed oxidative damage and are linked to the benefits of nutritionally derived polyphenol antioxidants.

Sugars such as glucose and fructose can react with certain amino acids such as lysine and arginine and certain DNA bases such as guanine to produce sugar adducts, in a process called glycation. These adducts can further rearrange to form reactive species which can then cross-link the structural proteins or DNA to similar biopolymers or other biomolecules such as non-structural proteins. People with diabetes, who have elevated blood sugar, develop senescence-associated disorders much earlier than the general population, but can delay such disorders by rigorous control of their blood sugar levels. There is evidence that sugar damage is linked to oxidant damage in a process termed glycoxidation.

Free radicals can damage proteins, lipids or DNA. Glycation mainly damages proteins. Damaged proteins and lipids accumulate in lysosomes as lipofuscin. Chemical damage to structural proteins can lead to loss of function; for example, damage to collagen of blood vessel walls can lead to vessel-wall stiffness and thus hypertension, and vessel wall thickening and reactive tissue formation (atherosclerosis); similar processes in the kidney can lead to renal failure. Damage to enzymes reduces cellular functionality. Lipid peroxidation of the inner mitochondrial membrane reduces the electric potential and the ability to generate energy. It is probably no accident that nearly all of the so-called "accelerated ageing diseases" are due to defective DNA repair enzymes.

It is believed that the impact of alcohol on ageing can be partly explained by alcohol's activation of the HPA axis, which stimulates glucocorticoid secretion; long-term exposure to which produces symptoms of ageing.

3.5 Reliability theory: Reliability theory suggests that biological systems start their adult life with a high load of initial damage. Reliability theory is a general theory about systems failure. It allows researchers to predict the age-related failure kinetics for a system of given architecture (reliability structure) and given reliability of its components. Reliability theory predicts that even those systems that are entirely composed of non-ageing elements (with a constant failure rate) will nevertheless deteriorate (fail more often) with age, if these systems

are redundant in irreplaceable elements. Ageing, therefore, is a direct consequence of systems redundancy.

Reliability theory also predicts the late-life mortality deceleration with subsequent leveling-off, as well as the late-life mortality plateaus, as an inevitable consequence of redundancy exhaustion at extreme old ages. The theory explains why mortality rates increase exponentially with age (the Gompertz law) in many species, by taking into account the initial flaws (defects) in newly formed systems. It also explains why organisms "prefer" to die according to the Gompertz law, while technical devices usually fail according to the Weibull (power) law. Reliability theory allows to specify conditions when organisms die according to the Weibull distribution: organisms should be relatively free of initial flaws and defects. The theory makes it possible to find a general failure law applicable to all adult and extreme old ages, where the Gompertz and the Weibull laws are just special cases of this more general failure law. The theory explains why relative differences in mortality rates of compared populations (within a given species) vanish with age (compensation law of mortality), and mortality convergence is observed due to the exhaustion of initial differences in redundancy levels.

3.6. Neuro-endocrine-immunological theories:

Senescence may also simply be a result of wear and tear overwhelming repair mechanisms. It is also possible that senescence is a mechanism to control the development and spread of cancer; if cells have built-in limits to how many times they can replicate, they must somehow overcome this before they can spread indefinitely.

Recently, early senescence has been alleged to be a possible unintended outcome of early cloning experiments. Most notably, the issue was raised in the case of Dolly the sheep, following her death from a contagious lung disease. The claim that Dolly's early death involved premature senescence has been vigorously contested (e.g. by Kerry Lynn Macintosh in her book, Illegal Beings: Human Clones and the Law), and Dolly's creator, Dr. Ian Wilmut has expressed the view that her illness and death were probably unrelated to the fact that she was a clone.

A set of rare hereditary (genetic) disorders, each called progeria, has been known for some time. Sufferers exhibit symptoms resembling accelerated ageing, including wrinkled skin. The cause of Hutchinson–Gilford progeria syndrome was reported in the journal Nature in May 2003. This report suggests that DNA damage, not oxidative stress, is the cause of this form of accelerated ageing.