Developmental Biology NINTH EDITION

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Paraxial and Intermediate Mesoderm

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Of physiology from top to toe I sing,
Not physiognomy alone or brain alone
is worthy for the Muse,
I say the form complete is worthier far,
The Female equally with the Male I sing.

WALT WHITMAN (1867)

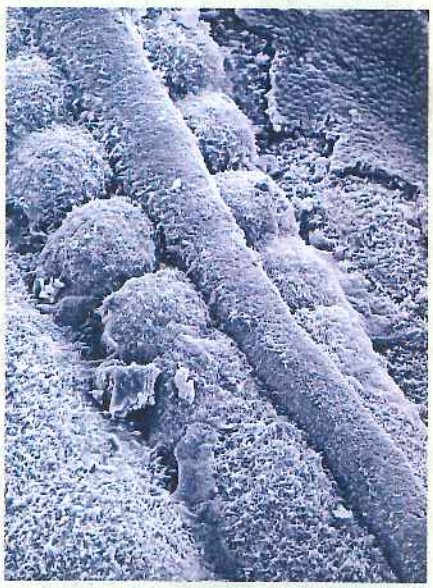
Built of 206 bones, the skeleton is a living cathedral of ivory vaults, ribs, and buttresses—a structure at once light and strong.

NATALIE ANGIER (1994)

IN CHAPTERS 9 AND 10 we followed the various tissues formed by the vertebrate ectoderm. In this chapter and the next, we will follow the development of the mesodermal and endodermal germ layers. We will see that the endoderm forms the lining of the digestive and respiratory tubes, with their associated organs. The mesoderm generates all the organs between the ectodermal wall and the endodermal tissues, as well as helping the ectoderm and endoderm to form their own tissues.

The trunk mesoderm of a neurula-stage embryo can be subdivided into four regions (Figure 11.1A):

1. The central region of trunk mesoderm is the **chordamesoderm**. This tissue forms the notochord, a transient organ whose major functions include inducing the formation of the neural tube and establishing the anterior-posterior body axis. The formation of the notochord on the future dorsal side of the embryo was discussed in Chapters 7 and 8.
2. Flanking the notochord on both sides is the **paraxial, or somitic, mesoderm**. The tissues developing from this region will be located in the back of the embryo, surrounding the spinal cord. The cells in this region will form somites—blocks of mesodermal cells on either side of the neural tube—which will produce muscle and many of the connective tissues of the back (dermis, muscle, and the vertebral and rib cartilage).
3. The **intermediate mesoderm** forms the urogenital system, consisting of the kidneys, the gonads, and their associated ducts. The outer (cortical) portion of the adrenal gland also derives from this region.
4. Farthest away from the notochord, the **lateral plate mesoderm** gives rise to the heart, blood vessels, and blood cells of the circulatory system, as well as to the lining of the body cavities and to all the mesodermal components of the limbs except the muscles. It also helps form a series of extraembryonic membranes that are important for transporting nutrients to the embryo.



These four subdivisions are thought to be specified along the mediolateral (center-to-side) axis by increasing amounts of BMPs (Pourquié et al. 1996; Tonegawa et al. 1997). The more lateral mesoderm of the chick embryo expresses higher levels of BMP4 than do the midline areas, and one can change the identity of the mesodermal tissue by altering BMP expression. While it is not known how this patterning is accomplished, it is thought that the different BMP concentrations may cause differential expression of the Forkhead (Fox) family of transcription factors. *Foxf1* is transcribed in those regions that will become the lateral plate and extraembryonic mesoderm, whereas *Foxc1* and *Foxc2* are expressed in the paraxial mesoderm that will form the somites (Wilm et al. 2004). If *Foxc1* and *Foxc2* are both deleted

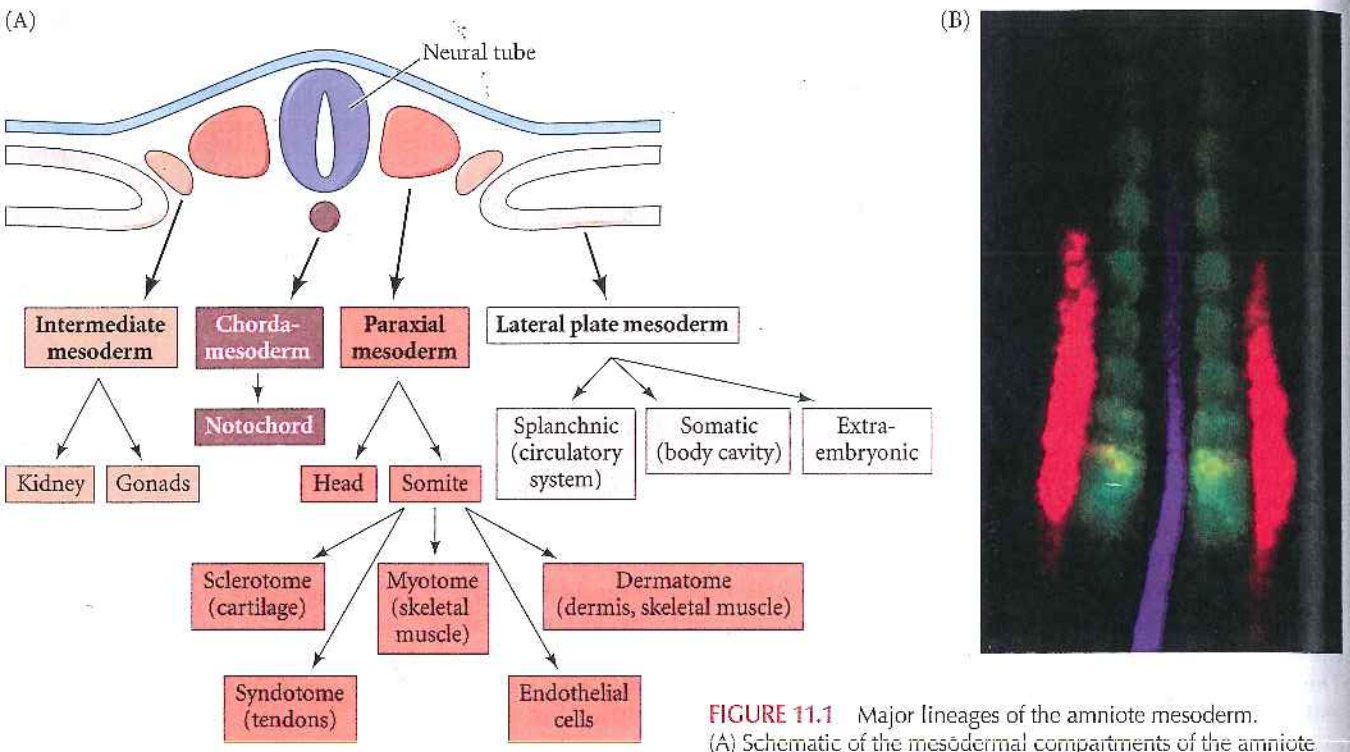


FIGURE 11.1 Major lineages of the amniote mesoderm. (A) Schematic of the mesodermal compartments of the amniote embryo. (B) Staining for the medial mesodermal compartments in the trunk of a 12-somite chick embryo (about 33 hours). In situ hybridization was performed with probes binding to *chordin* mRNA (blue) in the notochord, *paraxis* mRNA (green) in the somites, and *Pax2* mRNA (red) in the intermediate mesoderm. (B from Denkers et al. 2004, courtesy of T. J. Mauch.)

from the mouse genome, the paraxial mesoderm is respecified as intermediate mesoderm and initiates the expression of *Pax2*, which encodes a major transcription factor of the intermediate mesoderm (Figure 11.1B).

Anterior to the trunk mesoderm is a fifth mesodermal region, the **head mesoderm**, consisting of the unsegmented paraxial mesoderm and prechordal mesoderm. This region provides the head mesenchyme that forms much of the connective tissues and musculature of the face and eyes (Evans and Noden 2006). The muscles derived from the head mesoderm form differently than those formed from the somites. Not only do they have their own set of transcription factors, but the head and trunk muscles are affected by different types of muscular dystrophies (Emery 2002; Bothe and Dietrich 2006; Harel et al. 2009).

PARAXIAL MESODERM: THE SOMITES AND THEIR DERIVATIVES

One of the major tasks of gastrulation is to create a mesodermal layer between the endoderm and the ectoderm. As seen in Figure 11.2, the formation of mesodermal and endodermal tissues is not subsequent to neural tube formation but occurs synchronously. The notochord extends beneath the neural tube, from the base of the head into the tail. On either side of the neural tube lie thick bands of mesodermal cells. These bands of paraxial mesoderm are referred to either as the **segmental plate** (in chick embryos) or the **unsegmented mesoderm** (in other vertebrate embryos). As the primitive streak regresses and the neural folds begin to gather at the center of the embryo, the cells of the paraxial mesoderm will form **somites**. The paraxial mesoderm appears to be specified by the antagonism of BMP signaling by the Noggin protein. Noggin is usually synthesized by the early segmental plate mesoderm, and if Noggin-expressing cells are placed into the presumptive lateral plate mesoderm, the lateral plate tissue will be respecified into somite-forming paraxial mesoderm (Figure 11.3; Tonegawa and Takahashi 1998).

The mature somites contain three major compartments: the **sclerotome**, which forms the vertebrae and rib cartilage; the **myotome**, which forms the musculature of the back, rib cage, and ventral body wall; and the **dermamyotome**, which contains skeletal muscle progenitor cells, (including those that migrate into the limbs) and the cells that generate the dermis of the back. In addition, other, smaller compartments are formed from these three. The *syndetome* arising from the most dorsal sclerotome cells generates the tendons, while the most internal cells of the sclerotome (sometimes called the *arthrotome*) become the vertebral joints, the intervertebral discs, and the proximal

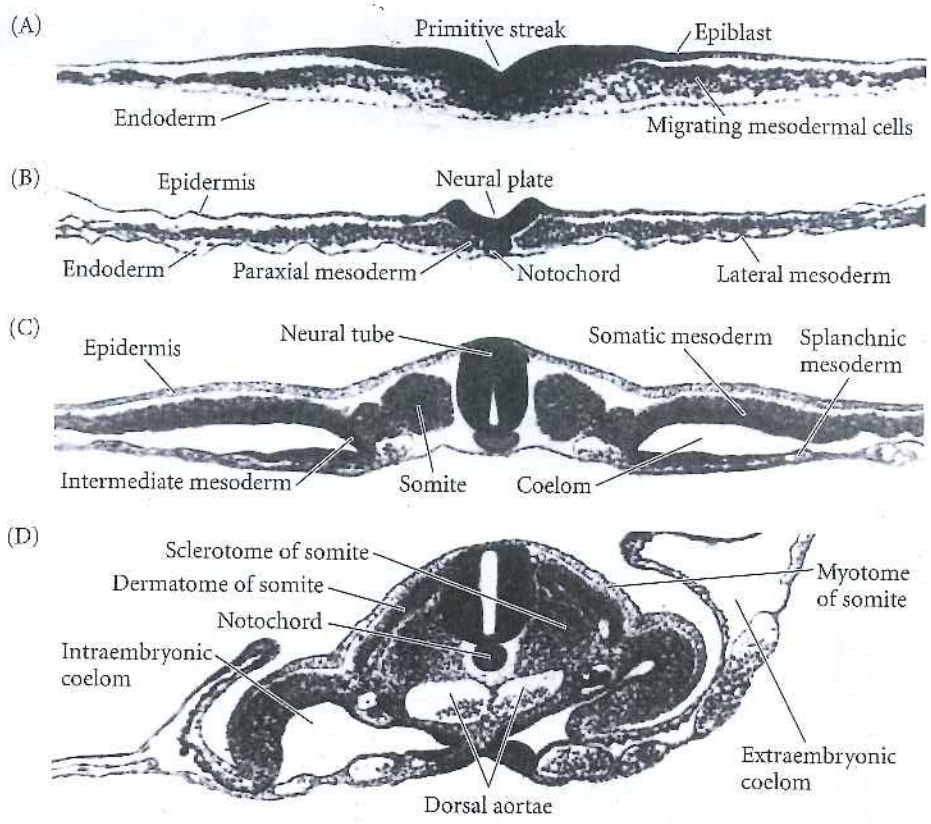


FIGURE 11.2 Gastrulation and neurulation in the chick embryo, focusing on the mesodermal component. (A) Primitive streak region, showing migrating mesodermal and endodermal precursors. (B) Formation of the notochord and paraxial mesoderm. (C,D) Differentiation of the somites, coelom, and the two aortae (which will eventually fuse). A–C, 24-hour embryos; D, 48-hour embryo.

portion of the ribs (Mittapalli et al. 2005; Christ et al. 2007). Moreover, an as-yet unnamed group of cells in the posterior sclerotome generates vascular cells of the dorsal aorta and intervertebral blood vessels (Table 11.1; Pardanaud et al. 1996; Sato et al. 2008).

See VADE MECUM
Mesoderm in the vertebrate embryo

Formation of the Somites

The important components of **somitogenesis** (somite formation) are (1) periodicity, (2) fissure formation (separation of the somites), (3) epithelialization, (4) specification, and (5) differentiation. The first somites appear in the anterior portion of the trunk, and new somites "bud off" from the rostral end of the presomitic mesoderm at regular intervals (Figure 11.4). Somite formation begins as paraxial mesoderm cells become organized into whorls of cells, sometimes called **somitomeres** (Meier 1979).

FIGURE 11.3 Specification of somites. Placing Noggin-secreting cells into a prospective region of chick lateral plate mesoderm will respecify that mesoderm into somite-forming paraxial mesoderm. Induced somites (bracketed) were detected by in situ hybridization with Pax3. (From Tonegawa and Takahashi 1998, courtesy of Y. Takahashi.)

The somitomeres become compacted and split apart as fissures separate them into discrete, immature somites. The mesenchymal cells making up the immature somite now change, with the outer cells joining into an epithelium while the inner cells remain mesenchymal. Because individual embryos in any species can develop at slightly different rates (as when chick embryos are incubated at slightly different temperatures), the number of somites present is usually the best indicator of how far development has proceeded.

TABLE 11.1 Derivatives of the somite

Traditional view	Current view
DERMAMYOTOME	
Myotome forms skeletal muscles	Lateral edges generate primary myotome that forms muscle
Dermatome forms back dermis	Central region forms muscle, muscle stem cells, dermis, brown fat cells
SCLEROTOME	
Forms vertebral and rib cartilage	Forms vertebral and rib cartilage
	Dorsal region forms tendons (syndetome)
	Medial region forms blood vessels and meninges
	Central mesenchymal region forms joints (arthrotome)
	Forms smooth muscle cells of dorsal aorta

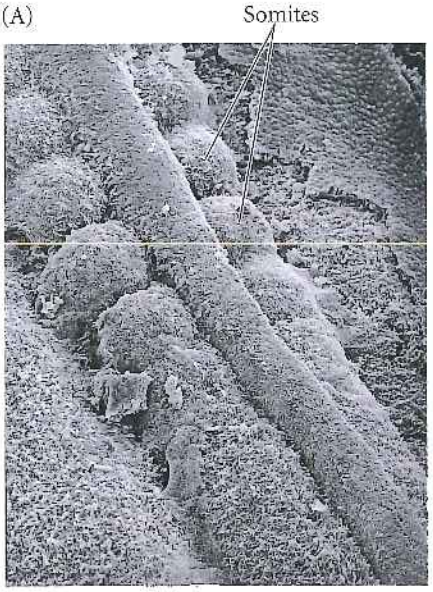
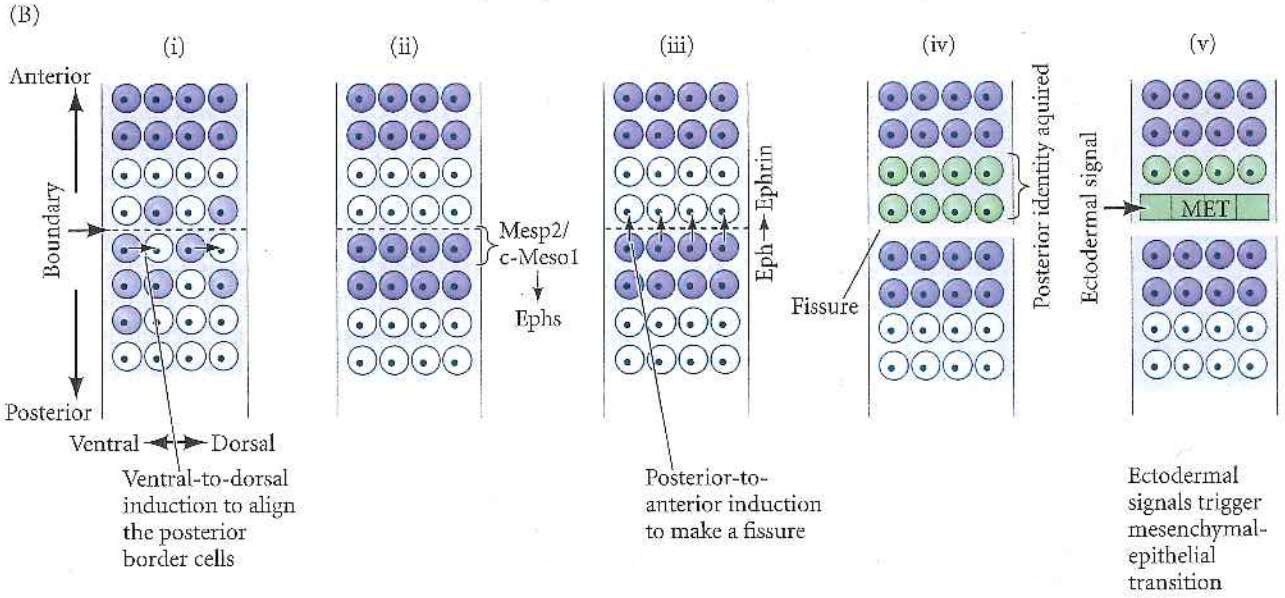


FIGURE 11.4 Formation of new somites. (A) Neural tube and somites seen by scanning electron microscopy. When the surface ectoderm is peeled away, well-formed somites are revealed, as well as paraxial mesoderm (bottom right) that has not yet separated into distinct somites. A rounding of the paraxial mesoderm into a somitomere is seen at the lower left, and neural crest cells can be seen migrating ventrally from the roof of the neural tube. (B) Sequential molecular and cellular events in somitogenesis. (i) At the boundary (determined by Notch signaling), a ventral-to-dorsal signal aligns the posterior border cells (i.e., those cells immediately posterior to the presumptive border. (ii, iii) Mesp2/c-Meso1 induces Ephs in the posterior border cells, and the Ephs induce ephrin in the cells across the border. This creates the fissure. (iv) As the fissure forms, a separate signal aligns the cells that will form the posterior boundary of the somite. (v) Ectodermal signals act on GTPases to coordinate the transition from mesenchymal to epithelial cell, completing the somite. (A courtesy of K. W. Tosney; B after Takahashi and Sato 2008.)



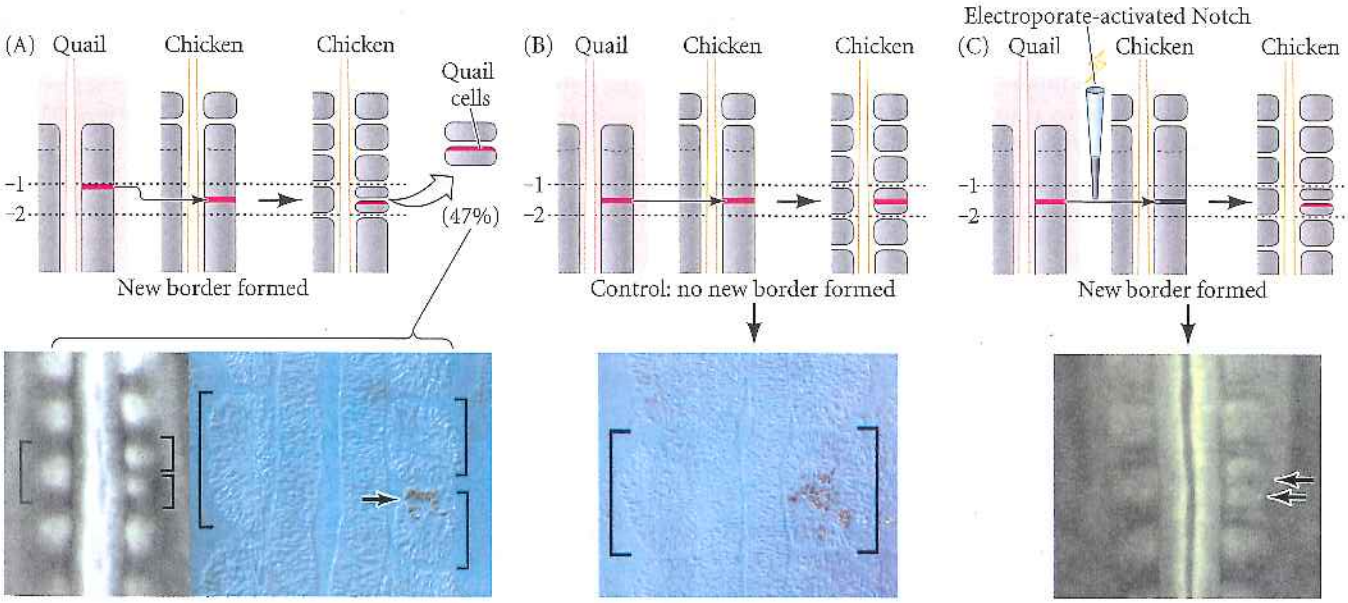
Periodicity of somite formation

Somite formation depends on a "clock and wave" mechanism, in which an oscillating signal (the "clock") is provided by the Notch and Wnt pathways, and a rostral-to-caudal gradient provides a moving "wave" of an FGF that sets the somite boundaries. In the chick embryo, a new somite is formed about every 90 minutes. In mouse embryos, this time frame is more variable (Tam 1981). However, somites appear at exactly the same time on both sides of the embryo, and the clock for somite formation is set when the cells first enter the presomitic mesoderm. If the presomitic mesoderm is inverted such that the caudal end is rostral and the rostral end faces the tail, somite formation will start from the caudal end and proceed rostrally. Even if isolated from the rest of the body, the presomitic mesoderm will segment at the appropriate time and in the right direction (Palmeirim et al. 1997).

Moreover, the number of somites is set at the initial stages of presomitic mesoderm formation. When *Xenopus* or mouse embryos are experimentally or genetically reduced in size, the number of somites remains the same (Tam 1981). The total number of somites formed is characteristic of a species (about 50 in chicks, 65 in mice, and as many as 500 in some snakes).

Where somites form: The Notch pathway

Although we do not completely understand the mechanisms controlling the temporal periodicity of somite formation, one of the key agents in determining where somites form is the Notch signaling pathway (see Aulehla and Pourquié 2008). When a small group of cells from a region constituting the posterior border at the presumptive somite boundary is transplanted into a region of unsegmented mesoderm that would not ordinarily be part of the boundary area, a new boundary is created. The transplanted boundary cells instruct the cells anterior to them to epithelialize and separate. Nonboundary cells will not induce border formation when transplanted to a nonborder area. However, these nonboundary cells can acquire boundary-forming ability if an activated Notch protein is electroporated into those cells (Figure 11.5A–C; Sato et al. 2002). Morimoto and colleagues (2005) have been able to



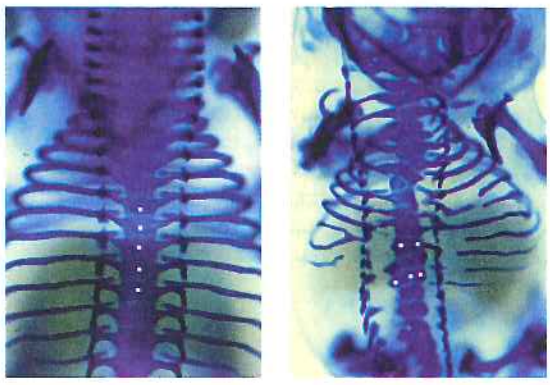


FIGURE 11.5 Notch signaling and somite formation. (A) Transplantation of a prospective somite boundary region into a nonboundary region creates a new boundary and a new somite. The transplanted quail cells can be identified by staining for a quail-specific protein. (B) Transplantation of nonboundary cells into a nonboundary region does not create a new boundary or a new somite. (C) Transplantation of a nonboundary region that has had Notch activated will cause a new somite boundary to occur. (D) Dorsal views of a control mouse and its littermate (E) with the *Delta-like3* gene (the gene encoding a Notch ligand) knocked out. The *Dll3* mutant has several ossification centers (white squares) in rows instead of in a column, and its ribs are malformed. (A–C after Sato et al. 2002, photographs courtesy of Y. Takahashi; D from Dunwoodie et al. 2002, courtesy of S. Dunwoodie.)

visualize the endogenous level of Notch activity in mouse embryos, and have shown that it oscillates in a segmentally defined pattern. The somite boundaries were formed at the interface between the Notch-expressing and Notch-nonexpressing areas.

Notch has also been implicated in somite fissioning by mutations. Segmentation defects have been found in mice that are mutant for important components of the Notch pathway. These include the Notch protein itself, as well as its ligands Delta-like1 and Delta-like3 (Dll1 and Dll3). Mutations affecting Notch signaling have been shown to be responsible for aberrant vertebral formation in mice and humans. In humans, individuals with spondylocostal dysplasia have numerous vertebral and rib defects that have been linked to mutations of the *Delta-like3* gene. Mice with knockouts of *Dll3* have a phenotype similar to that of the human syndrome (Figure 11.5D; Bulman et al. 2000; Dunwoodie et al. 2002).

Moreover, Notch signaling follows a remarkable wavelike pattern wherein the *Notch* gene becomes highly expressed in the posterior region of the forming somite, just anterior to the fissure. *Notch* genes are transcribed in a cyclic fashion and function as an autonomous segmentation "clock" (Palmeirim et al. 1997; Jouve et al. 2000, 2002). If Notch signaling determines the placement of somite formation, then Notch protein must control a cascade of gene expression that ultimately separates the tissues.

This wave can be illustrated by following the expression pattern of *hairy1*, a segmentation gene regulated by Notch activity. It is expressed in the presomitic segmental plate in a cyclic, wavelike manner, cresting every 90 minutes in the chick embryo (Figure 11.6). The caudal domain of the *hairy1* expression pattern rises anteriorly and then recedes like a wave, leaving a band of expression at what will become the posterior half of the somite. The caudal boundary of this domain is exactly where the transplantation experiments showed Notch expression to be important.

One of the most critical genes in somite formation is *Mesp2/c-Meso1* (the first name refers to the mouse homologue, the second to the chicken homologue). This gene is activated by Notch, and its protein product, a transcription factor, initiates the reactions that *suppress* Notch activity. This activation-suppression cycle causes Mesp2/c-Meso1 expression to oscillate in time and space. Wherever it is expressed, that site is the most anterior group of cells in the next somite, and the boundary forms immediately anterior to those cells (see Figure 11.4B). Mesp2/c-Meso1 induces Eph A4 (one of the compounds whose repulsive interaction separates the somites) in the rostral half of the somite (Saga et al. 1997; Watanabe et al. 2005). In the caudal (posterior) half of the somite, Mesp2/c-Meso1 induces the expression of the transcription factor Uncx4.1 (Takahashi et al. 2000; Saga 2007). In this way, the somite boundary is determined, and the somite is given anterior/posterior polarity at the same time. This, as we saw in Chapter 10, is critical for the migration patterns of neural crest cells and neurons.

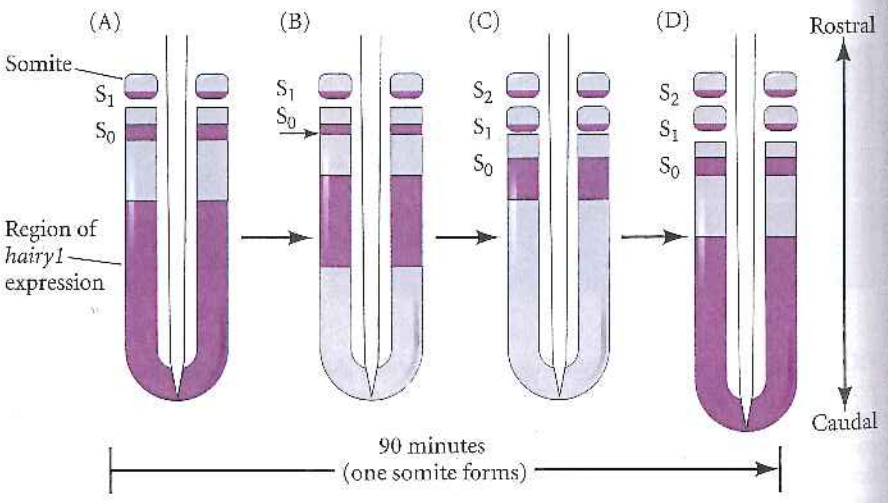


FIGURE 11.6 Somite formation correlates with the wavelike expression of the *hairy1* gene in the chick. (A) In the posterior portion of a chick embryo somite, S_1 has just budded off the presomitic mesoderm. Expression of the *hairy1* gene (purple) is seen in the caudal half of this somite, as well as in the posterior portion of the presomitic mesoderm and in a thin band that will form the caudal half of the next somite (S_0). (B) A caudal fissure (small arrow) begins to separate the new somite from the presomitic mesoderm. The posterior region of *hairy1* expression extends anteriorly. (C) The newly formed somite is now referred to as S_1; it retains the expression of *hairy1* in its caudal half, as the posterior domain of *hairy1* expression moves farther anteriorly and shortens. The former S_1 somite, now called S_2, undergoes differentiation. (D) The formation of somite S_1 is complete, and the anterior region of what had been the posterior *hairy1* expression pattern is now the anterior expression pattern. It will become the caudal domain of the next somite. The entire process takes 90 minutes.

SIDELIGHTS & SPECULATIONS

Coordinating Waves and Clocks in Somite Formation

Acyclic activation of Notch appears to be critical for forming the somites, but what controls Notch activation? The predominant model of somite formation is the clock-and-wavefront model, first proposed by Cooke and Zeeman (1976). In zebrafish, this been found to be relatively simple: the clock involves a negative feedback loop of the Notch signaling pathway. One of the proteins activated by the Notch protein is also able to inhibit *Notch*, which would establish such a negative feedback loop. When the inhibitor is degraded, *Notch* would become active again. Such a cycle would create a "clock" whereby the *Notch* gene would be turned on and off by a protein it itself induces. These off-and-on oscillations could provide the molecular basis for the periodicity of somite segmentation (Holley and Nüsslein-Volhard 2000; Jiang et al. 2000; Dale et al. 2003). More recent reports indicate that the Mesp2/c-Meso1 protein may be such a regulator of *Notch* (Morimoto et al. 2005). The output from this protein controls the ephrins that mediate the separation of the block of cells that form the somite.

The wave appears to be the gradient of Fgf8, which moves caudally as more cells are added to the posterior. As long as the unsegmented paraxial mesenchyme is in a region of relatively high Fgf8, the clock will not function. This appears to be due to the repression of Delta, the protein that is the major ligand of Notch. The binding of Fgf8 to its receptor enables the expression of the Her13.2 protein, which is necessary to inhibit the transcription of Delta (see Dequéant and Pourquié 2008). Interestingly, the Fgf8 signal can be perturbed by the right-left laterality signals being given at the same time, and the presence of retinoic acid is needed to insulate the somite wavefront from the laterality signals of Nodal proteins. If retinoic acid is not made, somitogenesis becomes asymmetric (Kawakami et al. 2005; Brend and Holley 2009).

In chicks and mice, the situation appears to be far more complicated. The Notch pathway still provides the clock, but the clock appears to be sensitive not only to Fgf but also to the Wnt signaling pathway, which also exhibits a posterior-to-anterior gradient and the retinoic acid gradient that extends in the opposite direction. The details of how the clock and wavefront are coordinated in amniotes have not been elucidated and are an extremely active area of research. It may be that the Fgf8 wavefront is merely providing the needed amount of β-catenin that can be localized into the nucleus by Wnt signaling. Alternatively, Fgf signaling may provide another clock whose oscillations form a segment when they coincide with both a Wnt clock and a Notch clock (Figure 11.7). This latter model has been proposed by Goldbeter and Pourquié (2008), who show that this might have the best fit mathematically.

One way to unravel the complex interactions of signaling molecules in amniotes may be to look at embryos that have extreme numbers of somites. Snake embryos, for instance, can have hundreds of somites. The pattern of gene expression in corn snake embryos indicates that they have the same Fgf8 and Wnt waves and Notch clockwork as chicks and mice; but the snake somite clock is four times faster relative to the embryo's growth rate, rapidly segregating off blocks of presomitic mesenchyme into more numerous, albeit smaller, somites (Gomez et al. 2008). If we can elucidate the alterations that are involved in regulating the speed of this clock, we might be able to figure out how the signaling pathways become integrated to give a single output at specific temporal and spatial intervals.

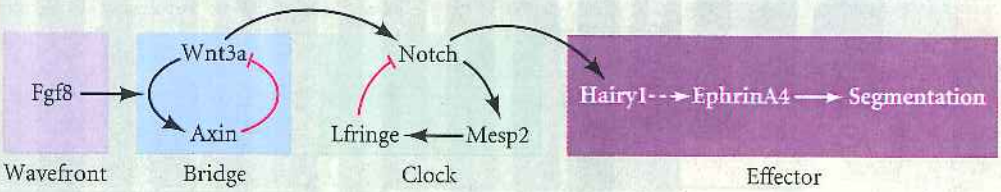


Figure 11.7 Hypothetical pathway for regulation of the clock through which an Fgf8 gradient regulates a Wnt oscillating clock, which in turn controls a Notch clock that can inhibit its own activity in a negative feedback loop. Different species might use different molecules in such a scheme.

Separation of somites from the unsegmented mesoderm

Two proteins whose roles appear to be critical for fissure formation and somite separation are the Eph tyrosine kinases and their ligands, the ephrin proteins. We saw in Chapter 10 that the Eph tyrosine kinase receptors and their ephrin ligands are able to elicit cell-cell repulsion between the posterior somite and migrating neural crest cells. The separation of the somite from the presomitic mesoderm occurs at the ephrin B2/Eph A4 border. In the zebrafish, the boundary between the most recently separated somite and the presomitic mesoderm forms between ephrin B2 in the posterior of the somite and Eph A4 in the most anterior portion of the presomitic mesoderm (Figure 11.8; Durbin et al. 1998). Eph A4 is restricted to the boundary area in chick embryos as well. Interfering with this signaling (by injecting embryos with mRNA encoding dominant negative Ephs) leads to the formation of abnormal somite boundaries.

In addition to the posterior-to-anterior induction of the fissure (from Eph proteins to ephrin proteins on their neighboring cells), a second signal originates from the ventral posterior cells of the somite, putting all the cells in register so that the cut is clean from the ventral to the doral aspects of the somite (Sato and Takahashi 2005).

Epithelialization of the somites

Several studies in the chick have shown that epithelialization occurs immediately after somitic fission occurs. As seen in Figure 11.4A, the cells of the newly formed somite are randomly organized as a mesenchymal mass. These cells have to be compacted into an outer epithelium and an internal mesenchyme (Figure 11.9). Ectodermal signals appear to cause the peripheral somitic cells to undergo mesenchymal-to-epithelial transition by lowering the Cdc42 levels in these cells. Low Cdc42 levels alter the cytoskeleton, allowing epithelial cells to form a box around the remaining mesenchymal cells, which have a higher level of Cdc42. Another small GTPase, Rac1, must be at a certain level that allows it to activate Paraxis, another transcription factor involved in epithelialization (Burgess et al. 1995; Barnes et al. 1997; Nakaya et al. 2004).

The epithelialization of each somite is stabilized by synthesis of the extracellular matrix protein fibronectin and the adhesion protein N-cadherin (Lash and Yamada 1986; Hatta et al. 1987; Saga et al. 1997; Linask et al. 1998). N-cadherin links the adjoining cells into an epithelium, while the fibronectin matrix acts alongside the Ephrin and Eph to promote the separation of the somites from each other (Martins et al. 2009).

Somite specification along the anterior-posterior axis

Although all somites look identical, they will form different structures. For instance, the somites that form the cervical vertebrae of the neck and lumbar vertebrae of the abdomen are not capable of forming ribs; ribs are generated only by the somites forming the thoracic vertebrae. Moreover, specification of the thoracic vertebrae occurs

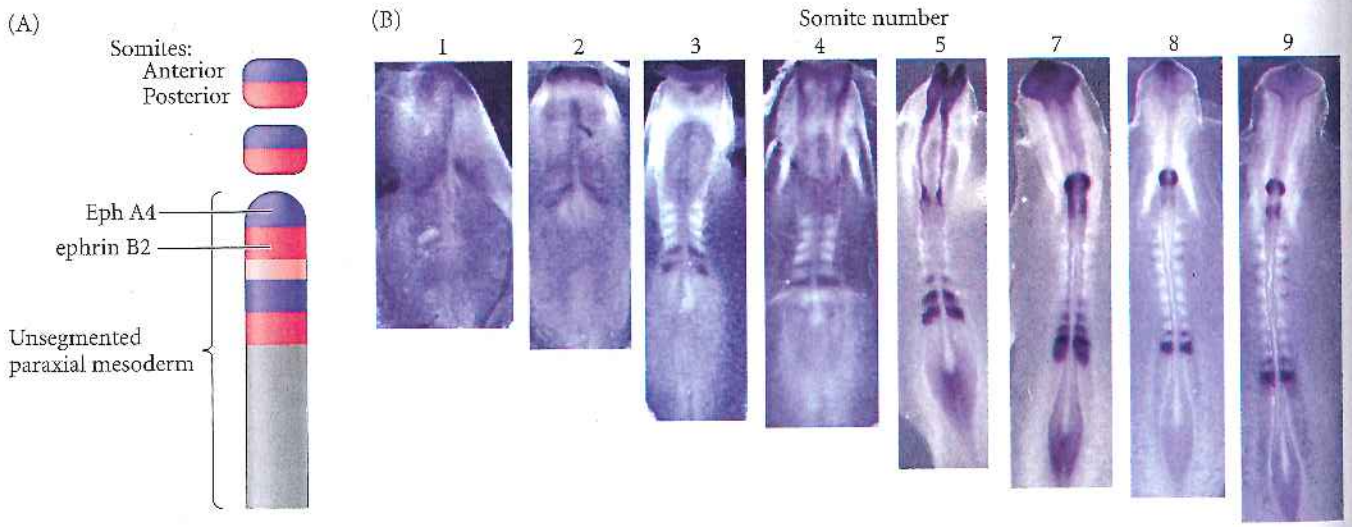


FIGURE 11.8 Ephrin and its receptor constitute a possible fissure site for somite formation. (A) Expression pattern of the receptor tyrosine kinase Eph A4 (blue) and its ligand, ephrin B2 (red), as somites develop. The somite boundary forms at the junction between the region of ephrin expression on the posterior of the last somite formed and the region of Eph A4 expression on the anterior of the next somite to form. In the presomitic mesoderm, the pattern is created anew as each somite buds off. The posteriormost region of the next somite to form does not express ephrin until that somite is ready to separate. (B) In situ hybridization showing Eph A4 (dark blue) expression as new somites form in the chick embryo. (A after Durbin et al. 1998; B courtesy of J. Kastner.)

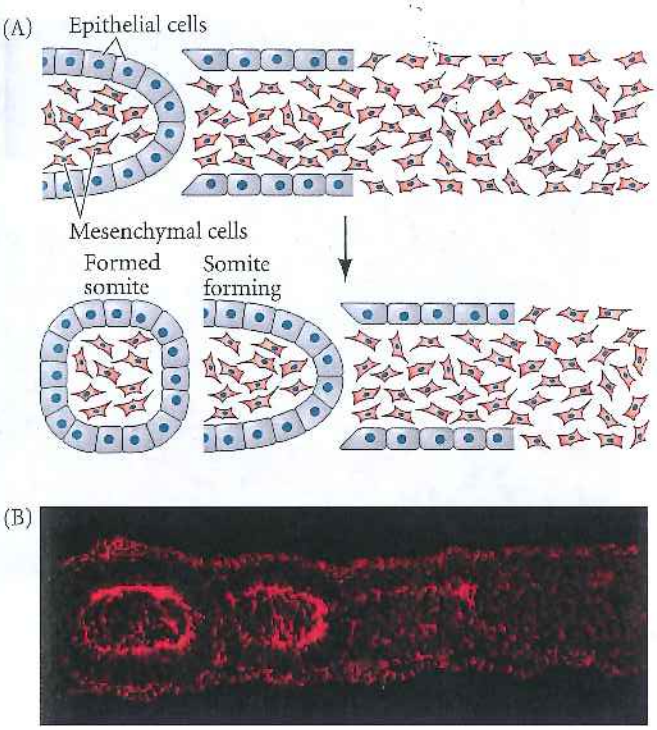


FIGURE 11.9 Epithelialization and de-epithelialization in somites of a chick embryo. (A) Changes in cell shape from mesenchymal (pink) to epithelial (gray) cells when a somite forms from presomitic mesenchyme. A formed somite is surrounded by epithelial cells, with mesenchymal cells remaining inside. In chickens, epithelialization occurs first at the posterior edge of the somite, with the anterior edge becoming epithelial later. (B) Changes in cell polarity as somites form are revealed by staining that visualizes F-actin accumulation (red). (After Nakaya et al. 2004; B courtesy of Y. Takahashi.)

very early in development. The segmental plate mesoderm is determined by its position along the anterior-posterior axis before somitogenesis. If one isolates the region of chick segmental plate that will give rise to a thoracic somite and transplants this mesoderm into the cervical (neck) region of a younger embryo, the host embryo will develop ribs in its neck—but only on the side where the thoracic mesoderm has been transplanted (Figure 11.10; Kieny et al. 1972; Nowicki and Burke 2000).

The somites are specified according to the Hox genes they express (see Chapter 8). These Hox genes are active in the segmental plate mesoderm before it becomes organized into somites (Carapuço et al. 2005). Mice that are homozygous for a loss-of-function mutation of *Hoxc8* convert a lumbar vertebra into an extra thoracic vertebra, complete with ribs (see Figure 8.32). The Hox genes are activated concomitantly with somite formation, and the embryo appears to "count somites" in setting the expression boundaries of the Hox genes. If Fgf8 levels are manipulated to create extra (albeit smaller) somites, the appropriate Hox gene expression will be activated in the appropriately numbered somite, even if it is in a different position along the anterior-posterior axis. Moreover, when mutations affect the autonomous segmentation clock, they also affect the activation of the appropriate Hox genes (Dubrulle et al. 2001; Zakany et al. 2001). Once established, each somite retains its pattern of Hox gene expression, even if that somite is transplanted into another region of the embryo (Nowicki and Burke 2000). The regulation of the Hox genes by the segmentation clock should allow coordination between the formation and the specification of the new segments.

Differentiation of the somites

In contrast to the early commitment of the presomitic segmental plate mesoderm along the anterior-posterior axis, the commitment of the cells *within* a somite occurs relative-

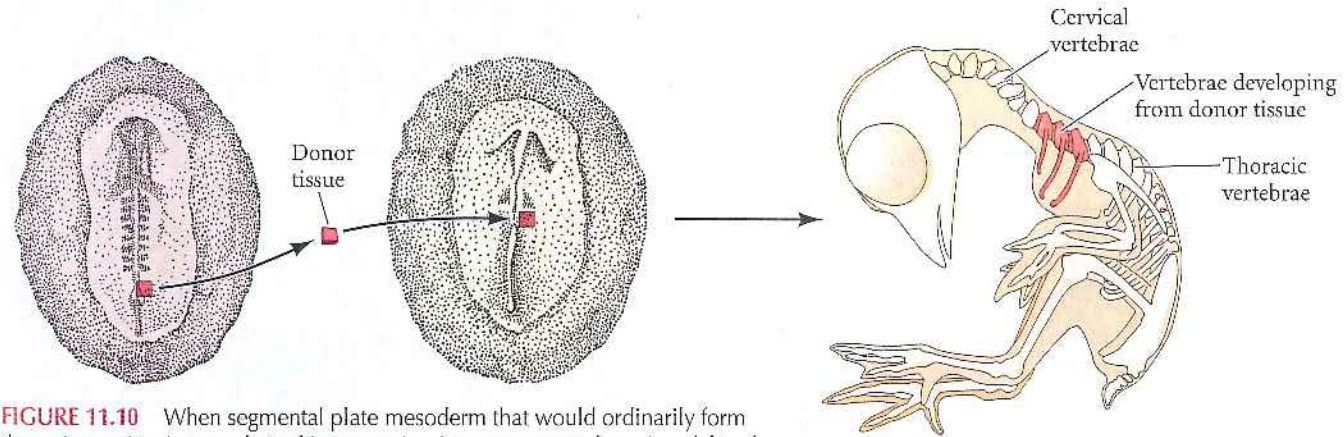
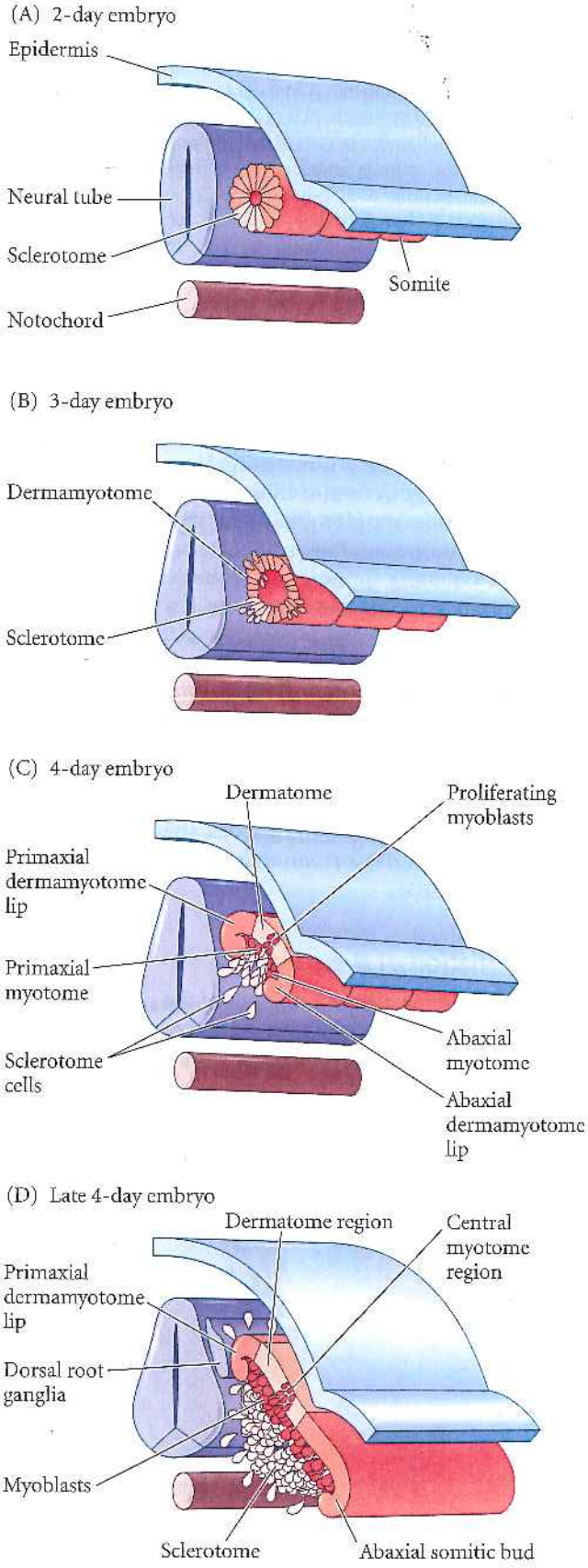


FIGURE 11.10 When segmental plate mesoderm that would ordinarily form thoracic somites is transplanted into a region in a younger embryo (caudal to the first somite) that would ordinarily give rise to cervical (neck) somites, the grafted mesoderm differentiates according to its original position and forms ribs in the neck. (After Kieny et al. 1972.)



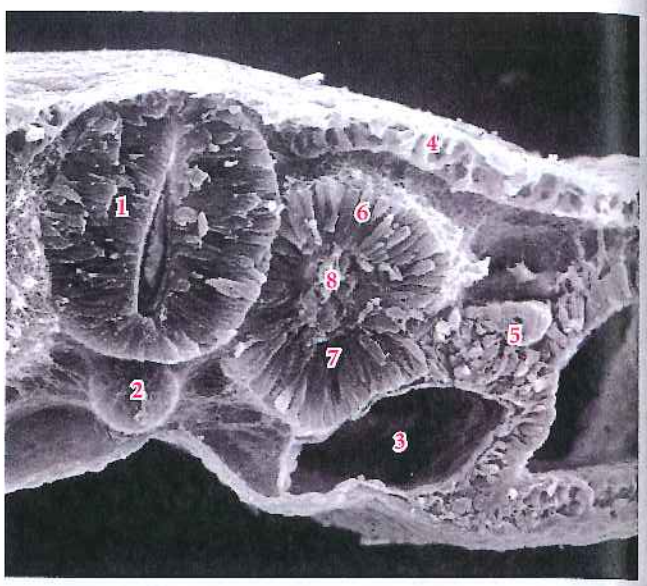
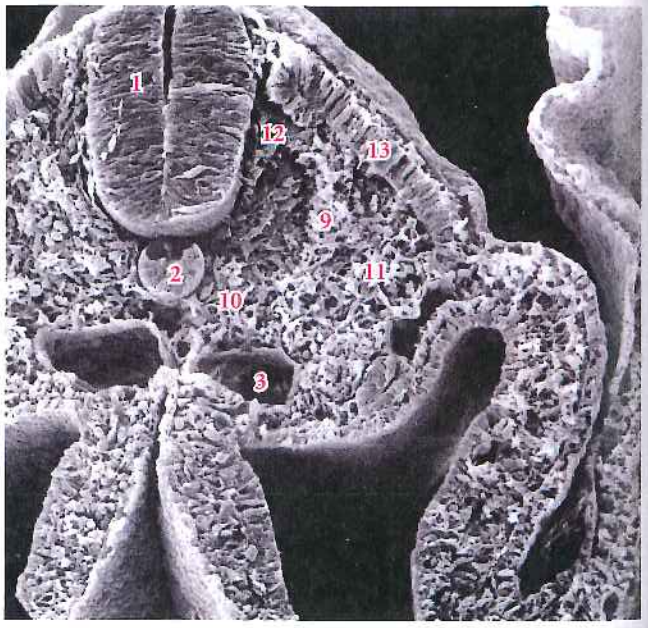


FIGURE 11.11 Transverse section through the trunk of a chick embryo on days 2–4. (A) In the 2-day somite, the sclerotome cells can be distinguished from the rest of the somite. (B) On day 3, the sclerotome cells lose their adhesion to one another and migrate toward the neural tube. (C) On day 4, the remaining cells divide. The medial cells form a primaxial myotome beneath the dermamyotome, while the lateral cells form an abaxial myotome. (D) A layer of muscle cell precursors (the myotome) forms beneath the epithelial dermamyotome. (E,F) Scanning electron micrographs correspond to (A) and (D), respectively; 1, neural tube; 2, notochord; 3, dorsal aorta; 4, surface ectoderm; 5, intermediate mesoderm; 6, dorsal half of somite; 7, ventral half of somite; 8, somitocoel/arthrotome; 9, central sclerotome; 10, ventral sclerotome; 11, lateral sclerotome; 12, dorsal sclerotome;. 13, dermamyotome. (A,B after Langman 1981; C,D after Ordahl 1993; E,F from Christ et al. 2007, courtesy of H. J. Jacob and B. Christ.)



of its cells can become any of the somite-derived structures. These structures include:

- The cartilage of the vertebrae and ribs
- The muscles of the rib cage, limbs, abdominal wall, back, and tongue
- The tendons that connect the muscles to the bones
- The dermis of the dorsal skin
- Vascular cells that contribute to the formation of the aorta and the intervertebral blood vessels
- The cellular sheaths, or *meninges*, of the spinal cord that protect the central nervous system

Thus, the somite contains a population of multipotent cells whose specification depends on their location within the somite. Paracrine factors from the surrounding tissues (neural tube, notochord, epidermis, and intermediate mesoderm) will have profound influences on the regions of the somite adjacent to them. As the somite matures, its various regions become committed to forming only certain cell types. The ventral-medial cells of the somite (those cells closest to the neural tube and notochord) undergo mitosis, lose their round epithelial characteristics, and become mesenchymal cells again. The portion of the somite that gives rise to these cells is the sclerotome, and these mesenchymal cells ultimately become the cartilage cells (chondrocytes) of the vertebrae and a major part of each rib (Figure 11.11A,B; see also Figure 11.2).

The remaining epithelial portion of the somite is the dermamyotome. Fate mapping with chick-quail chimeras (Ordahl and Le Douarin 1992; Brand-Saberi et al. 1996; Kato and Aoyama 1998) has revealed that the dermamyotome is arranged into three regions (Figure 11.11C,D). The cells in the two lateral portions of this epithelium (i.e., the dorsomedial and ventrolateral lips closest to and farthest from the neural tube, respectively) are the myotomes and will form muscle cells. The central region, the dermatome, will form back dermis and several other derivatives. In the lateral myotomes, muscle precursor cells will migrate beneath the dermamyotome to produce a lower layer of muscle precursor cells, the **myoblasts**. Those myoblasts in the myotome closest to the neural tube form the centrally located **primaxial muscles**,* which include the intercostal musculature between the ribs and the deep muscles of the back; those myoblasts formed in the region farthest from the neural tube produce the **abaxial muscles** of the body wall, limbs, and tongue (Figure 11.12). The boundary between the primaxial and abaxial muscles and between the somite-derived and lateral plate-derived dermis is called the **lateral somitic frontier** (Christ and Ordahl 1995; Burke and Nowicki 2003; Nowicki et al. 2003.) Various transcription factors distinguish the primaxial and abaxial muscles.

The central portion of the dermamyotome has traditionally been called the **dermatome**, since its major prod-

*The terms *primaxial* and *abaxial* are used here to designate the muscles from the medial and lateral portions of the somite, respectively. The terms *epaxial* and *hypaxial* are commonly used, but these terms are derived from secondary modifications of the adult anatomy (the hypaxial muscles being innervated by the ventral regions of the spinal cord) rather than from the somitic myotome lineages.

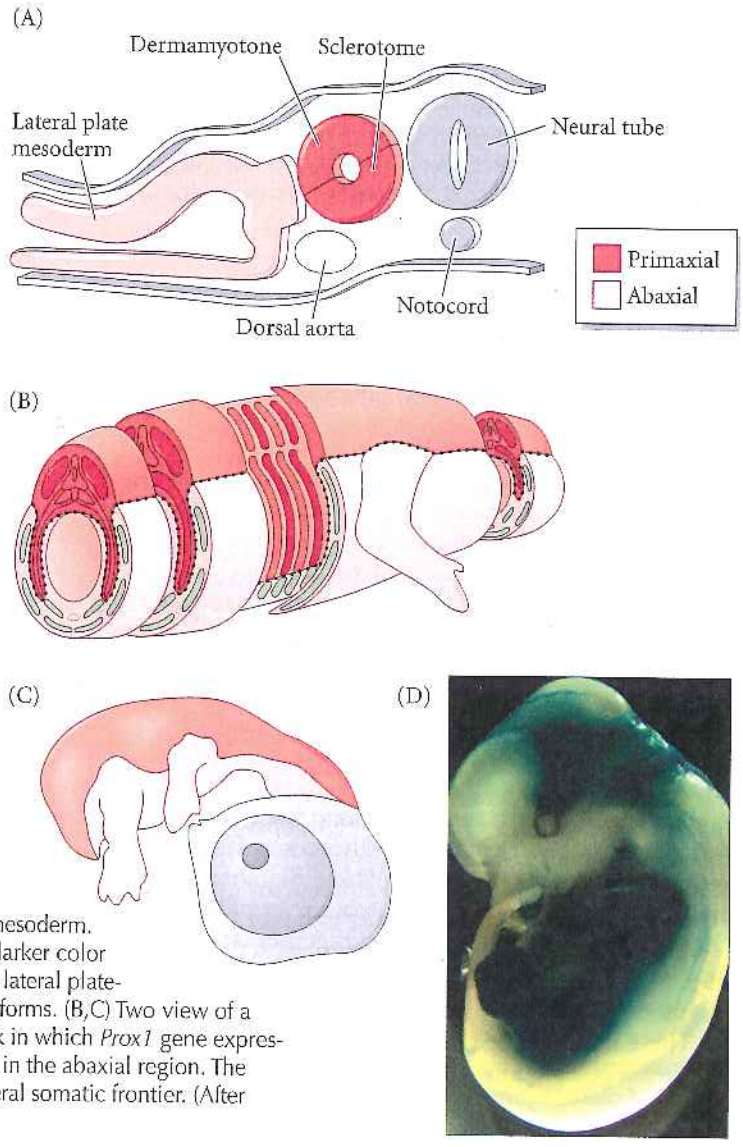


FIGURE 11.12 Primaxial and abaxial domains of vertebrate mesoderm. (A–C) Color-coded diagrams of chick development where the darker color represents somite-derived mesoderm and the lighter represents lateral plate-derived mesoderm. (A) Early-stage chick embryo as mesoderm forms. (B,C) Two view of a day 9 chick embryo, showing regionalization. (D) Day 13 chick in which *Prox1* gene expression is shown by dark stain. In the trunk, this gene is expressed in the abaxial region. The boundary between the stained and unstained regions is the lateral somatic frontier. (After Winslow et al. 2007; D courtesy of A. C. Burke.)

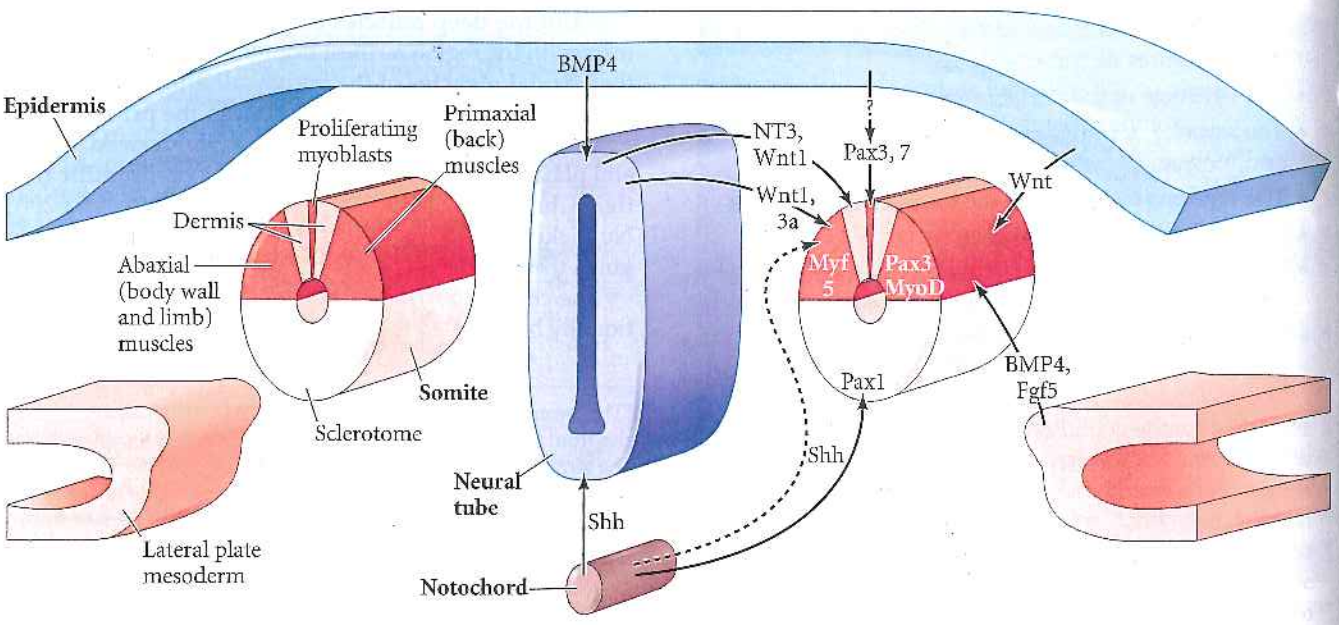


FIGURE 11.13 Model of major postulated interactions in the patterning of the somite. A combination of Wnts (probably Wnt1 and Wnt3a) is induced by BMP4 in the dorsal neural tube. These Wnt proteins, in combination with low concentrations of Sonic hedgehog from the notochord and floor plate, induce the primaxial myotome, which synthesizes the myogenic transcription factor Myf5. High concentrations of Shh induce Pax1 expression in those cells fated to become the sclerotome. Certain concentrations of neurotrophin-3 (NT3) from the dorsal neural tube appear to specify the dermatome, while Wnt proteins from the epidermis, in conjunction with BMP4 and Fgf5 from the lateral plate mesoderm, are thought to induce the primaxial myotome. (After Cossu et al. 1996b.)

uct is the precursors of the dermis of the back. (The dermis of the ventral portion of the body is derived from the lateral plate, and the dermis of the head and neck comes, at least in part, from the cranial neural crest.) However, recent studies have shown that this central region of the dermamyotome also gives rise to a third population of muscle cells (Gros et al. 2005; Relaix et al. 2005). Therefore, some researchers (Christ and Ordahl 1995; Christ et al. 2007) prefer to retain the term *dermamyotome* (or *central dermamyotome*) for this epithelial region. Soon, however, this part of the somite also undergoes an epithelial-to-mesenchymal transition (EMT). FGF signals from the myotome activate the transcription of the *Snail* gene, whose product is a well-known regulator of EMT (see Chapters 3 and 10; Delfini et al. 2009). During this EMT, the mitotic spindles of these epithelial cells are realigned so that cell division takes place along the dorsal-ventral axis. One daughter cell enters the ventral region and becomes part of the myotome, while the other daughter cell locates dorsally to become a precursor of the dermis. The N-cadherin holding the cells together is downregulated and the cells go their separate ways, with the remaining N-cadherin found only on the cells entering the myotome (Ben-Yair and Kalcheim 2005).

These muscle precursor cells that delaminate from the epithelial plate to join the primary myotome cells remain undifferentiated, and they proliferate rapidly to account for most of the myoblast cells. While most of these progenitor cells differentiate to form muscles, some remain undifferentiated and surround the mature muscle cells. These undifferentiated cells become the *satellite cells* responsible for postnatal muscle growth and muscle repair.

Determination of the sclerotome

Like the proverbial piece of real estate, the destiny of a somitic region depends on three things: location, location, and location. As shown in Figure 11.13, the locations of the somitic regions place them close to different signaling centers such as the notochord (source of Sonic hedgehog and Noggin), neural tube (source of Wnts and BMPs), and surface epithelium (also a source of Wnts and BMPs).

The specification of the somite is accomplished by the interaction of several tissues. The ventromedial portion of the somite is induced to become the sclerotome by paracrine factors (especially Noggin and Sonic hedgehog) secreted from the notochord (Fan and Tessier-Lavigne 1994; Johnson et al. 1994). If portions of the notochord are transplanted next to other regions of the somite, those regions will also become sclerotome cells. Paracrine factors induce the presumptive sclerotome cells to express the transcription factor Pax1, which is required for their epithelial-to-

mesenchymal transition and subsequent differentiation into cartilage (Smith and Tuan 1996). In this EMT, the epithelial cells lose N-cadherin expression and become motile (Sosic et al. 1997). Sclerotome cells also express I-mf, an inhibitor of the myogenic (muscle-forming) family of transcription factors (Chen et al. 1996).

The sclerotome contains several regions, each of which becomes specified according to its location. While most sclerotome cells become the prescursors of the vertebral and rib cartilage, the medial sclerotome cells closest to the neural tube generate the meninges (coverings) of the spinal cord as well as giving rise to blood vessels that will provide the spinal cord with nutrients and oxygen (Halata et al. 1990; Nimmagadda et al. 2007). The cells in the center of the somite (which remain mesenchymal) also contribute to the sclerotome, becoming the vertebral joints, the intervertebral discs, and the portions of the ribs closest to the vertebrae (Mittapalli et al. 2005; Christ et al. 2007). This region of the somite has been called the **arthrotome**.

The notochord, with its secretion of Sonic hedgehog, is critical for sclerotome formation. Moreover, we will also see that it produces compounds that direct the migration of sclerotome cells to the center of the embryo to form the vertebrae. But what happens to the notochord, that central mesodermal structure that induced the nervous system and caused the sclerotome to form? After it has provided the axial integrity of the early embryo and has induced the formation of the dorsal neural tube, most of it degenerates by apoptosis. This apoptosis is probably signaled by mechanical forces. Wherever the sclerotome cells have formed a vertebral body, the notochordal cells die. However, between the vertebrae, the notochordal cells form part of the intervertebral discs, the nuclei pulposi (Aszódi et al. 1998; Guehring et al. 2009). These are the spinal discs that "slip" in certain back injuries.

Determination of the central dermamyotome

The central dermamyotome generates muscle precursors as well as the dermal cells that constitute the connective tissue layer of the *dorsal* skin. The dermis of the *ventral* and *lateral* sides of the body is derived from the lateral plate mesoderm that forms the body wall. There is a sharp demarcation between the somite- and lateral plate-derived dermis. This corresponds to the lateral somitic frontier (see Figure 11.12; Nowicki et al. 2003; Shearman and Burke 2009), a boundary that may have medical importance for the spread of skin diseases (such as viruses that cause rashes only in the chest and belly but not in the back).

The maintenance of the central dermamyotome depends on Wnt6 coming from the epidermis (Christ et al. 2007), and its epithelial-to-mesenchymal transition appears to be regulated by two factors secreted by the neural tube: neurotrophin-3 (NT3) and Wnt1. Antibodies that block the activities of NT3 prevent the conversion of epithelial dermatome into the loose dermal mesenchyme that migrates beneath the epidermis (Brill et al. 1995). Removing or rotating the neural tube prevents this dermis from forming (Takahashi et al. 1992; Olivera-Martinez et al. 2002). The Wnt signals from the epidermis promote the differentiation of the dorsally migrating central dermamyotome cells into dermis (Atit et al. 2006).

But muscle precursor cells and dermal cells are not the only derivatives of the central dermamyotome. Atit and her colleagues (2006) have shown that **brown adipose cells** ("brown fat") are also somite-derived and appear to come from the central dermamyotome. Brown fat plays active roles in energy utilization by burning fat (unlike the better known adipose tissue, or "white fat," which stores fat). Tseng and colleagues (2008) have found that skeletal muscle and brown fat cells share the same somitic precursor that originally expresses *bHLH proteins*. In brown fat precursor cells, the transcription factor PRDM16 is induced (probably by BMP7); PRDM16 appears to be critical for the conversion of myoblasts to brown fat cells, as it activates a battery of genes that are specific for the fat-burning metabolism of brown adipocytes (Kajimura et al. 2009).

Determination of the myotome

All the skeletal musculature in the vertebrate body (with the exception of the head muscles) comes from the dermamyotome of the somite. In similar ways, the myotome is induced in two different places by at least two distinct signals. Studies using transplantation and knockout mice indicate that the primaxial myoblasts coming from the medial portion of the somite are induced by factors from the neural tube—probably Wnt1 and Wnt3a from the dorsal region and low levels of Sonic hedgehog from the ventral region (Münsterberg et al. 1995; Stern et al. 1995; Borycki et al. 2000). The abaxial myoblasts coming from the lateral edge of the somite are probably specified by a combination of Wnt proteins from the epidermis and signals from the lateral plate mesoderm (see Figure 11.13; Cossu et al. 1996a; Pourquié et al. 1996; Dietrich et al. 1998). These proteins, which probably include Scatter factor (a protein that induces epithelial-to-mesenchymal transition), cause the myoblasts to migrate away from the dorsal region and delay their differentiation until they are in a more ventral position. These myoblasts are also the cells that form the muscles of the limb (Chapter 13).

In addition to positive signals, there are inhibitory signals preventing the positive signals from affecting an inappropriate group of cells. For example, Sonic hedgehog and Noggin both inhibit BMP4 (Marcelle et al. 1997). Indeed, one model of myotome determination holds that the two conditions needed to produce muscle precursors in the somite are (1) the presence of Wnt signals and (2) the absence of BMPs (Reshef et al. 1998). Recent studies (Gerhart et al. 2006) have traced the development of these BMP-inhibiting centers to cells that arise prior to somite formation. They found a small population of surface epiblast cells

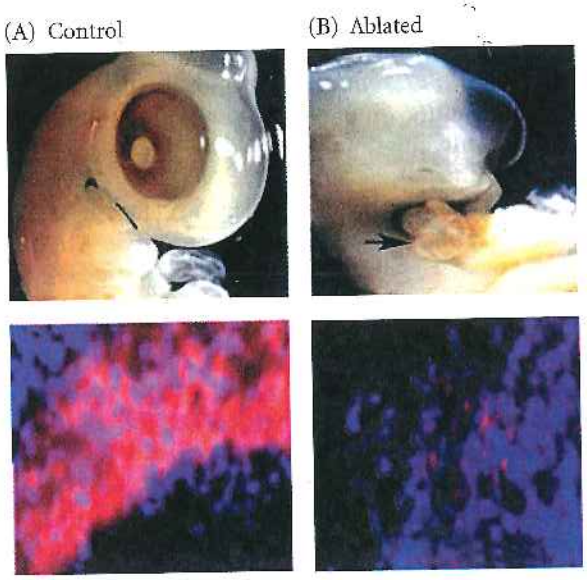


FIGURE 11.14 Ablating Noggin-secreting epiblast cells results in severe muscle defects. Noggin-secreting epiblast cells were ablated in Stage 2 chick embryos using antibodies against G8. (A) The control embryo has normal morphology and abundant staining of myosin (lower photograph) in the muscles. (B) Embryos whose Noggin-secreting epiblast cells are ablated have severe eye defects, severely reduced somatic musculature, and the herniation of abdominal organs through the thin abdominal wall. Severely reduced musculature (sparse myosin in lower photograph) is characteristic of these embryos. (From Gerhart et al. 2006, courtesy of J. Gerhart and M. George-Weinstein.)

that express the mRNA for MyoD but do not translate this message into protein. These particular cells migrate to become paraxial mesoderm and specifically sort out to the dorsomedial and ventrolateral lips of the dermamyotome. There, they synthesize and secrete the BMP inhibitor Noggin to promote the differentiation of myoblasts. If these particular cells are removed from the epiblast, there is a decrease in the skeletal musculature throughout the body, and the ventral body wall is so weak that the heart and abdominal organs often are herniated through it (Figure 11.14). (This defect can be prevented by implanting Noggin-releasing beads into the somites lacking these cells.)

See WEBSITE 11.1
Calling the competence of the somite into question

See WEBSITE 11.2 Cranial paraxial mesoderm

Myogenesis: The Generation of Muscle

Myogenic bHLH proteins

What do these Wnt signals activate in the absence of BMPs? As we have seen, muscle cells come from two cell lineages in the somite, the primaxial and the abaxial. In both lineages, paracrine factors instruct cells to become muscles by inducing them to synthesize the Myf5 and MyoD proteins (see Figure 11.13; Maroto et al. 1997; Tajbakhsh et al. 1997; Pownall et al. 2002). MyoD and Myf5 belong to a family of transcription factors called the **bHLH** (basic helix-loop-helix) **proteins** (sometimes also referred to as **myogenic regulatory factors**, or **MRFs**). The proteins of this family all bind to similar sites on the DNA and activate muscle-specific genes. The mechanism of induction of bHLHs differs slightly between the primaxial and abaxial lineages and between different vertebrate classes. In the lateral portion of the mouse dermamyotome, which forms the abaxial muscles, factors from the surrounding environment induce the Pax3 transcription factor. In the absence of other inhibitory transcription factors (such as those found in the sclerotome cells), Pax3 activates the *myoD* and *myf5* genes (Buckingham et al. 2006). In the medial region of the dermamyotome, which forms the primaxial (epaxial) muscles, MyoD is induced by the Myf5 protein.

In the formation of skeletal muscles, MyoD establishes a temporal cascade of gene activation. First, it can bind directly to certain regulatory regions to activate gene expression. For instance, the MyoD protein appears to directly activate the muscle-specific creatine phosphokinase gene by binding to the DNA immediately upstream from it (Lassar et al. 1989). There are also two MyoD-binding sites on the DNA adjacent to the genes encoding a subunit of the chicken muscle acetylcholine receptor (Piette et al. 1990). MyoD also directly activates its own gene. Therefore, once the *myoD* gene is activated, its protein product binds to the DNA immediately upstream of *myoD* and keeps this gene active.

Second, MyoD can activate other genes whose products act as cofactors for MyoDs binding to a later group of enhancers. For instance, MyoD activates the *p38* gene (which is not muscle-specific) and the *Mef2* gene. The p38 protein facilitates the binding of MyoD and Mef2 to a new set of enhancers, activating a second set of muscle-specific genes (Penn et al. 2004).

Although Pax3 is found in several other cell types, the myogenic bHLH proteins are specific to muscle cells. Any cell making a myogenic bHLH transcription factor such as MyoD or Myf5 becomes committed to forming a muscle cell. Transfection of genes encoding any of these myogenic proteins into a wide range of cultured cells converts those cells into muscles (Thayer et al. 1989; Weintraub et al. 1989).

Specification of muscle progenitor cells

As any athlete or sports fan knows, adult muscles are capable of limited regeneration following injury. The new myofibers come from sets of stem cells or progenitor cells that reside alongside the adult muscle fibers. One type of putative stem cell, the **satellite cell**, is found within the basal lamina of mature myofibers. Satellite cells respond to injury or exercise by proliferating into myogenic cells that fuse and form new muscle fibers; these cells may be stem cells with the capacity to generate daughter cells for

renewal or differentiation. Lineage tracing using chick-quail chimeras indicates that these are somite-derived muscle progenitor cells (see Figure 11.15) that have not fused and remain potentially available throughout adult life (Armand et al. 1983).

In 2005, the source of mouse and chick satellite cells was determined to be the central part of the dermamyotome (Ben-Yair and Kalcheim 2005; Gros et al. 2005; Kassar-Duchossoy et al. 2005; Relaix et al. 2005). While the myoblast-forming cells of the dermamyotome form at the lips and express Myf5 and MyoD, the cells that enter into the myotome from the central region usually express Pax3 and Pax7 and do not initially express the bHLH transcription factors. The combination of Pax3 and Pax7 appears to inhibit MyoD expression and muscle differentiation in these cells, and Pax7 protects the satellite cells against apoptosis (Olguin and Olwin 2004; Kassar-Duchossoy et al. 2005; Buckingham et al. 2006).

Injury or exercise causes the satellite cells to enter the cell cycle, produce the bHLH transcription factors, and fuse with the existing muscle fibers (see Gilbert and Epel 2009). Recent experimentation has shown that these satellite cells are not a homogeneous population but contain both stem cells and progenitor cells. The satellite cells that express Pax7 but not Myf5 ($Pax7^+/Myf5^-$ cells) appear to be stem cells that can divide asynchronously to produce two types of cells: another $Pax7^+/Myf5^-$ stem cell and a $Pax7^+/Myf5^+$ satellite cell. This latter cell differentiates into muscle. The $Pax7^+/Myf5^-$ cells, when transplanted into other muscles, contribute to the stem cell population there (Kuang et al. 2007). Muscle stem cell research is a controversial field, and the cell types responsible for muscle regeneration and repair are being intensely explored for therapeutic purposes (Darabi et al. 2008; Buckingham and Vincent 2009).

Myoblast fusion

The myotome cells producing the myogenic bHLH proteins are the myoblasts—committed muscle cell precursors. Experiments with chimeric mice and cultured myoblasts showed that these cells align and fuse to form the multinucleated myotubes characteristic of muscle tissue. Thus, the multinucleated myotube cells are the product of several myoblasts joining together and dissolving the cell membranes between them (Konigsberg 1963; Mintz and Baker 1967; Richardson et al. 2008). By the time a mouse is born, it has the adult number of myofibers, and these multinucleated myofibers grow during the first week by the fusion of singly nucleated myoblasts (Ontell et al. 1988). After the first week, muscle cells can still continue to grow by the fusion of satellite cells into existing myofibers and by an increase in contractile proteins within the myofibers.

Muscle cell fusion begins when the myoblasts leave the cell cycle. As long as particular growth factors (particularly FGFs) are present, myoblasts will proliferate without differentiating. When these factors are depleted, the myoblasts stop dividing, secrete fibronectin onto their extracellular matrix, and bind to it through $\alpha5\beta1$ integrin, their major fibronectin receptor (Menko and Boettiger 1987; Boettiger et al. 1995). If this adhesion is experimentally blocked, no further muscle development ensues, so it appears that the signal from the integrin-fibronectin attachment is critical for instructing myoblasts to differentiate into muscle cells (Figure 11.15).

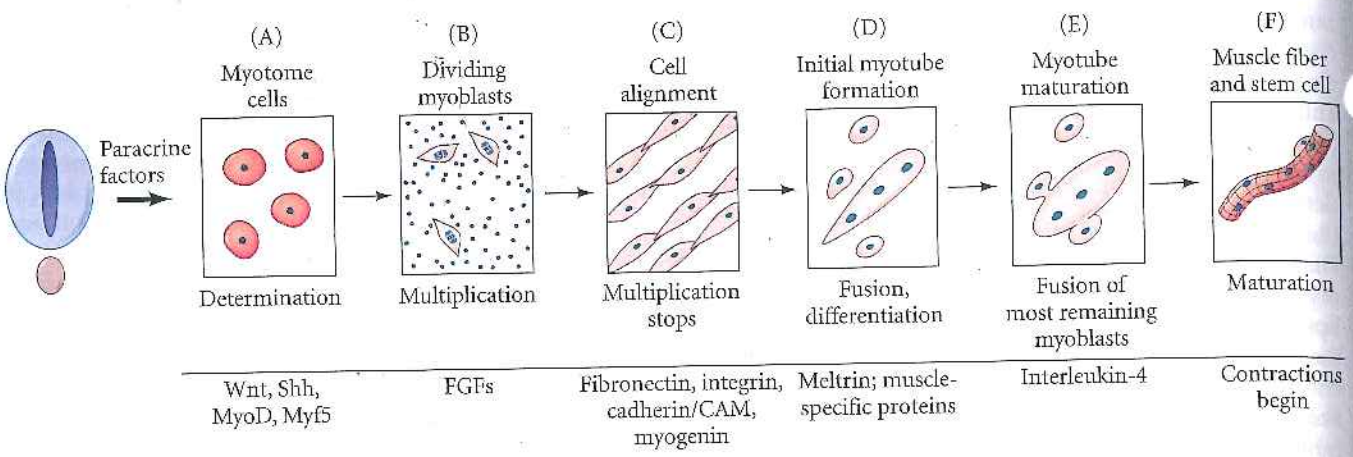
The second step is the alignment of the myoblasts into chains. This step is mediated by cell membrane glycoproteins, including several cadherins (Knudsen 1985; Knudsen et al. 1990). Recognition and alignment between cells take place only if the cells are myoblasts. Fusion can occur even between chick and rat myoblasts in culture (Yaffe and Feldman 1965); the identity of the species is not critical. The internal cytoplasm is also rearranged in preparation for the fusion, with actin regulating the regions of contact between the cells (Duan and Gallagher 2009).

The third step is the cell fusion event itself. As in most membrane fusions, calcium ions are critical, and fusion can be activated by calcium ionophores, such as A23187, that carry Ca^{2+} across cell membranes (Shainberg et al. 1969; David et al. 1981). Fusion appears to be mediated by a set of metalloproteinases called **meltrins**. These proteins were discovered during a search for myoblast proteins that would be homologous to fertilin, a protein implicated in sperm-egg membrane fusion. Yagami-Hiromasa and colleagues (1995) found that one of these, meltrin-α, is expressed in myoblasts at about the same time that fusion begins, and that antisense RNA to the meltrin-α message inhibited fusion when added to myoblasts. As the myoblasts become capable of fusing, another myogenic bHLH protein—**myogenin**—becomes active. Myogenin binds to the regulatory region of several muscle-specific genes and activates their expression. Thus, while MyoD and Myf5 are active in the lineage specification of muscle cells, myogenin appears to mediate muscle cell differentiation (Bergstrom and Tapscott 2001).

The last step of cell fusion involves the re-sealing ("healing") of the apposed membranes. This is accomplished by proteins such as myoferlin and dysferlin, which appear to stabilize phospholipids (Doherty et al. 2005). These proteins are similar to those that reseal the membranes at axon nerve synapses after membrane vesicle fusion releases neurotransmitters.

After the original fusion of myoblasts to become a myotube, the myotube secretes interleukin-4 (IL4). IL4 is a paracrine factor that was originally identified as being an important signaling molecule in the adult immune system, and until 2003 it was not known to play a role in the embryo. However, Horsely and colleagues (2003) found that IL4 secreted by the new myotubes recruits other myoblasts to fuse with the tube, thereby forming the mature myotube (see Figure 11.15).

The number of muscle fibers in the embryo and the growth of these fibers after birth appear to be negatively regulated by **myostatin**, a member of the TGF-β family of



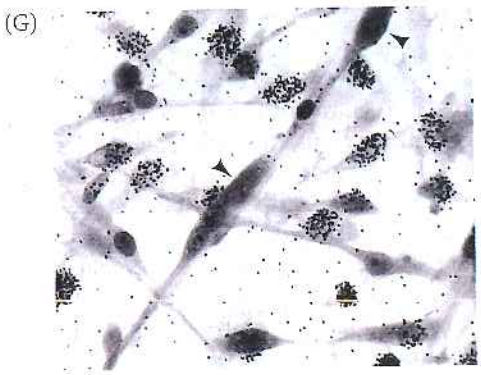


FIGURE 11.15 Conversion of myoblasts into muscles in culture. (A) Determination of myotome cells by paracrine factors. (B) Committed myoblasts divide in the presence of growth factors (primarily FGFs) but show no obvious muscle-specific proteins. (C–E) When the growth factors are used up, the myoblasts cease dividing, align, and fuse into myotubes. (F) The myotubes become organized into muscle fibers that spontaneously contract. (G) Autoradiograph showing DNA synthesis in myoblasts and the exit of fusing cells from the cell cycle. Phospholipase C can "freeze" myoblasts after they have aligned with other myoblasts, but before their membranes fuse. Cultured myoblasts were treated with phospholipase C and then exposed to radioactive thymidine. Unattached myoblasts continued to divide and thus incorporated the radioactive thymidine into their DNA. Aligned (but not yet fused) cells (arrowheads) did not incorporate the label. (A–F after Wolpert 1998; G from Nameroff and Munar 1976, courtesy of M. Nameroff.)

paracrine factors (McPherron et al. 1997; Lee 2004). Myostatin is made by developing and adult skeletal muscle and most probably works in an autocrine fashion. As mentioned in Chapter 2, *myostatin* loss-of-function mutations allow both hyperplasia (more fibers) and hypertrophy (larger fibers) of the muscle. These changes give rise to Herculean phenotypes in dogs,* cattle, mice, and humans (see Figure 2.30).

See WEBSITE 11.3 Muscle formation

Osteogenesis: The Development of Bones

Three distinct lineages generate the skeleton. The somites generate the axial (vertebral) skeleton, the lateral plate mesoderm generates the limb skeleton, and the cranial neural crest gives rise to the pharyngeal arch and craniofacial bones and cartilage. There are two major modes of bone formation, or **osteogenesis**, and both involve the transformation of preexisting mesenchymal tissue into bone tissue. The direct conversion of mesenchymal tissue into bone is called **intramembranous ossification** and was discussed in Chapter 10. In other cases, the mesenchymal cells differentiate into cartilage, which is later replaced by bone. The process by which a cartilage intermediate is formed and then replaced by bone cells is called **endochondral ossification**. Endochondral ossification is seen predominantly in the vertebral column, ribs, pelvis, and limbs.

Endochondral ossification

Endochondral ossification involves the formation of cartilage tissue from aggregated mesenchymal cells and the subsequent replacement of cartilage tissue by bone (Horton 1990). This is the type of bone formation characteristic of the vertebrae, ribs, and limbs. The vertebrae and ribs form from the somites, while the limb bones (to be discussed in Chapter 13) form from the lateral plate mesoderm.

The process of endochondral ossification can be divided into five stages. First, the mesenchymal cells commit to becoming cartilage cells (Figure 11.16A). This commitment is stimulated by Sonic hedgehog, which induces nearby sclerotome cells to express the Pax1 transcription factor (Cserjesi et al. 1995; Sosic et al. 1997). Pax1 initiates a cascade that is dependent on external paracrine factors and internal transcription factors.

During the second phase of endochondral ossification, the committed mesenchyme cells condense into compact nodules and differentiate into chondrocytes, or cartilage

*A loss-of-function mutation in the *myostatin* gene has found its way into whippets bred for dog racing. In these dogs, the homozygous loss-of-function condition is not advantageous, but heterozygotes have more muscle power and are significantly overrepresented among the top racers (Mosher et al. 2007).

cells (Figure 11.16B). BMPs appear to be critical in this stage. They are responsible for inducing the expression of the adhesion molecules N-cadherin and N-CAM and the transcription factor Sox9. N-cadherin appears to be important in the initiation of these condensations, and N-CAM seems to be critical for maintaining them (Oberlender and Tuan 1994; Hall and Miyake 1995). Sox9 activates other transcription factors as well as the genes encoding collagen 2 and agrican, which are critical in cartilage function. In humans, mutations of the *SOX9* gene cause camptomelic dysplasia, a rare disorder of skeletal development that results in deformities of most of the bones of the body. Most affected babies die from respiratory failure due to poorly formed tracheal and rib cartilage (Wright et al. 1995).

During the third phase of endochondral ossification, the chondrocytes proliferate rapidly to form the cartilage model for the bone (Figure 11.16C). As they divide, the chondrocytes secrete a cartilage-specific extracellular matrix. In the fourth phase, the chondrocytes stop dividing and increase their volume dramatically, becoming **hypertrophic chondrocytes** (Figure 11.16D). This step appears to be mediated by the transcription factor Runx2 (also called Cbfa1), which is necessary for the development of both intramembranous and endochondral bone (see Figure 10.11). Runx2 is itself regulated by histone deacetylase-4 (HDAC4), a form of chromatin restructuring enzyme that is expressed solely in the prehypertrophic cartilage. If HDAC4 is overexpressed in the cartilaginous ribs or limbs, ossification is seriously delayed; if the *HDAC4* gene is knocked out of the mouse genome, the limbs and ribs ossify prematurely (Vega et al. 2004).

These large chondrocytes alter the matrix they produce (by adding collagen X and more fibronectin) to enable it to become mineralized (calcified) by calcium phosphate. They also secrete the angiogenesis factor VEGF, which can transform mesodermal mesenchyme cells into blood vessels (see Chapter 12; Gerber et al. 1999; Haigh et al. 2000). A number of events lead to the hypertrophy and mineralization (calcification) of the chondrocytes, including an initial switch from aerobic to anaerobic respiration that alters chondrocyte cell metabolism and mitochondrial energy potential (Shapiro et al. 1982). Hypertrophic chondrocytes secrete numerous small, membrane-bound vesicles into the extracellular matrix. These vesicles contain enzymes that are active in the generation of calcium carbonate crystals, called *hydroxyapatite*, which mineralize the cartilaginous matrix (Wu et al. 1997). The hypertrophic chondrocytes, with their metabolism and mitochondrial membranes altered, then die by apoptosis (Hatori et al. 1995; Rajpurohit et al. 1999).

In the fifth phase, the blood vessels induced by VEGF invade the cartilage model (Figure 11.16E–G). As the hypertrophic chondrocytes die, the cells that surround the cartilage model differentiate into osteoblasts. The replacement of chondrocytes by bone cells depends on the mineralization of the extracellular matrix. This remodeling releases VEGF, and more blood vessels are made around the dying cartilage. The blood vessels bring in both osteoblasts and

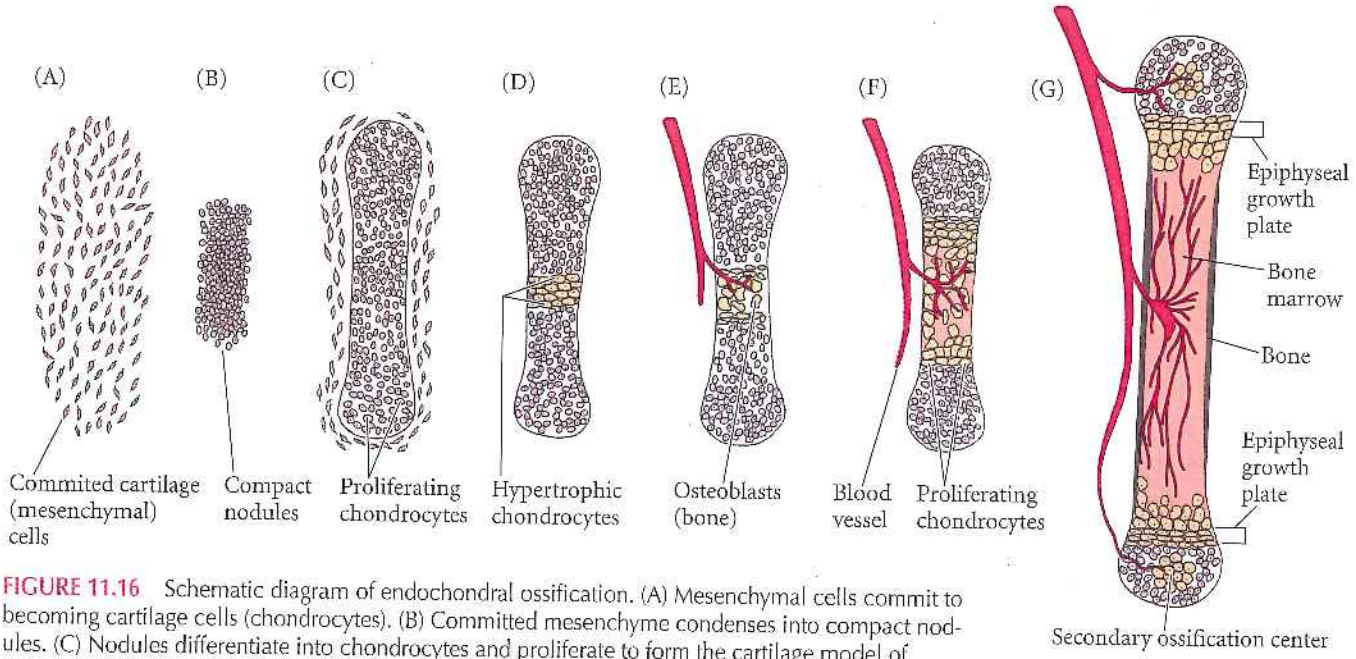


FIGURE 11.16 Schematic diagram of endochondral ossification. (A) Mesenchymal cells commit to becoming cartilage cells (chondrocytes). (B) Committed mesenchyme condenses into compact nodules. (C) Nodules differentiate into chondrocytes and proliferate to form the cartilage model of bone. (D) Chondrocytes undergo hypertrophy and apoptosis while they change and mineralize their extracellular matrix. (E) Apoptosis of chondrocytes allows blood vessels to enter. (F) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (G) Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification centers also form as blood vessels enter near the tips of the bone. (After Horton 1990.)

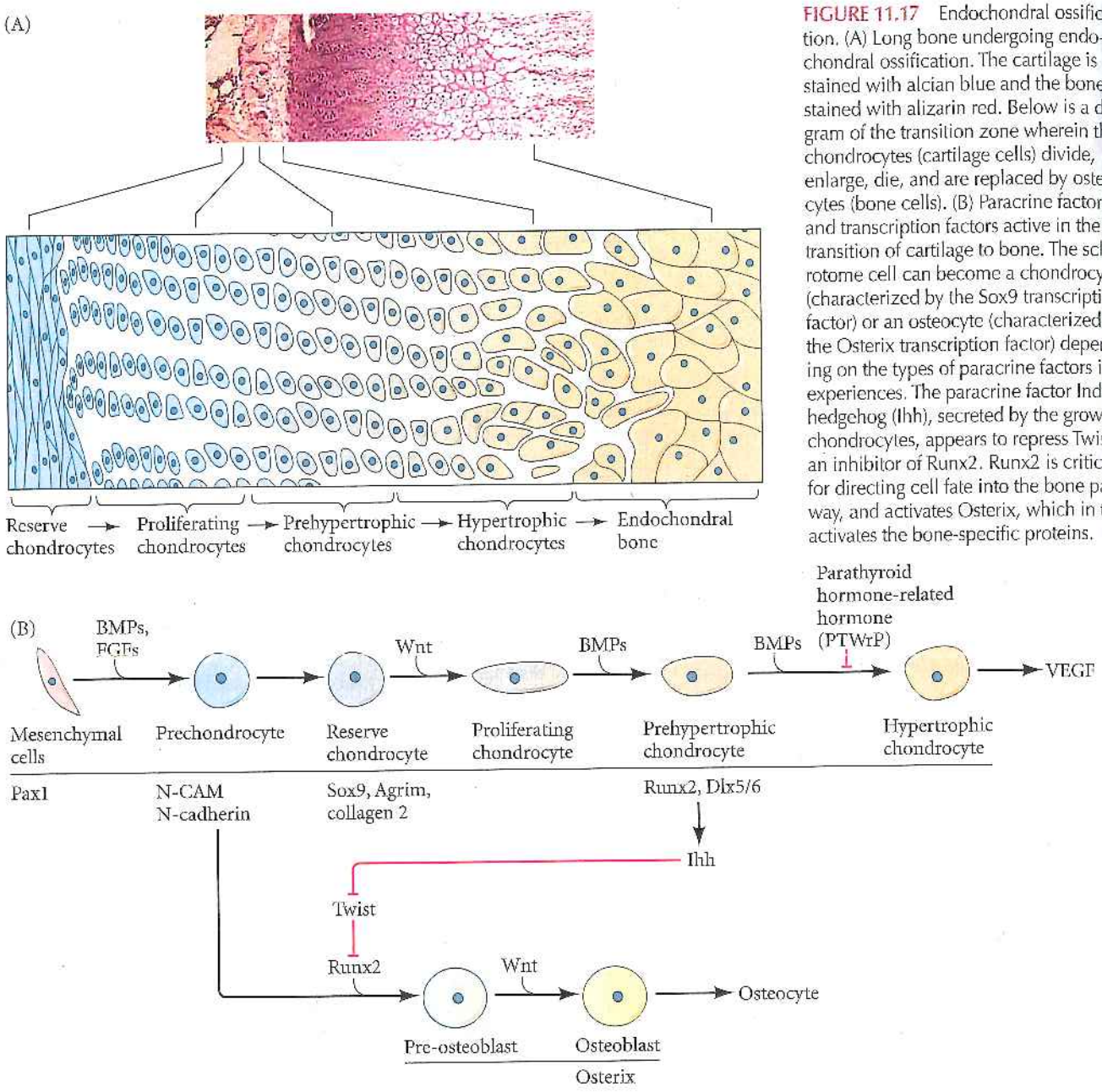


FIGURE 11.17 Endochondral ossification. (A) Long bone undergoing endochondral ossification. The cartilage is stained with alcian blue and the bone is stained with alizarin red. Below is a diagram of the transition zone wherein the chondrocytes (cartilage cells) divide, enlarge, die, and are replaced by osteocytes (bone cells). (B) Paracrine factors and transcription factors active in the transition of cartilage to bone. The sclerotome cell can become a chondrocyte (characterized by the Sox9 transcription factor) or an osteocyte (characterized by the Osterix transcription factor) depending on the types of paracrine factors it experiences. The paracrine factor Indian hedgehog (Ihh), secreted by the growing chondrocytes, appears to repress Twist, an inhibitor of Runx2. Runx2 is critical for directing cell fate into the bone pathway, and activates Osterix, which in turn activates the bone-specific proteins.

chondroclasts (which eat the debris of the apoptotic chondrocytes). If the blood vessels are inhibited from forming, bone development is significantly delayed (Yin et al. 2002; see Karsenty and Wagner 2002).

The osteoblasts begin forming bone matrix on the partially degraded matrix and construct a bone collar around the calcified cartilage matrix (Bruder and Caplan 1989; Hatori et al. 1995; St-Jacques et al. 1999). It is thought that the osteoblasts are derived from same sclerotomal precursors as the chondrocytes (Figure 11.17). The osteoblasts form when Indian hedgehog (secreted by the prehypertrophic chondrocytes) causes a relatively immature cell (probably prechondrocyte) to produce the transcription factor Runx2. Runx2 allows the cell to make bone matrix but keeps the cell from becoming fully differentiated. Moreover, this osteoblast becomes responsive to Wnt signals that upregulate the transcription factor Osterix (Nakashima et al. 2002; Hu et al. 2005). Osterix instructs the cells to become bone. New bone material is added peripherally from the internal surface of the **periosteum**, a fibrous sheath containing connective tissue, capillaries, and bone progenitor cells and that covers the developing bone. At the same time, there is a hollowing out of the internal region of the bone to form the bone marrow cavity. This destruction of bone tissue is

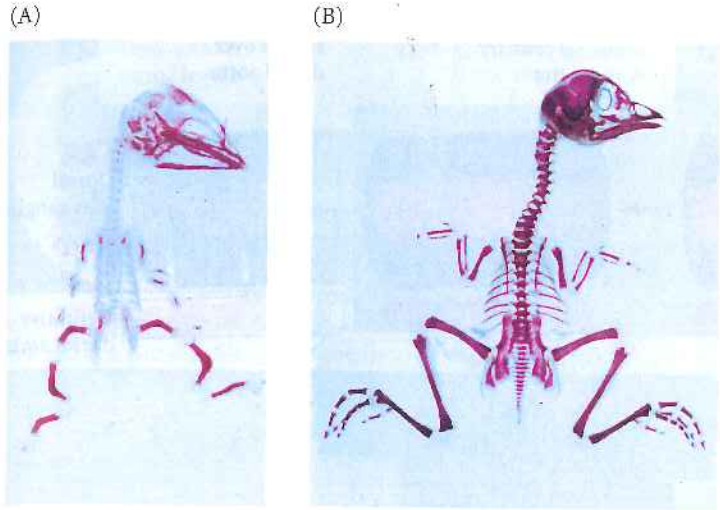


FIGURE 11.18 Skeletal mineralization in 19-day chick embryos that developed (A) in shell-less culture and (B) inside an egg during normal incubation. The embryos were fixed and stained with alizarin red to show the calcified bone matrix. (From Tuan and Lynch 1983, courtesy of R. Tuan.)

carried out by **osteoclasts**, multinucleated cells that enter the bone through the blood vessels (Kahn and Simmons 1975; Manolagas and Jilka 1995). Osteoclasts are not derived from the somite; rather, they are derived from a blood cell lineage (in the lateral plate mesoderm) and come from the same precursors as macrophage blood cells (Ash et al. 1980; Blair et al. 1986).

The importance of the mineralized extracellular matrix for bone differentiation is clearly illustrated in the developing skeleton of the chick embryo, which uses the calcium carbonate of the egg's shell as its calcium source. During development, the circulatory system of the chick embryo translocates about 120 mg of calcium from the shell to the skeleton (Tuan 1987). When chick embryos are removed from their shells at day 3 and grown in plastic wrap for the duration of their development, much of the cartilaginous skeleton fails to mature into bony tissue (Figure 11.18; Tuan and Lynch 1983).

See WEBSITE 11.4 Paracrine factors, their receptors, and human bone growth

Vertebrae formation

The notochord appears to induce its surrounding mesenchyme cells to secrete epimorphin, and this epimorphin attracts sclerotome cells to the region around the notochord and neural tube, where they begin to condense and differentiate into cartilage (Oka et al. 2006). However, before the sclerotome cells form a vertebra, they must split into a rostral (anterior) and a caudal segment (Figure 11.19). As the motor neurons from the neural tube grow laterally to innervate the newly forming muscles, the rostral segment of each sclerotome recombines with the caudal segment of the next anterior sclerotome to form the vertebral rudiment (Remak 1850; Aoyama and Asamoto 2000; Morin-Kensicki et al. 2002). As we will see in our discussion of tendons, this **resegmentation** enables the muscles to coordinate the movement of the skeleton, permitting the body to move

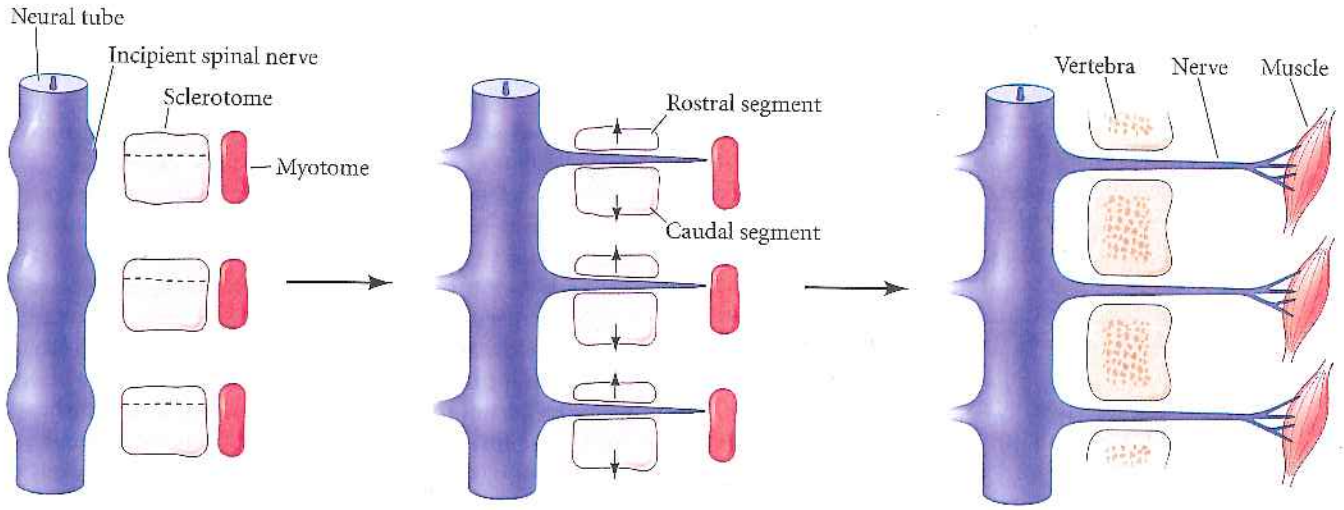


FIGURE 11.19 Respecification of the sclerotome to form each vertebra. Each sclerotome splits into a rostral and caudal segment. As the spinal neurons grow outward to innervate the muscles from the myotome, the rostral segment of each sclerotome combines with the caudal segment of the next anterior sclerotome to form a vertebral rudiment. (After Larson 1998.)

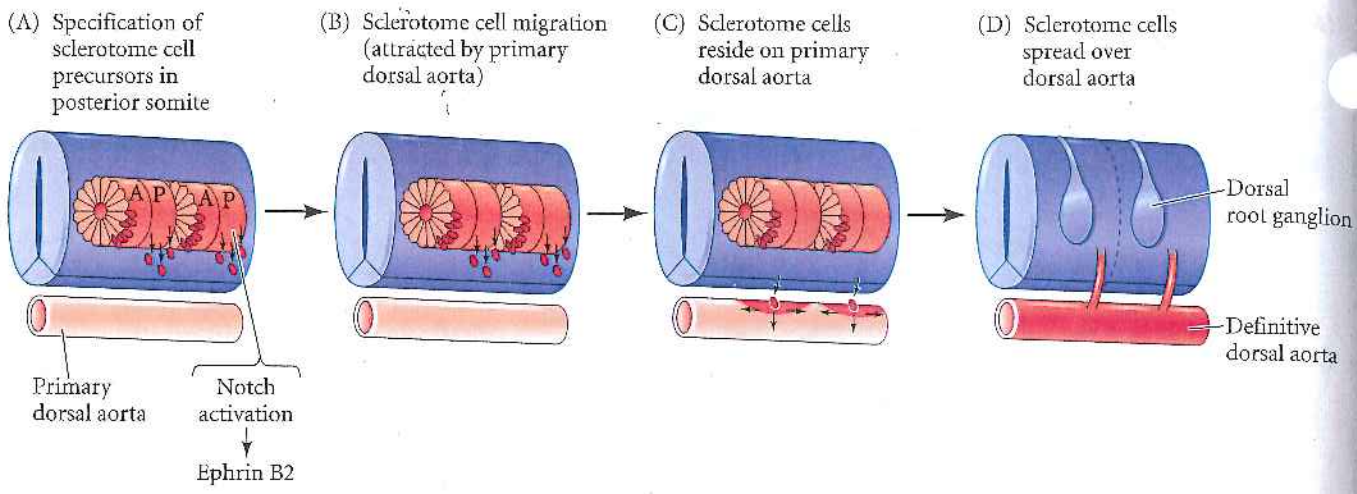


FIGURE 11.20 Model showing contribution of somitic cells to the dorsal aorta. (A) At an early stage, the primary dorsal aorta is of lateral plate origin (pink). A subpopulation of sclerotome cells becomes specified by Notch in the posterior half of somites as endothelial precursors. (B) Chemoattractants made in the primary dorsal aorta cause these cells to migrate through the somite to the aorta. (C) The sclerotome cells take up residence in the dorsal region of the vessel. (D) These cells then spread along both the anterior-posterior and dorsal-ventral axes, ultimately occupying the entire region of the aorta. The primary aortic endothelial cells become blood cell precursors. (After Sato et al. 2008.)

laterally. The resegmentation of somites to allow coordinated movement is reminiscent of the strategy used by insects when constructing segments out of parasegments (see Chapter 6). The bending and twisting movements of the spine are permitted by the intervertebral (synovial) joints that form from the arthrotome region of the sclerotome. Removal of these sclerotome cells leads to the failure of synovial joints to form and to the fusion of adjacent vertebrae (Mittapalli et al. 2005).

Dorsal Aorta Formation

While most of the circulatory system of the early amniote embryo is directed outside the embryo (to obtain nutrients from the yolk or placenta), the intraembryonic circulatory system begins with the formation of the dorsal aorta. The dorsal aorta is composed of two cell layers: an internal lining of endothelial cells surrounded concentrically by a layer of smooth muscle cells. While these two layers of blood vessels are usually derived from the lateral plate mesoderm (and this will be an important subject of Chapter 12), the posterior sclerotome provides the endothelial cells and smooth muscle cells for the dorsal aorta and intervertebral blood vessels (Pardanaud et al. 1996; Wiegreffe et al. 2007). The presumptive endothelial cells are formed by Notch signaling, and they are then instructed to migrate ventrally by a chemoattractant that has been made by the primary dorsal aorta, a transitory structure made by the lateral plate mesoderm. Eventually, the endothelial cells from the sclerotome replace the cells of the primary dorsal aorta (which will become part of the blood stem cell population) (Figure 11.20; Pouget et al. 2008; Sato et al. 2008).

Tendon Formation: The Syndetome

The most dorsal part of the sclerotome will become the fourth compartment of the somite, the **syndetome** (Greek *syn*, "connected"). Since the tendons connect muscles to bones, it is not surprising that the syndetome is derived from the most dorsal portion of the sclerotome—that is, from sclerotome cells adjacent to the muscle-forming myotome. The tendon-forming cells of the syndetome can be visualized by their expression of the *scleraxis* gene (Figure 11.21; Schweitzer et al. 2001; Brent et al. 2003). Because there is no obvious morphological distinction between the sclerotome and syndetome cells (they are both mesenchymal), our knowledge of this somitic compartment had to wait until we had molecular markers (Pax1 for the sclerotome, scleraxis for the syndetome) that could distinguish them and allow one to follow their cells' fates.

The syndetome is made from the myotome's secretion of Fgf8 onto the immediately subjacent row of sclerotome cells (Figure 11.22A; Brent et al. 2003; Brent and Tabin 2004). Other transcription factors limit the expression of scleraxis to the anterior and posterior portions of the syndetome, causing two stripes of scleraxis expression. Meanwhile, the developing cartilage cells, under the influence of Sonic hedgehog from the notochord and floorplate, synthesize Sox5 and Sox6—transcription factors that block scleraxis transcription (Figure 11.22B). In this way, the cartilage protects itself from any spread of the Fgf8 signal. The tendons then associate with the muscles directly above them and with the skeleton (including the ribs) on either side of them (Figure 11.22C; Brent et al. 2005).