10th EDITION

Ten Cate's Oral Histology

Development, Structure, and Function

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PREFACE

The first edition of *Oral Histology: Development, Structure, and Function* appeared in 1980 and was edited by A.R. Ten Cate. The textbook was subsequently renamed *Ten Cate's Oral Histology: Development, Structure, and Function* (6th edition) in 2003 in recognition of his contributions to oral histology and oral health. The present edition celebrates 44 years of a didactic style that remains fully relevant today and that has helped train multiple generations of oral health practitioners and researchers throughout the world.

The scope of this new edition remains to provide a solid treatise in oral histology with emphasis on structure–function relationships. Molecular concepts are integrated to help understand genes and mechanisms implicated in embryogenesis, development, cell function, and matrix events. The updates in information, in some cases, may appear subtle, and in others they are more significant. Boxed texts by key protagonists have been added to provide broader pictures, present novel concepts that may appear opposing and will likely continue to evolve, and discuss topics of clinical relevance.

Finally, I sincerely believe that, within the limits and purpose of an educational text, it is most important to keep an open mind.

Like previous editions, this 10th edition is intended to serve as a learning guide for students in a variety of disciplines. Although coverage is exhaustive, the text has been structured such that individual chapters and even selected sections can be used independently—in this regard, the digital edition will greatly facilitate the search and identification of information of specific interest. The focus continues to be on learning and understanding concepts rather than on memorization of detail, particularly numeric values. Thus dental hygienists, medical students, undergraduate and graduate dental students, and oral health researchers will find a degree of coverage suited for their respective needs.

Finally, a major objective is to sensitize students to the concept that, in addition to being pertinent to clinical practice, better understanding of the development and biology of oral tissues is expected to engender novel therapeutic approaches based on biologics that will likely be used by oral health practitioners in the foreseeable future. As progress is logically bound to occur in the coming years, the future practice of dentistry will inevitably undergo a shift from the traditional restorative approach to one more oriented toward the medical management of patients.

ACKNOWLEDGMENTS

The present edition builds on material from previous editions provided over the years by various contributors. I am most grateful to P. Mark Bartold, Paolo Bianco, Anne C. Dale, Jack G. Dale, Dale R. Eisenmann, Donald H. Enlow, Michael W. Finkelstein, Eric Freeman, Arthur R. Hand, Stéphane Roy, Paul T. Sharpe, Martha J. Somerman, Christopher A. Squier, Calvin D. Torneck, and S. William Whitson for their excellent past contributions. More recently, Shingo Kuroda, Matthieu Schmittbuhl, Eiji Tanaka, and Daniel Turgeon have also contributed.

Although every effort has been made to have a text free of factual and editorial errors, a few may still have managed to slip through, and for this I apologize. Timely identification of such slips is important, as the digital age now permits corrections to be carried on continuously through digital editions and, in some cases, in new batches of printed textbooks rather than having to wait for a new edition. Educators and students are most welcome to contact me should they find any inaccuracy or ambiguous text or want to share new perspectives.

The personnel who have over the years contributed to generating much of the illustration material deserve special thanks, as the quality of illustrations is ultimately a reflection of their personal talent. I thank Dainelys Guadarrama Bello and Katia J. Ponce for their general assistance with the assembly of text and figures. At Elsevier, I thank Kelly Skelton (Senior Content Strategist), Sneha Kashyap (Senior Content Development Specialist), and Sindhuraj Thulasingam (Project Manager) for their assistance and patience throughout preparation of this 10th edition.

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Structure of the Oral Tissues: An Overview

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OUTLINE

This chapter presents an overview of the histology of the tooth and its supporting tissues (Fig. 1.1) and the salivary glands, bones of the jaw, and articulations between the jaws (temporomandibular joints [TMJs]) as a basis for subsequent detailed consideration.

THE TOOTH

Teeth constitute approximately 20% of the surface area of the mouth, the upper teeth significantly more than the lower teeth. Mastication is the function most associated with the human dentition, but teeth also are essential for proper speech. In the Animal Kingdom, teeth have important roles as weapons of attack and defense. Teeth must be hard and firmly attached to the bones of the jaws to fulfill most of these functions. In most submammalian vertebrates the teeth are fused directly to the jawbone. Although this construction provides a firm attachment, such teeth frequently are broken and lost during normal function. In these cases, many successional teeth form to compensate for tooth loss and to ensure continued function of the dentition.

The tooth proper consists of a hard, inert, acellular enamel formed by epithelial cells and supported by the less mineralized, more resilient, and vital hard connective tissue dentin, which is formed and supported by the dental pulp, a soft connective tissue (Fig. 1.2; see also Fig. 1.1). In mammals, teeth are attached to the jaw by tooth-supporting connective tissues consisting of cementum, periodontal ligament (PDL), and alveolar bone, which provide enough flexibility to withstand the forces of mastication. In human beings and most mammals, a limited succession of teeth still occurs, not to compensate for continual loss of teeth but to accommodate the growth of the face and jaws. The face and jaws of a human child are small and consequently can carry fewer teeth of smaller size. These smaller teeth constitute the deciduous or primary dentition. A large increase in the size of the jaws occurs with growth, necessitating not only more teeth but also larger ones. Because the size of teeth cannot increase after they are formed, the deciduous dentition becomes inadequate and must be replaced by a permanent or secondary dentition consisting of more and larger teeth.

Anatomically, the tooth consists of a crown and a root (see Figs 1.1 and 1.2); the junction between the two is the cervical margin. The term *clinical crown* denotes that part of the tooth that is visible in the oral cavity. Although teeth vary considerably in shape and size (e.g., an incisor compared with a molar), histologically they are similar.

Enamel

Enamel is an eccentric hard tissue because of its origin, its chemically distinct nature of the various noncollagenous matrix proteins expressed by ameloblasts, and its large mineral crystals. Enamel has evolved as an epithelial-derived protective covering for the crown of the teeth (see Figs. 1.1 and 1.2). The enamel is the most highly mineralized tissue in the body, consisting of more than 96% inorganic material in the form of apatite crystals and traces of organic material. The cells responsible for the formation of enamel (ameloblasts) cover the entire surface of the layer as it forms but are lost as the tooth emerges into the oral cavity. The loss of these cells renders enamel a nonvital and insensitive matrix that, when destroyed by any means (usually wear or caries), cannot be replaced or regenerated. To compensate for this inherent limitation, enamel has acquired a high degree of mineralization and a complex organization. These structural and compositional features allow enamel to withstand large masticatory forces and continual assaults by acids from food and bacterial sources. The apatite crystals within enamel pack together differentially to create a structure of enamel rods separated by interrod enamel (Fig. 1.3). Although enamel is a dead tissue in a strict biologic sense, it is permeable; ionic exchange can occur between the enamel and the environment of the oral cavity, in particular the saliva.

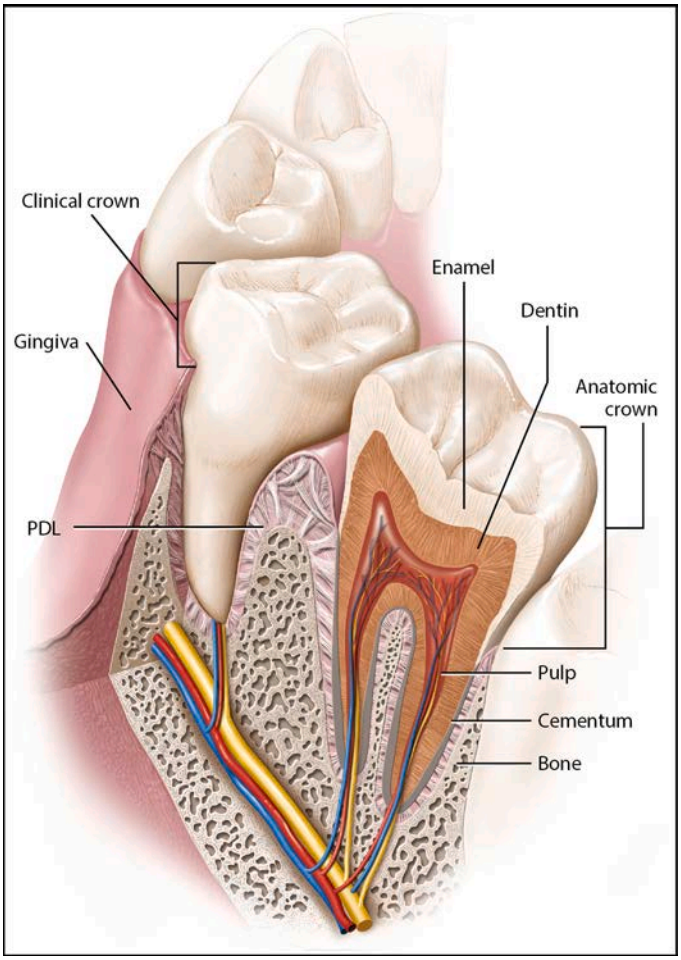


Fig. 1.1 The tooth and its supporting structure. *PDL*, Periodontal ligament.

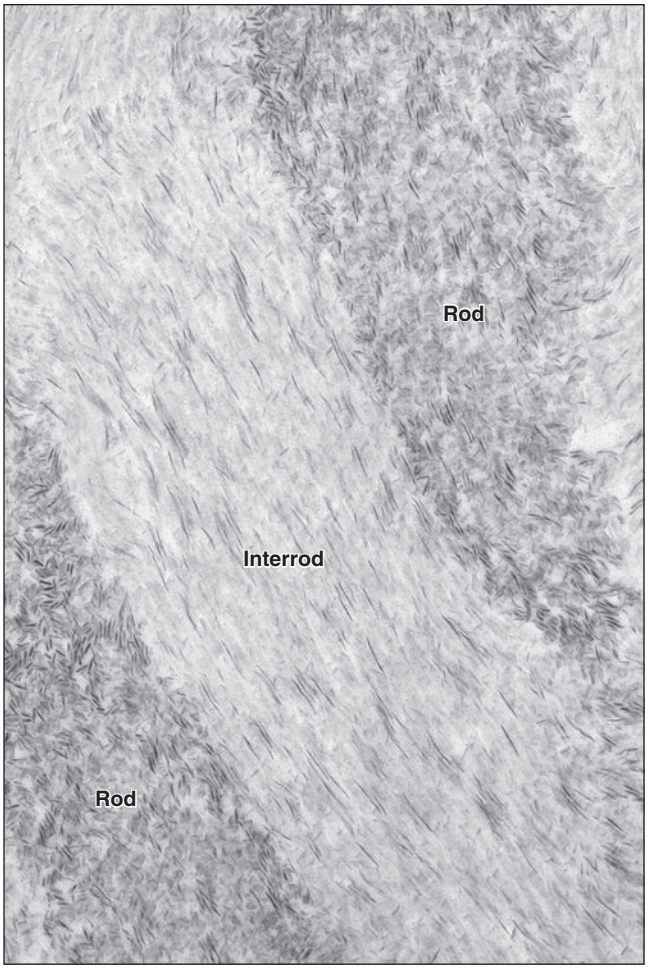


Fig. 1.3 Enamel. Electron micrograph showing that enamel consists of crystallites organized into rod and interrod enamel.

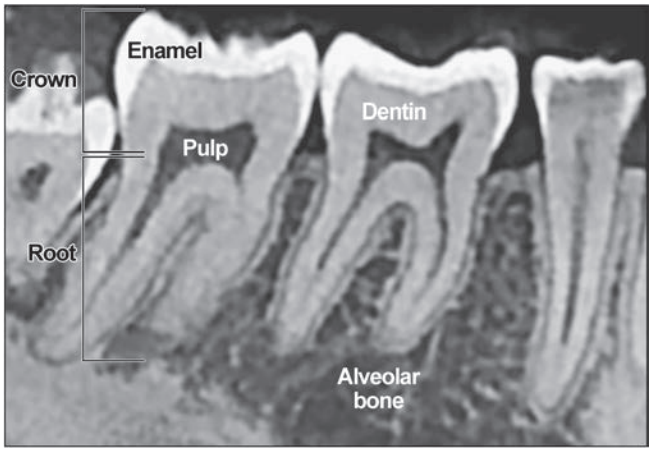


Fig. 1.2 Vertical cone beam computed tomography slice of mandibular molars and premolars. (Courtesy M. Schmittbuhl.)

Dentin

Because of its exceptionally high mineral content, enamel is a brittle tissue that cannot withstand the forces of mastication without fracture unless it has the support of a more resilient tissue, such as dentin. Dentin forms the bulk of the tooth, supports the enamel, and compensates for its brittleness.

Dentin is a mineralized, elastic, yellow-white, avascular tissue enclosing the central pulp chamber (Fig. 1.4; see also Figs. 1.1 and 1.2). The mineral is also apatite, and the organic component is mainly the fibrillar protein collagen. A characteristic feature of dentin is its permeation by closely packed tubules traversing its entire thickness and containing the cytoplasmic extensions of the cells that once formed it and later maintain it (see Fig. 1.4B). These cells are called *odontoblasts*; their cell bodies are aligned along the inner edge of the dentin, where they form the peripheral boundary of the dental pulp (see Fig. 1.4A). The very existence of odontoblasts makes dentin a vastly different tissue from enamel. Dentin is a sensitive tissue, and, more importantly, it is capable of repair because odontoblasts or cells in the pulp can be stimulated to deposit more dentin as the occasion demands.

Pulp

The central pulp chamber, enclosed by dentin, is filled with a soft connective tissue called *pulp* (see Fig. 1.4A). Dentin is a hard tissue; the pulp is soft (and is lost in dried teeth, leaving a clearly recognizable empty chamber). Despite distinctive histologic features, dentin and pulp are related embryologically and functionally and should be considered together. This unity is exemplified by the classic functions of pulp: It is (1) formative in that it produces the dentin that surrounds it; (2) nutritive in that it nourishes the avascular dentin; (3) protective in that it carries nerves that give dentin its sensitivity; and (4) reparative in that it is capable of producing new dentin when required.

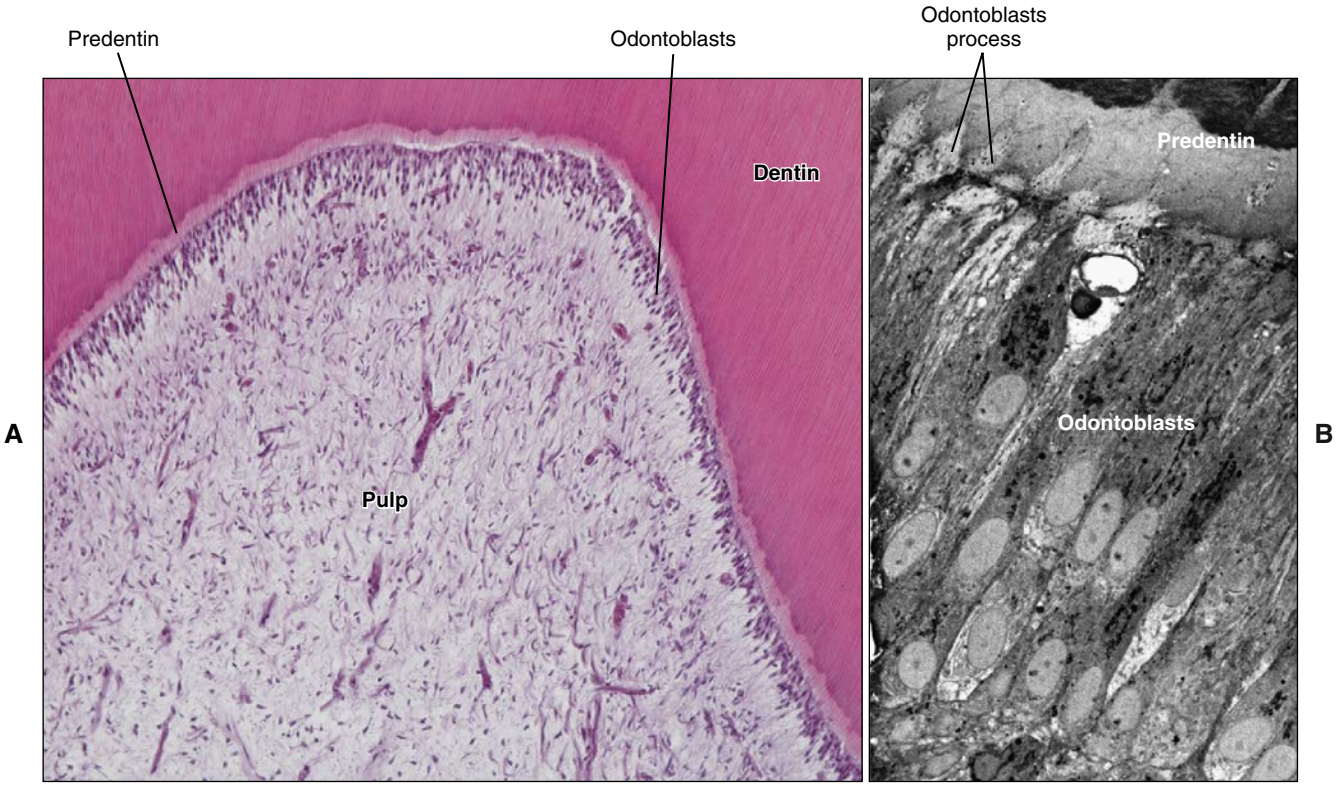


Fig. 1.4 Dentin and pulp. (A) The odontoblasts (cells that form dentin) line the pulp. (B) At higher magnification, these cells show processes extending into dentin.

In summary, the tooth proper consists of two hard tissues: the acellular enamel and the supporting dentin. The latter is a specialized connective tissue, the formative cells of which are in the pulp. These tissues bestow on teeth the properties of hardness and resilience. Their indestructibility also gives teeth special importance in paleontology and forensic science, for example, as a means of identification.

SUPPORTING TISSUES OF THE TOOTH

The tooth is attached to the jaw by a specialized supporting apparatus that consists of the alveolar bone, the PDL, and the cementum, all of which are protected by the gingiva (Fig. 1.5; also see Fig. 1.1).

Periodontal Ligament

The PDL is a highly specialized connective tissue situated between the tooth and the alveolar bone (see Fig. 1.5). The principal function of the PDL is to connect the tooth to the jaw, which it must do in such a way that the tooth will withstand the considerable forces of mastication. This requirement is met by the collagen fiber bundles that span the distance between the bone and the tooth and by ground substance between them. At one extremity the fibers of the PDL are embedded in bone; at the other extremity they are embedded in cementum. Each collagen fiber bundle is much like a spliced rope in which individual strands can be remodeled continually without the overall fiber losing its architecture and function. In this way the collagen fiber bundles can adapt to the stresses placed on them. The PDL has another important function, a sensory one. Tooth enamel is an inert tissue and therefore insensitive, yet the moment teeth come into contact with each other, we know it. Part of this sense of discrimination is provided by sensory receptors within the PDL.

Cementum

Cementum covers the roots of the teeth and is interlocked firmly with the dentin of the root (see Figs. 1.1, 1.2, and 1.5B). Cementum is a mineralized connective tissue similar to bone except that it is avascular; the mineral is also apatite, and the organic matrix contains collagen. The cells that form cementum are called *cementoblasts.*

The two main types of cementum are cellular and acellular. The cementum attached to the root dentin and covering the upper (cervical) portion of the root is acellular and thus is called *acellular (primary) cementum.* The lower (apical) portion of the root is covered by cellular (secondary) cementum. In this case, cementoblasts become trapped in lacunae within their own matrix, much like osteocytes occupy lacunae in bone; these entrapped cells are now called *cementocytes.* Acellular cementum anchors PDL fiber bundles to the tooth; cellular cementum has an adaptive role. Bone, the PDL, and cementum together form a functional unit of special importance when orthodontic tooth movement is undertaken.

ORAL MUCOSA

The oral cavity is lined by a mucous membrane that consists of two layers: an epithelium and subjacent connective tissue (the lamina propria) (Fig. 1.6). Although its major functions are lining and protecting, the mucosa also is modified to serve as an exceptionally mobile tissue that permits free movement of the lip and cheek muscles. In other locations it serves as the organ of taste.

Histologically, the oral mucosa can be classified into three types: (1) masticatory, (2) lining, and (3) specialized. The masticatory mucosa covers the gingiva and hard palate. The masticatory mucosa is bound down tightly by the lamina propria to the underlying bone (see Fig. 1.6B),

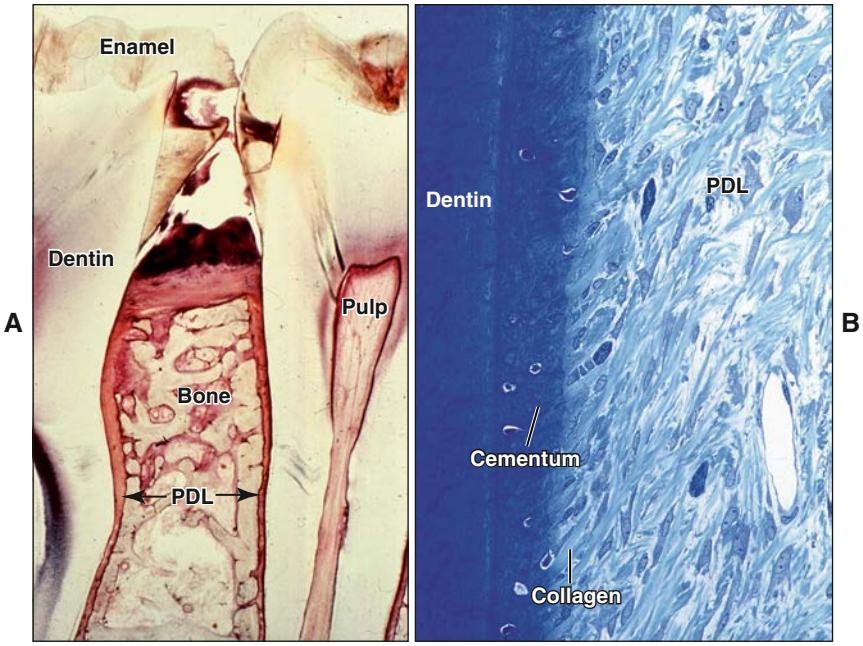


Fig. 1.5 Histologic sections of the periodontal ligament *(PDL).* (A) Supporting apparatus of the tooth in longitudinal section. (B) At higher magnification, note the fibrocellular nature of the PDL.

and the covering epithelium is keratinized to withstand the constant pounding of food during mastication. The lining mucosa, by contrast, must be as flexible as possible to perform its function of protection. The epithelium is not keratinized; the lamina propria is structured for mobility and is not tightly bound to underlying structures (see Fig. 1.6C). The dorsal surface of the tongue is covered by a specialized mucosa consisting of a highly extensible masticatory mucosa containing papillae and taste buds.

A unique feature of the oral mucosa is that the teeth perforate it. This anatomic feature has profound implications in the initiation of periodontal disease. The teeth are the only structures that perforate epithelium anywhere in the body. Nails and hair are epithelial appendages around which epithelial continuity is always maintained. This perforation by teeth means that a sealing junction must be established between the gum and the tooth.

The mucosa immediately surrounding an erupted tooth is the gingiva. In functional terms the gingiva consists of two parts: (1) the part facing the oral cavity, which is masticatory mucosa, and (2) the part facing the tooth, which is involved in attaching the gingiva to the tooth and forms part of the periodontium. The junction of the oral mucosa and the tooth is permeable, and thus antigens can pass easily through it and initiate inflammation in gum tissue (marginal gingivitis).

SALIVARY GLANDS

Saliva is a complex fluid that, in health, almost continually bathes the parts of the tooth exposed within the oral cavity. Consequently, saliva represents the immediate environment of the tooth. Saliva is produced by three paired sets of major salivary glands (parotid, submandibular, and sublingual) and by the many minor salivary glands scattered throughout the oral cavity. A precise account of the composition of saliva is difficult because not only are the secretions of each of the major and minor salivary glands different, but their volume may vary at any given time. In recognition of this variability, the term *mixed saliva* has been used to describe the fluid of the oral cavity. Regardless of its precise composition, saliva has several functions. Saliva moistens the mouth, facilitates speech, lubricates food, and helps with taste by acting as a solvent for food molecules. Saliva also contains a digestive enzyme (amylase). Saliva not only dilutes noxious material mistakenly taken into the mouth, it also cleanses the mouth. Furthermore, it contains antibodies and antimicrobial substances, and by virtue of its buffering capacity plays an important role in maintaining the pH of the oral cavity.

The basic histologic structure of the major salivary glands is similar. A salivary gland may be likened to a bunch of grapes. Each so-called grape is the acinus (terminal secretory unit), which is a mass of secretory cells surrounding a central space. The spaces of the acini open into ducts running through the gland that are called successively the *intercalated, striated,* and *excretory ducts* (Fig. 1.7), analogous to the stalks and stems of a bunch of grapes. These ducts are more than passive conduits, however; their lining cells have a function in determining the final composition of saliva.

The ducts and acini constitute the parenchyma of the gland, the whole of which is invested by a connective tissue stroma carrying blood vessels and nerves. This connective tissue supports each individual acinus and divides the gland into a series of lobes or lobules, finally encapsulating it (Fig. 1.8).

BONES OF THE JAW

As stated, teeth are attached to bone by the PDL (see Figs 1.1 and 1.5A). This bone, the alveolar bone, constitutes the alveolar process, which is in continuity with the basal bone of the jaws. The alveolar process forms in relation to teeth. When teeth are lost, the alveolar process is gradually lost as well, creating the characteristic facial profile of the edentulous person whose chin and nose approximate because of a reduction in facial height. Although the histologic structure of the alveolar process is essentially the same as that of the basal bone, practically it is necessary to distinguish between the two. The position of teeth and supporting tissues, which include the alveolar process, can be modified easily by orthodontic therapy. However, modification of

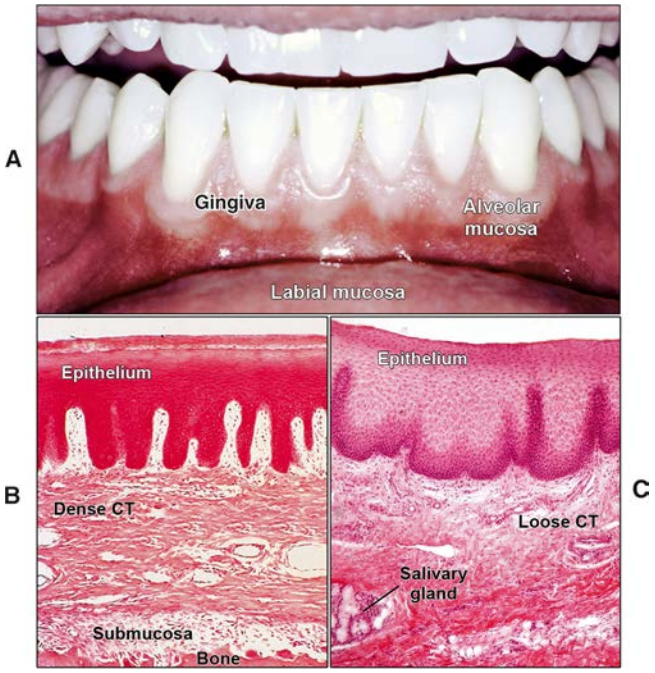


Fig. 1.6 Oral mucosa. (A) Note the difference between tightly bound mucosa of the gingiva (gum) and mobile mucosa of the labial sulcus (alveolar mucosa). (B) In histologic sections, the gingival epithelium is seen to be supported by dense connective tissue *(CT),* whereas the epithelium of the lip (C) is supported by a much looser connective tissue.

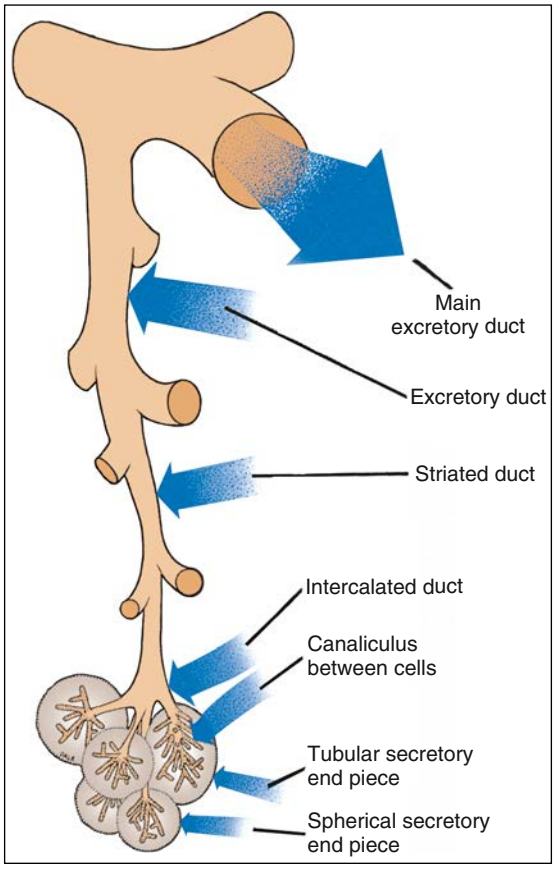


Fig. 1.7 Diagrammatic illustration of the ductal system of a salivary gland.

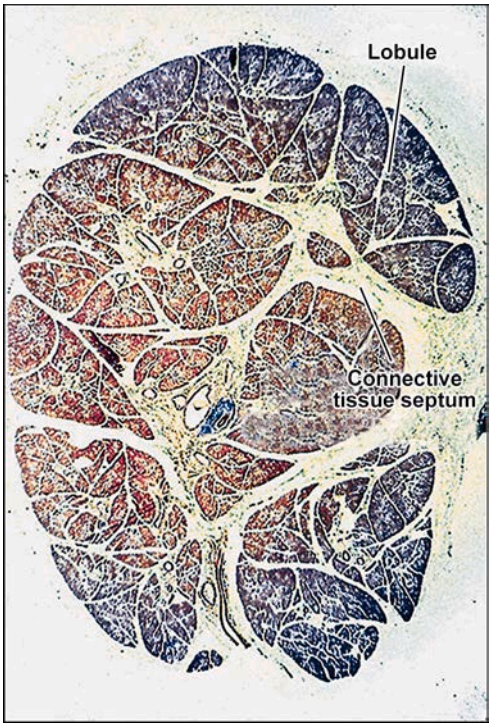


Fig. 1.8 Low-power photomicrograph of a salivary gland showing its lobular organization.

the position of the basal bone is usually much more difficult; this can be achieved only by influencing its growth. The way these bones grow is thus important in determining the position of the jaws and teeth.

TEMPOROMANDIBULAR JOINT

The relationship between the bones of the upper and lower jaws is maintained by the articulation of the condylar process of the mandible with the glenoid fossa of the temporal bone. This articulation, the TMJ, is a synovial joint with special features that permit the complex movements associated with mastication. The specialization of the TMJ is reflected in its histologic appearance (Fig. 1.9). The TMJ cavity is formed by a fibrous capsule lined with a synovial membrane and is separated into two compartments by an extension of the capsule to form a specialized movable disk. The articular surfaces of the bone are covered not by hyaline cartilage but by a fibrous layer that is a continuation of the periosteum covering the individual bones. A simplified way to understand the function of the TMJ is to consider it as a joint with the articular disk being a movable articular surface.

HARD TISSUE FORMATION

The hard tissues of the body—bone, cementum, dentin, and enamel—are associated with the functioning tooth. Because the practice of dentistry involves manipulation of these tissues, a detailed knowledge of them is obligatory (and each is discussed separately in later chapters). The purposes of this section are (1) to explain that a number of common features are associated with hard tissue formation, even though the final products are structurally distinct; (2) to indicate that the functional role of a number of these features is still not fully understood; and (3) to describe the common mechanism of hard tissue breakdown.

Three (i.e., bone, cementum, and dentin) of the four hard tissues in the body have many similarities in their composition and formation. They are specialized connective tissues, and collagen (principally type

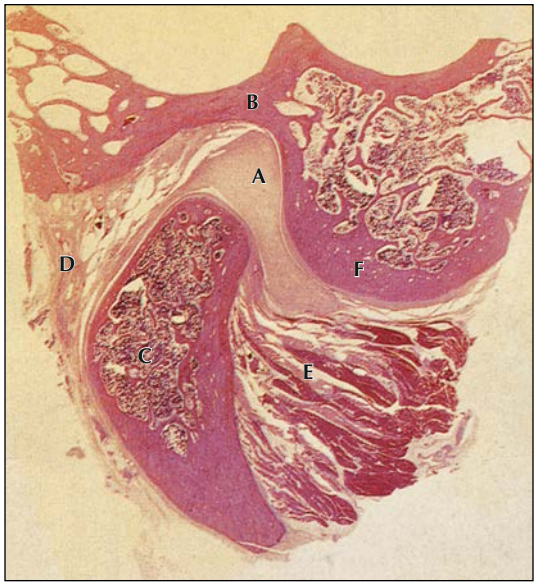


Fig. 1.9 Sagittal section through the temporomandibular joint. The disc (dividing the joint cavity into upper and lower compartments) is apparent. *A,* Intraarticular disc; *B,* mandibular (glenoid) fossa; *C,* condyle of mandible; *D,* capsule; *E,* lateral pterygoid muscle; *F,* articular eminence. (From Berkovitz BKB, et al.: *Oral anatomy, histology, and embryology,* ed 3, London, 2002, Mosby.)

I) plays a large role in determining their structure. Although enamel is not a connective tissue, and no collagen is involved in its makeup, its formation still follows many of the principles involved in the formation of hard connective tissue. Hard tissue formation may be summarized as the production by cells of an organic matrix capable of accommodating mineral. This rather simple concept, however, embraces complex events. How mineralization takes place is presented later.

The Organic Matrix in Hard Tissues

A hallmark of calcified tissues is the various matrix proteins that attract and organize calcium and phosphate ions into a structured mineral phase based on carbonated apatite. The formative blast cells of calcified tissues produce the organic matrix constituents that interact with the mineral phase. These cells specialize in protein synthesis and secretion, and they exhibit a polarized organization for vectorial secretion and appositional deposition of matrix proteins.

Of great interest is the fact that the proteins involved in these hard tissues, with one exception (enamel), are similar, comprising a predominant supporting meshwork of type I collagen with various added noncollagenous proteins functioning primarily as modulators of mineralization. Table 1.1 provides a comparative analysis of the characteristics of the various calcified tissues. This basic similarity of constituents is consistent with the general role of collagen-based hard tissues in providing rigid structural support and protection of soft tissues in vertebrates. Enamel has evolved to function specifically as an abrasion-resistant, protective coating that relies on its uniquely large mineral crystals for function. The organic matrix of enamel consists essentially of noncollagenous proteins that have no scaffolding role. However, enamel is not the only calcified tissue without collagen. Mineralization of cementum situated along the cervical margin of the tooth occurs within a matrix composed largely of noncollagenous matrix proteins also found in bone. In invertebrates, the shell of mollusks consists of laminae of calcium carbonate separated by a thin layer of organic material (acidic macromolecules, etc.).

Mineral

The inorganic component of mineralized tissues consists of hydroxyapatite, represented as $Ca_{10}(PO_4)_6(OH)_2$ and which has undergone substitutions with other ions. This formula indicates only the atomic content of a conceptual entity known as the *unit cell,* which is the least number of calcium, phosphate, and hydroxyl ions able to establish stable relationships. The unit cell of biologic apatite is hexagonal; when stacked together, these cells form the lattice of a crystal. The number of repetitions of this arrangement produces crystals of various sizes. Generally, the crystals are described as needlelike or platelike and, in the case of enamel, as long, thin ribbons. An unstable amorphous calcium phosphate phase may precede the formation of crystals.

Each apatite crystal has three compartments: the crystal interior, the crystal surface, and a layer of water called the *hydration shell,* all of which are available for the exchange of ions. Thus magnesium and sodium can substitute in the calcium position, fluoride and chloride in the hydroxyl position, and carbonate in the hydroxyl and phosphate positions. Fluoride substitution decreases the solubility of the crystals, whereas carbonate increases it. Magnesium inhibits crystal growth. The apatite crystal can retain its structural configuration while accommodating these substitutions.

In summary, biologic apatite is built on a definite ionic lattice pattern that permits considerable variation in its composition through substitution, exchange, and adsorption of ions. This pattern of ionic variability reflects the immediate environment of the crystal and is used clinically to modify the structure of crystals by exposing them to a fluoride-rich environment.

MINERALIZATION

Over the past few years there has been a shift in the perception of biologic mineralization, from a physiologic process highly dependent on sustained active promotion to one relying more on rate-limiting activities, including release from inhibition of mineralization (Box 1.1). Essentially, when calcium phosphate deposition is initiated, the crux is then to control spontaneous precipitation from tissue fluids supersaturated in calcium and phosphate ions and to limit it to well-defined sites. Formative cells achieve this by creating microenvironments that facilitate mineral ion handling and by secreting proteins that stabilize calcium and phosphate ions in body fluids and/or control their deposition onto a receptive extracellular matrix. Genome sequencing and gene mapping have shown that several of these proteins are located on the same chromosome and that there is synteny across several species.

Collectively, these proteins are referred to as the secretory calcium-binding phosphoprotein gene cluster that comprises (1) salivary proteins, (2) some enamel matrix proteins, and (3) bone/cementum/dentin matrix proteins. These proteins derive from the duplication and diversification of a common ancestral gene during evolution, with an enamel-related gene as an early intermediate in the process.

Initiation of Mineralization

Two mechanisms have been proposed for initiating mineralization of hard connective tissue. The first involves a structure called the *matrix vesicle* (Fig. 1.10), and the second is *heterogeneous nucleation.*

Matrix vesicles, first reported in 1967, have had an interesting history since their discovery, initially questioned as an artifact of tissue preparation. They are found in initial dentin, cementum, bone, and cartilage but not tooth enamel. Their abundance varies, and their detectability can be physiologically modulated (hypocalcemia); thus they appear to represent a *bona fide* entity but their exact role is questioned.

TABLE 1.1 Comparative Relationship Between Vertebrate Hard Tissues

	Enamel	Dentin	Fibrillar Cementum	Bone
Major Matrix Proteins				
Types	Amelogenin (several isoforms)	Collagen (type I) (+ type III, traces of V, VI)	Collagen (type I) (+ type III, XII, traces of V, VI, XIV)	Collagen (type I) (+ type III, traces of V, XII, XIV)
Conformation	Globular supramolecular aggregates; ribbons?	Random fibrils	Fibrils • Bundles (AEFC) • Sheets (CIFC)	Fibrils as random • Random (woven) • Sheets (lamellar)
Other Matrix Proteins				
Types	Nonamelogenins 1. Ameloblastin 2. Enamelin 3. Sulfated protein	Noncollagenous 1. Dentin sialophosphoprotein as transcript • Dentin glycoprotein • Dentin phosphoprotein • Dentin sialoprotein 2. Dentin matrix protein 1 3. Bone sialoprotein 4. Osteopontin 5. Osteocalcin 6. Osteonectin 7. Matrix extracellular phosphoglycoprotein	Noncollagenous 1. Bone sialoprotein 2. Osteopontin 3. Osteocalcin 4. Osteonectin 5. Dentin matrix protein 1 6. Dentin sialoprotein	Noncollagenous 1. Bone sialoprotein 2. Osteopontin 3. Osteocalcin 4. Osteonectin 5. Bone acidic glycoprotein-75 6. Dentin matrix protein 1 7. Dentin sialophosphoprotein as transcript 8. Matrix extracellular phosphoglycoprotein
Status of matrix proteins	Degraded along with amelogenins	Remain in matrix; also some present in peritubular dentin	Remain in matrix, but some may be degraded; also present in resting lines	Remain in matrix, but some may be degraded; also present in resting and reversal lines
Proteoglycans	Controversial	SLRP	SLRP	SLRP
Matrix Proteinases	1. MMP-20 (enamelysin) 2. KLK-4	Collagen-processing enzymes and others needed to degrade matrix	Collagen-processing enzymes and others needed to degrade matrix	Collagen-processing enzymes and others needed to degrade matrix
Mineral	Hydroxyapatite >90% ribbons (R) expand (mature crystallites can be millimeters in length)	Hydroxyapatite 67% Uniform small plates	Hydroxyapatite 45%–50% Uniform small plates	Hydroxyapatite 50%–60% Uniform small plates
Location of mineral	Between amelogenin nanospheres; related to ribbons?	Inside, at periphery, and between type I collagen fibril	Inside, at periphery, and between type I collagen fibril	Inside, at periphery, and between type I collagen fibril
Nucleated from	Controversial—Amelogenins? Nonamelogenins? Dentin?	Matrix vesicles then moving mineralization front, although additional mechanisms are most likely involved	Matrix vesicles then moving mineralization front, although additional mechanisms are most likely involved	Matrix vesicles then moving mineralization front, although additional mechanisms are most likely involved (See Boxes 1.1 and 1.2)
Prematrix	None present; crystallites abut plasma membrane of ameloblasts	Always present	Always present; usually very thin	Present only during formative phase

Continued

TABLE 1.1 Comparative Relationship Between Vertebrate Hard Tissues—cont'd

	Enamel	Dentin	Fibrillar Cementum	Bone
Growth Type				
	Appositional	Appositional	Appositional	Appositional
Cells				
Formative	Ameloblasts very tall and thin; multiple morphologies	Odontoblasts tall with long cytoplasmic processes	Cementoblasts short	Osteoblasts short
Microenvironment	Putatively sealed by secretory and ruffle-ended ameloblasts; leaky relative to smooth-ended ameloblasts	Incomplete, leaky junctions; cells act as limiting membrane	Cells widely spaced	No junctions at the level of the cell body; cells act as limiting membrane
Lifespan of formative cells	Limited to time until crown erupts	For life of tooth with gradual loss as pulp chamber occludes	Probably for life of tooth	Limited; associated with appositional growth phase
Maintenance	None	Odontoblast process	Cementocytes	Osteocytes
Lifespan of maintenance cells	NA	For life of tooth, with gradual loss as pulp chamber occludes	Limited by overall thickness of the layer	Long until area of bone undergoes turnover
Degradative	None per se; cells secrete proteinases	Odontoclasts	Odontoclasts/cementoclasts	Osteoclasts (limited lifespan)

Dentin, fibrillar cementum, and bone are collagen-based tissues. Enamel is outside rather than inside the body. Enamel, dentin, and cementum are not vascularized, and they do not turn over. Enamel, dentin, and primary cementum are acellular, but dentin contains the large, arborizing processes of odontoblasts embedded in the matrix.

AEFC, Acellular extrinsic fiber cementum; *CIFC*, cellular intrinsic fiber cementum; *KLK-4*, kallikrein-4; *MMP*, metalloproteinase; *NA*, not applicable; *SLRP*, small leucine-rich proteoglycans (biglycan, decorin).

From Nanci A, Smith CE: Matrix-mediated mineralization in enamel and the collagen-based hard tissues. In Goldberg M, Boskey A, Robinson C, editors: *Chemistry and biology of mineralized tissues*, Rosemont, IL, 1999, American Academy of Orthopedic Surgeons.

BOX 1.1 The Stenciling Principle of Extracellular Matrix Mineralization

The stenciling principle pertains to multilevel regulation of biologic mineralization within an extracellular matrix, as occurs in bones and teeth.[1] It describes a double-negative process whereby inhibition of inhibitors (i.e., release from inhibition at specific sites [stenciling]) activates/promotes mineralization, whereas the default condition of inhibition alone (without release) prevents mineralization elsewhere in soft connective tissues. It acts across multiple levels from the macroscale (skeleton/dentition vs. soft connective tissues) to the mesoscale (e.g., entheses and the tooth attachment complex where the soft periodontal ligament is situated between mineralized tooth cementum and mineralized alveolar bone), and to the microscale (mineral tessellation). It refers to inhibition of mineralization by both small molecules and proteins and then second-level inhibition of these inhibitors by enzymes that degrade the inhibitors to permit and carefully regulate mineralization. The stenciling principle for extracellular matrix mineralization derives from the original pivotal paradigm for negative regulation discovered by Francois Jacob and Jacques Monod in the 1950s and published in 1961. Their paradigm at that time of the double-negative "repressing a repressor" to induce an activation effect—originally explaining genetic regulation of enzyme expression in bacteria—continues today and explains many processes in developmental biology, cancer biology, and even ecology.

The stenciling principle that promotes mineralization of extracellular matrices is best exemplified by two well-documented (mostly in bone, but also to some degree in tooth dentin) enzyme and inhibitor substrate relationships. The first inhibition-of-an-inhibitor relationship exists as an enzyme-substrate pair consisting of the enzyme tissue-nonspecific alkaline phosphatase (TNAP, TNSALP, ALPL), which degrades the inhibitory substrate small biomolecule pyrophosphate (PP_i). The second inhibition-of-an-inhibitor relationship exists as an enzyme-substrate pair consisting of the enzyme phosphate-regulating endopeptidase homolog X-linked (PHEX), which degrades the inhibitory protein osteopontin (OPN). It is thought that these enzyme-substrate pairs may act sequentially in the order given earlier, with the former providing for initial release from inhibition of mineralization and the latter providing for subsequent finer regulatory control over mineralization of the extracellular matrix. It is expected that other as yet unknown inhibitor-inhibitor pairs exist, and there indeed may be some tissue specificity to other pairs.

To achieve mineralization, the stenciling principle has at its core the notion that cell- and tissue-specific expression of the inhibitor-degrading enzymes occurs by differentiated resident cells at sites of skeletal and dental extracellular matrix production. In the presence of local inhibitory extracellular PP_i and OPN, in concert with appropriate levels of circulating (systemic) calcium and phosphorus mineral ions required for mineralization, gene expression patterns encoding for enzyme production/activity locally stencil early mineralization patterns and trajectories into the extracellular matrix. Mineralization initiates through degradation of inhibitory PP_i by TNAP, then continues through degradation of inhibitory OPN by PHEX. Given the ubiquity of inhibitory PP_i in most, if not all, tissue fluids, it can be considered that this widespread small biomolecule generally inhibits mineralization everywhere as a default pathway of inhibition except for skeletal and dental sites that will be released from this inhibition (stenciled) by PP_i-degrading TNAP. Thus the cells expressing TNAP are osteoblasts and osteocytes in bone, and odontoblasts, cementoblasts, and cementocytes in teeth. Once triggered in the extracellular matrix, mineralization trajectories are further stenciled (regulated) and propagated by OPN-degrading PHEX. Among connective tissue

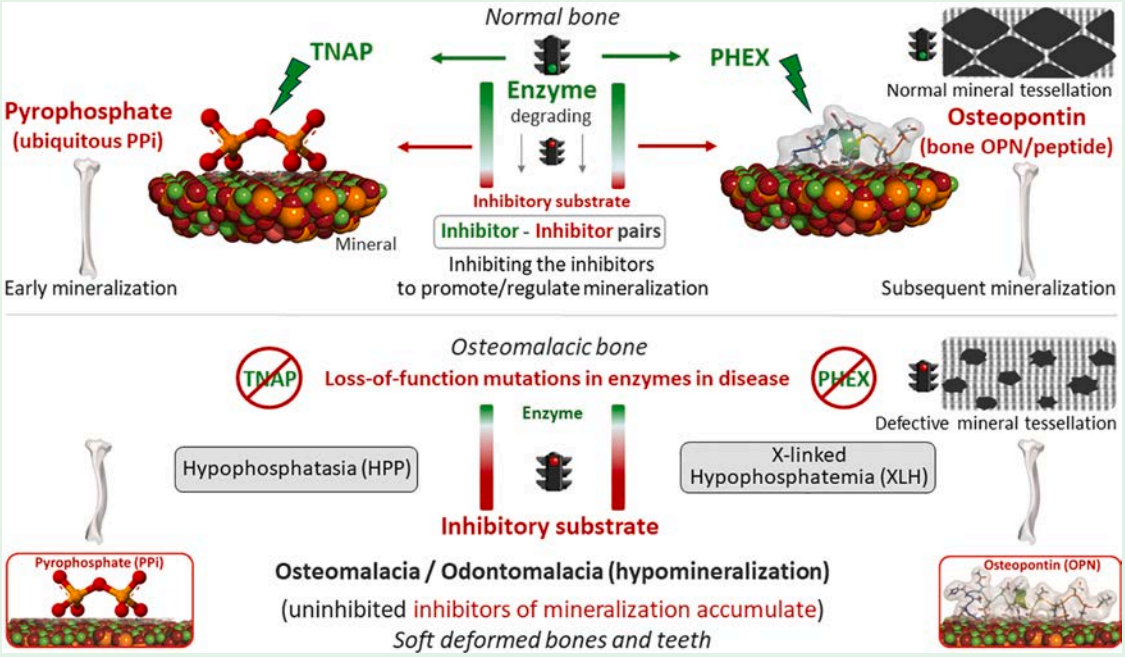
BOX 1.1 The Stenciling Principle of Extracellular Matrix Mineralization—cont'd

cells, inhibitory OPN is produced most abundantly by bone and tooth cells as a major noncollagenous protein of their respective extracellular matrices, and thus its removal (degradation) is required for release from OPN inhibition such that continued bulk mineralization of extracellular matrix occurs. As part of the PHEX-OPN mineral-stenciling axis, but acting at a very fine scale, is the regulation of mineralization that occurs at the extensive cell-matrix interface found along the lacuno-canalicular network in which osteocytes and their cell processes reside.

In bone, the stenciling principle connects to the patterning of mineralization at the micrometer scale by describing how mineral propagation from small mineralization foci located in the collagenous osteoid subsequently forms a repeating, space-filling structural motif for the mineral, termed *crossfibrillar mineral tessellation*. The pattern first appears at the mineralization front and extends into the bone across its lamellar structure. The microscale mineral formations have been called *tesselles* (French, "tiles"), and they geometrically approximate irregular prolate ellipsoids. Although the tesselles are closely packed, they remain discrete with no complete fusion against their adjacent, abutting tesselle neighbors.

Deviations From the Stenciling Principle Cause Mineralization Defects in Skeletal and Dental Diseases

Hypomineralization of bones and teeth and defective and incomplete bone tessellation structure can be seen in certain single-gene mutation (monogenic) mineralization diseases (osteomalacias/odontomalacias). Consistent with the notions of the stenciling principle, this subset of the skeletal dysplasias manifests as bones and teeth that are soft (hypomineralized) and deformed because of defective mineralization originating from the enzyme mutations. This is caused, in part (there is also renal phosphate wasting in the hypophosphatemias), by an abnormal, unbalanced enzyme-substrate relationship stemming from an inactivating mutation in the enzyme gene that leads to decreased enzyme activity and causes mineralization-inhibiting substrate to accumulate in the extracellular matrix. For two cases of osteomalacia/odontomalacia, the gene for TNAP enzyme is mutated in the case of hypophosphatasia, and the gene for PHEX is mutated in the case of X-linked hypophosphatemia (XLH). Reduced or absent ability of the enzyme to remove its respective mineralization-inhibiting substrate from the extracellular matrix results in local accumulation of the inhibitor, and in the case of XLH, altered systemic mineral ion homeostatic controls, both of which ultimately reduce bone and tooth mineralization to compromise skeletal and dental structure and function. Particularly well-documented for XLH, mutations in the PHEX gene result in increased inhibitory OPN in the extracellular matrix, which prevents nascent mineral tesselles from enlarging such that they do not properly abut against one another and fail to pack into a three-dimensional tessellation pattern that would ensure appropriate stiffness (Box 1.1 Fig. 1). This results in debilitating, incomplete mineral tessellation at the microscale that clinically renders bones deformable under loading.



Box 1.1 Fig. 1 Stenciling principle for bone mineralization.

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[1] From Buss DJ, et al.: Mineral tessellation in bone and the stenciling principle for extracellular matrix mineralization, *J Struct Biol* 214(1):107823, 2022. https://doi.org/10.1016/j.jsb.2021.107823.

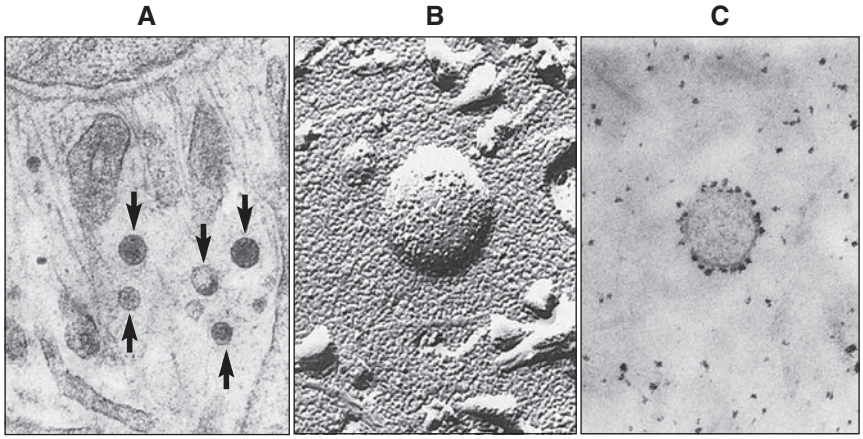


Fig. 1.10 (A) Matrix vesicles *(arrows)* as seen with an electron microscope. (B) Freeze fracture of the vesicle, showing many intramembranous particles thought to represent enzymes. (C) Histochemical demonstration of calcium-adenosine triphosphatase activity on the surface of the vesicle. (From Sasaki T, Garant PR: Structure and organization of odontoblasts, *Anat Rec* 22:235–249, 1996.)

A widely accepted view is that the matrix vesicle is a small, membrane-bound structure that buds off from the cell to form an independent unit within the first-formed organic matrix of hard tissues during initial mineralization. They provide a microenvironment in which proposed mechanisms for initial mineralization exist. Thus it contains alkaline phosphatase, calcium-adenosine triphosphatase, metalloproteinases, proteoglycans, and anionic phospholipids, which can bind calcium and inorganic phosphate and thereby form calcium–inorganic phosphate phospholipid complexes. The first morphologic evidence of a crystallite is seen within this vesicle. These membrane-bound extracellular vesicles have received much attention in the past few years for the biomolecules they contain, including nucleic acids, their ability to affect both physiologic and pathologic cell behavior, and their diagnostic and therapeutic potential.

In the second mechanism, during the formation of collagen-based calcified tissues, deposition of apatite crystals is catalyzed by charged amino acid side chains that line hole zones and channels of molecular assemblages of collagen fibrils (Figs. 1.11 and 1.12). These residues are thought to bind calcium and phosphate ions from solution and bring these ions into proximity so that they may interact to form initial prenucleation clusters and calcium phosphate nuclei inside and on the surface of fibrils. Osteocalcin has also been found at both sites, suggesting it could participate in nucleation and stabilization of mineral ions or phases. Altogether, this event leads to nucleation and the growth of crystals aligned inside collagen or disposed in random arrangements outside fibrils. This process is schematically represented in Fig. 1.13. Eventually, intrafibrillar and extrafibrillar collagen spaces are entirely mineralized, although extrafibrillar volume may vary with species, tissue, and speed of formation of the mineralizing matrix.

The role of type I collagen to mediate vertebrate mineral deposition may thus be modified to include contributions from certain noncollagenous proteins and possibly other molecules. More detailed discussion of the role of collagen in mineralization is presented by Landis et al. (see Recommended Reading). Neither of the two abovementioned mechanisms is involved in the mineralization of enamel; matrix vesicles are absent, and enamel contains no collagen. Initiation of enamel mineralization is believed to be achieved by crystal growth from the already mineralized dentin, by matrix proteins secreted by the ameloblasts, or by both processes.

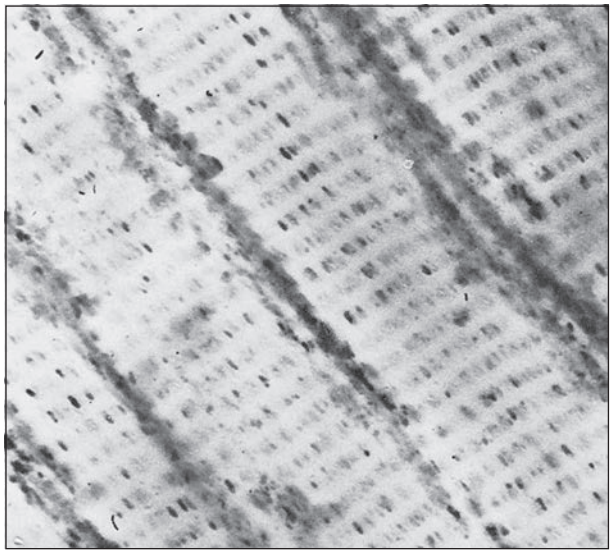


Fig. 1.11 Electron micrograph showing the disposition of crystals in collagen fiber bundles. The gaps in the collagen fibrils are where mineral has been deposited. (From Nylen MU, et al.: Mineralization of turkey leg tendon. II. Collagen-mineral relations revealed by electron and x-ray microscopy. In Sognnaes RF, editor: *Calcification in biological systems* [pub no 64], Washington, DC, 1960, American Association for the Advancement of Science, pp 129–142.)

Crystal Growth

When an apatite crystal has been initiated, its initial growth is rapid but then slows. Several factors influence crystal growth and composition, but especially important is the immediate environment of the growing crystal. For example, noncollagenous proteins can bind selectively to different surfaces of the crystal, preventing further growth and thereby determining the final size of the crystal. The accumulation of inorganic pyrophosphoric acid (pyrophosphate [PP_i]) at the crystal surface also blocks further growth.

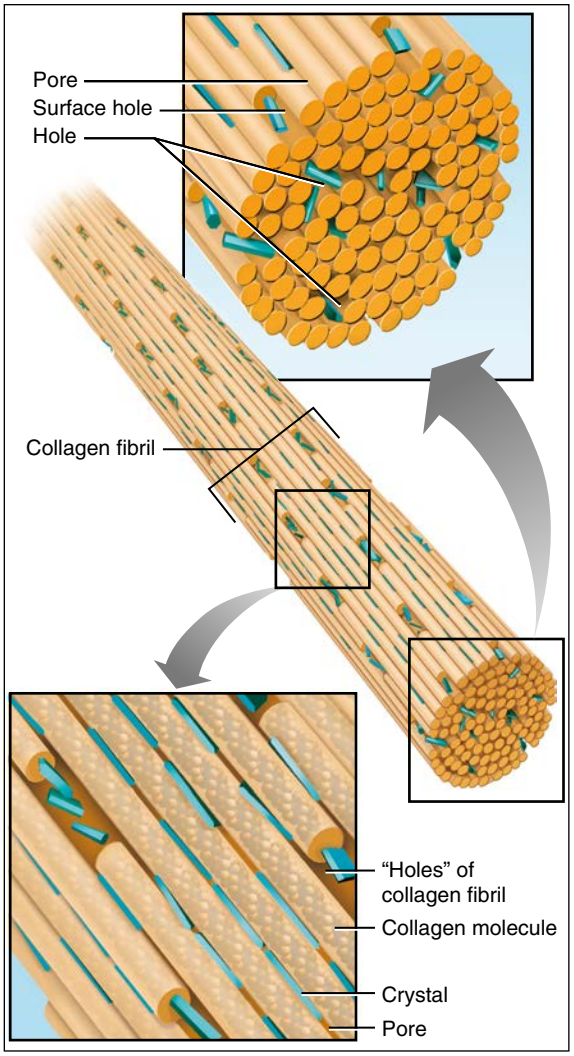


Fig. 1.12 Schematic illustration of the localization of mineral within the collagen fibril. (Redrawn from Glimcher MJ: On the form and function of bone: from molecules to organ. Wolffs law revisited, 1981. In Veis A, editor: *The chemistry and biology of mineralized connective tissues*, Amsterdam, 1981, Elsevier, pp 616–673.)

Alkaline Phosphatase

Alkaline phosphatase activity is always associated with the production of a mineralized tissue, and the implicated isozymes are part of the mammalian alkaline phosphatase gene family. Because the major isozyme is found in several other tissues, the isozyme is referred to as *tissue-nonspecific alkaline phosphatase*. In all cases, alkaline phosphatase exhibits a similar pattern of distribution and is involved with the blood vessels and cell membrane of hard tissue–forming cells. In hard connective tissues, alkaline phosphatase also is found in the organic matrix, associated with matrix vesicles (when present) and occurring freely within the matrix.

Although the enzyme alkaline phosphatase has a clearcut function, its role in mineralization is not yet fully defined. A precise description of this role is complicated by at least two factors. First, the term *alkaline phosphatase* is nonspecific, describing enzymes that have the capacity to cleave phosphate groups from substrates to provide phosphate ions at mineralization sites, most efficiently at an alkaline pH. Second, the enzyme may have more than one distinct function in mineralization.

The extracellular activity of alkaline phosphatase at mineralization sites occurs where continuing crystal growth is taking place. At these sites the enzyme is believed to have the function of cleaving PP_i. Hydroxyapatite crystals in contact with serum or tissue fluids are prevented from growing larger because PP_i ions are deposited on their surfaces, inhibiting further growth. Alkaline phosphatase activity breaks down PP_i, thereby permitting crystal growth to proceed.

The stenciling principle describes cell- and tissue-specific enzyme expression and activity to create at least two nested circuits of inhibition of inhibitors (or release from inhibition) for extracellular matrix mineralization—one that first permits mineralization and a second that sculpts in more detail hydroxyapatite crystal growth patterns. Together these act to ensure that mineralization in the skeleton and dentition occurs in the right locations and to the right extent. Both circuits arise from resident cell activity that also includes extracellular release of mineralization inhibitors (see Box 1.1).

Transport of Mineral Ions to Mineralization Sites

Although the subject has been studied extensively, the mechanism(s) whereby large amounts of phosphate and calcium are delivered to calcification sites is still not fully resolved. Mineral ions can reach a mineralization front by movement through or between cells. Tissue fluid is supersaturated in these ions, and it is possible that fluid simply needs to percolate between cells to reach the organic matrix where local factors then would permit mineralization. A priori, this mechanism is more likely to occur between cells, such as osteoblasts and odontoblasts, that have no complete tight junctions and where serum proteins, such as albumin, can be found in the osteoid and predentin matrix they produce. This also applies to cementoblasts that frequently are separated from each other by PDL fibers entering cementum. Several facts, however, complicate such a simple explanation. For example, hormones influence the movement of calcium in and out of bone. Thus it has been proposed that osteoblasts and odontoblasts form a sort of limiting membrane that would regulate ion influx into their respectable tissues.

The situation would seem more straightforward for enamel, where tight junctions between secretory stage ameloblasts restrict the passage of calcium. It has been concluded that during the secretory phase of enamel formation, some calcium likely passes between cells but that the majority of calcium entry into enamel occurs through a transcellular route. The situation is different during the maturation stage.

The possibility of transcellular transport is dictated by a particular circumstance: The cytosolic free calcium ion concentration cannot exceed 10^{-6} mol/L because a greater concentration would cause calcium to inhibit critical cellular functions leading to cell death. Two mechanisms have been proposed that permit transcellular transport of calcium without exceeding this critical threshold concentration. The first suggests that, as calcium enters the cell through specific calcium channels, it is sequestered by calcium-binding proteins that, in turn, are transported through the cell to the site of release. The second suggests that a continuous and constant flow of calcium ions occurs across the cell without the concentration ever exceeding 10^{-6} mol/L. Finally, intracellular compartments (e.g., endoplasmic reticulum and mitochondria) also play a role in calcium handling. Calcium has been localized to these structures not only in hard tissue–forming cells but

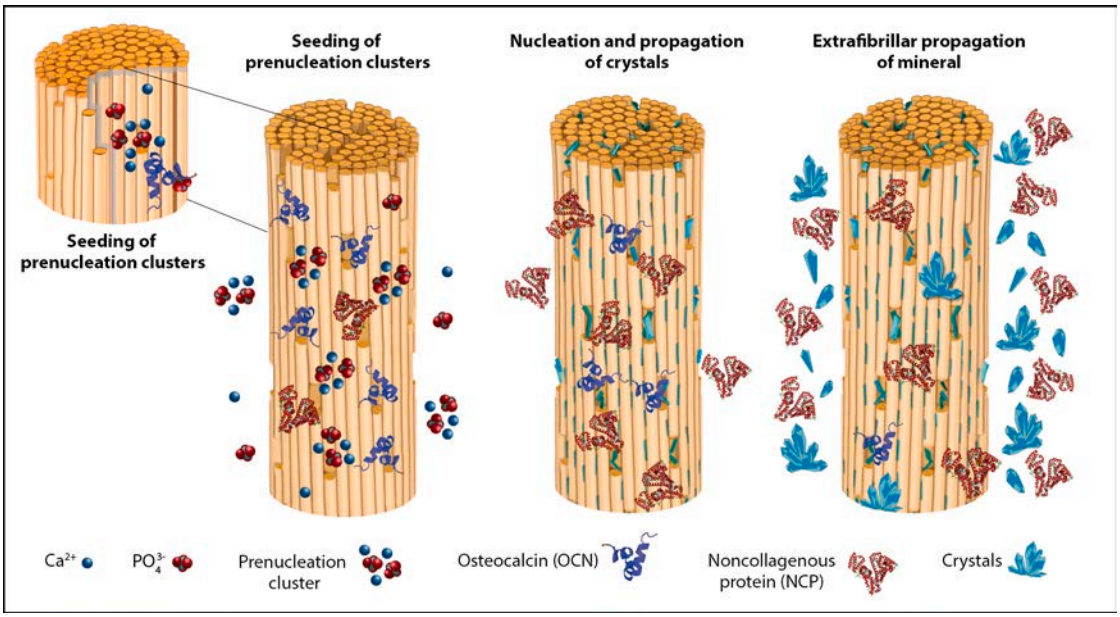


Fig. 1.13 Diagrammatic representation of intrafibrillar and extrafibrillar mineralization of collagen.

also in most other cells, and it is believed that the sequestration of calcium to these organelles is a safety device to control the calcium concentration of the cytosol.

Recent data from cryogenically preserved embryonal chicken bone (Box 1.2) have further shown the presence of numerous intracellular vesicles containing mineral precursors in osteocytes as well as in osteoblasts and preosteocytes. A series of nanochannels associated with the osteocyte lacunar system, has also been put in evidence for the passive transport of mineral precursor to the site of mineralization. The generalized presence and functionality of these nanochannels in other species, however, remain to be demonstrated.

HARD TISSUE DEGRADATION

Bone is remodeling constantly by an orchestrated interplay between removal of old bone and its replacement by new bone. Formative and destructive phases result from the activity of cells derived from two separate lineages. The osteoblasts, originating from mesenchyme in the case of long bones, are responsible for bone formation, whereas osteoclasts, originating from the blood (monocyte/macrophage lineage), destroy focal areas of bone as part of normal maintenance. Enamel under ameloblasts undergoes removal of matrix proteins by a process of extracellular enzymatic processing similar to that in the resorption lacuna under osteoclasts. The exact extent of the degradation of its organic matrix constituents and the exact manner by which their fragments leave the site of resorption are still not fully defined; in bone, transcytosis is involved (see Chapter 6). Such tissues as cementum and dentin do not normally undergo turnover, but all hard tissues of the tooth can be resorbed under certain normal eruptive conditions (e.g., deciduous teeth) and under certain pathologic conditions, including excessive physical forces and inflammation. The cells involved in their resorption have similar characteristics to osteoclasts but generally are referred to as *odontoclasts* (see Chapter 10).

SUMMARY OF HARD TISSUE FORMATION

Formative cells situated close to a good blood supply, producing an organic matrix capable of accepting mineral (apatite). These cells thus have the cytologic features of cells that actively synthesize and secrete protein. Mineralization in the connective hard tissues entails an initial nucleation mechanism involving a cell-derived matrix vesicle and the control of spontaneous mineral precipitation from supersaturated tissue fluids. After initial nucleation, further mineralization is achieved in relation to the collagen fiber and spread of mineral within and between fibers. In enamel, mineralization initiates either in relation to preexisting apatite crystals of dentin or enamel matrix proteins. Alkaline phosphatase is associated with mineralization, but its role is still not fully understood. The breakdown of hard tissue involves the macrophage system, which produces a characteristic multinucleated giant cell, the osteoclast. To break down hard tissue, this cell attaches to mineralized tissue and creates a sealed environment that is first acidified to demineralize the hard tissue. After exposure to the acidic environment, the organic matrix is broken down by proteolytic enzymes. In enamel, the challenge is to maintain a relatively neutral pH environment that will prevent mineral dissolution and allow optimal activity of the enzymes that break down the organic matrix components.

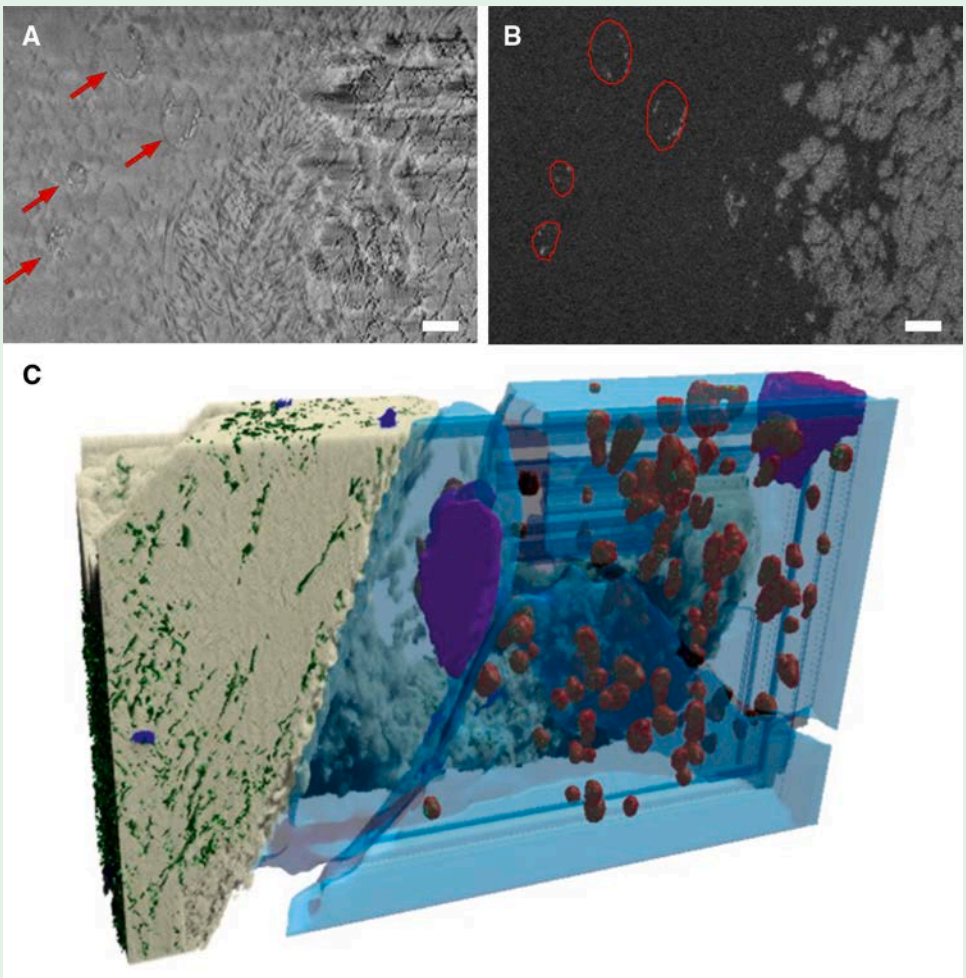
BOX 1.2 Mineralization Logistics During Bone Formation

During the formation and development of skeletal tissues, large amounts of calcium ions need to be transported from the bloodstream to the sites of mineralization. These ions must overcome huge distances and be moved through extracellular and intracellular compartments where calcium is highly regulated and cannot exceed millimolar and micromolar concentrations, respectively. Indeed, cells must maintain a very low calcium concentration in their cytosol that is crucial for their intracellular signaling functions. Yet, calcium concentration eventually reaches molar concentrations in the fully mineralized tissue. Thus the transportation of mineralization precursors in growing bone represents a major challenge.

New data acquired in the femur of the fast-growing chick embryo by focused ion beam with scanning electron microscopy (FIB-SEM) under cryogenic condition have shed some light on the understanding of how vertebrates overcome this major logistic problem.[1] The three-dimensional visualization of large volumes at high resolution and in a close-to-native state has revealed the presence of numerous intracellular vesicles containing mineral precursors (Box 1.2 Fig. 1) in osteocytes as well as in osteoblasts/preosteocytes. Based on the quantification of the volume of the different structures from the segmented data (see Box 1.2 Fig. 1C), an intracellular density of 0.037 vesicles per $\mu m\mu^3$ on average can be estimated, and the mineral precursors found inside these vesicles occupy less than 10% of the volume.

Based on these FIB-SEM data and on the osteocytic lacunar density established using microCT, one can attempt to interpret the process of biomineralization in forming bone in a dynamic manner. To provide the calcium needed to mineralize the amount of bone tissue synthesized during 1 day, the available intracellular vesicles need to be transported at a velocity of 0.27 µm/s. Such a high velocity, similar to one of molecular motors involved in vesicle transport, suggests that the vesicles containing mineral precursors are trafficked through the cellular network by active cellular processes. In addition, an interconnected network of nanochannel of approximately 40 nm in diameter and in relation with the canaliculi was imaged. These nanochannels could facilitate the passive transport of the mineral precursor over the last 1 or 2 µm to the site of mineralization after being externalized by the cells; however, no extracellular vesicles were observed in the cryo-FIB/SEM experiments.

Although the presence of intracellular vesicles containing mineral precursors has been observed in various animal models,[2–6] our quantitative 3D study proposes an alternative calcium transport mechanism that requires revisiting the prominent existing theory on bone mineralization, the Matrix Vesicle-Mediated Mineralization, at least in chick embryos. Initially formulated by Anderson[7] and Bonucci,[8] this model suggests that chondrocytes/osteoblasts release small vesicles into the extracellular matrix, initiating the nucleation and growth of hydroxyapatite crystals for tissue mineralization. These vesicles have also been observed in initial dentin and cementum but not in tooth enamel. In addition, they are readily apparent among the abundant mineralization foci present in the osteoid seam of trabecular bone in a rat model of hypocalcemia, suggesting that they can be physiologically modulated.[9] Irrespective of the fact that no vesicles have been observed in the extracellular matrix in this study, matrix vesicles are characterized by a small diameter (20 to 200 nm) and a relatively lower occurrence,[10] prompting concerns about their ability to meet the essential mineralization demands for chick embryo development.



Box 1.2 Fig. 1 Scanning electron microscope images acquired in cryomode of (A) mixed in-lens/secondary electron detector *(arrows)* showing the presence of intracellular vesicles *(arrows)*. Mineral precursors are found inside some of the vesicles and appear brighter in the corresponding backscattered electron detector image *(contour of the vesicle is shown in red)* (scale bar: 1 µm). (C) Perspective rendering of three-dimensional segmented data showing the different structural features: Numerous vesicles containing mineral precursors *(red)* are found inside the cells *(light blue)*. Nuclei are shown in purple. Canaliculi *(dark blue)* penetrate the mineralized bone matrix *(light yellow)*, the latter being also composed of an extensive network of nanochannels *(green)*.

(Continued)

BOX 1.2 Mineralization Logistics During Bone Formation—cont'd

However, the density of matrix vesicles has not been firmly established, making a direct comparison precarious. It remains conceivable, though, that both mechanisms—intracellular and matrix vesicles—may act simultaneously in different regions of the long chick bone.

Hence this study revisited the embryonal bone mineralization from the viewpoint of calcium transportation. The new proposed model (Box 1.2 Fig. 2), based on experimental and conceptual data, suggests that bone mineralization is enabled by different transport mechanisms in which calcium ions first transit in the vasculature, probably regulated by the action of calcium-binding proteins. Active transport of calcium-loaded vesicles within the osteocytic cellular network is then at play, allowing mineral ions to be trafficked over long distance (tens of micrometers). Finally, diffusion through a nanochannel network could be the way to bridge the last micrometers. Many open questions remain, such as the specific mechanism by which calcium ions are packaged into intracellular vesicles and then released from them into the matrix. Moreover, the observations have been made in embryonal chicken skeleton, and it cannot be excluded that different skeletal materials use different strategies in the mineralization process.

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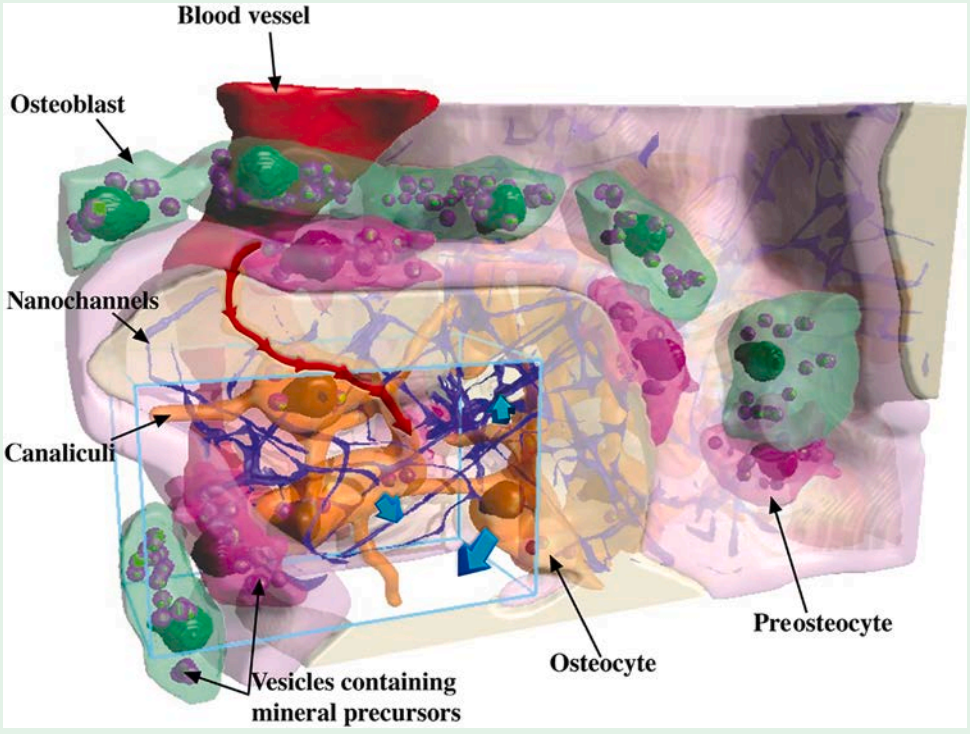

Box 1.2 Fig. 2 Schematic representation of the new model of calcium transportation during mineralization of a forming bone showing the active transportation of vesicles containing mineral precursors *(red arrows)* that subsequently shed their content into the matrix. This mineral content is then passively transported by diffusion through the nanochannels network to finally reach the sites of mineralization.

RECOMMENDED READING

Landis WJ, editor, et al.: *Current concepts of the mineralization of type I collagen in vertebrate tissues*, 1st ed., United Kingdom, 2021, Taylor & Francis Group.

2

General Embryology

Antonio Nanci

OUTLINE

This chapter provides basic general embryology information needed to explain the development of the head, particularly the structures in and around the mouth. It supplies a background for understanding (1) origins of the tissues associated with facial and dental development and (2) the cause of many congenital defects that manifest in these tissues.

GERM CELL FORMATION AND FERTILIZATION

The human somatic (body) cell contains 46 chromosomes, 46 being the diploid number for the cell. Two of these are sex chromosomes; the remaining are autosomes. Each chromosome is paired so that every cell has 22 homologous sets of paired autosomes, with one sex chromosome derived from the mother and one from the father. The sex chromosomes, designated X and Y, are paired as XX in the female and XY in the male.

Fertilization is the fusion of male and female germ cells (the spermatozoa and ova, collectively called *gametes*) to form a zygote, which commences the formation of a new individual. Germ cells are required to have half as many chromosomes (the haploid number) so that, on fertilization, the original complement of 46 chromosomes will be reestablished in the new somatic cell. The process that produces germ cells with half the number of chromosomes of the somatic cell is called *meiosis.* Mitosis describes the division of somatic cells.

Before mitotic cell division begins, DNA is first replicated during the synthetic phase of the cell cycle so that the amount of DNA is doubled to a value known as tetraploid (four times the amount of DNA found in the germ cell). During mitosis the chromosomes containing this tetraploid amount of DNA are split and distributed equally between the two resulting cells; thus both daughter cells have a diploid DNA quantity and chromosome number, which duplicates the parent cell exactly.

Meiosis, by contrast, involves two sets of cell divisions occurring in quick succession. Before the first division, DNA is replicated to the tetraploid value (as in mitosis). In the first division the number of chromosomes is halved, and each daughter cell contains a diploid amount of DNA. The second division involves the splitting and separation of the chromosomes, resulting in four cells; thus the final composition of each cell is haploid with respect to its DNA value and its chromosome number.

Meiosis is discussed in this textbook because the process occasionally malfunctions by producing zygotes with an abnormal number of chromosomes and individuals with congenital defects that sometimes affect the mouth and teeth. For example, an abnormal number of chromosomes can result from the failure to separate a homologous chromosome pair during meiosis so that the daughter cells contain 24 or 22 chromosomes. If, on fertilization, a gamete containing 24 chromosomes fuses with a normal gamete (containing 23 chromosomes), the resulting zygote will possess 47 chromosomes; one homologous pair has a third component. Thus the cells are trisomic for a given pair of chromosomes. If one member of the homologous chromosome pair is missing, a rare condition known as *monosomy* prevails. The best-known example of trisomy is Down syndrome (trisomy 21). Among features of Down syndrome are facial clefts, a shortened palate, a protruding and fissured tongue, and delayed eruption of teeth.

Approximately 10% of all human malformations are caused by an alteration in a single gene. Such alterations are transmitted in several ways, of which two are of special importance. First, if the malformation results from autosomal dominant inheritance, the affected gene generally is inherited from only one parent. The trait usually appears in every generation and can be transmitted by the affected parent to statistically half of the children. Examples of autosomal dominant conditions include achondroplasia, cleidocranial dysostosis, osteogenesis imperfecta, and dentinogenesis imperfecta; the latter two conditions result in abnormal formation of the dental hard tissues. Dentinogenesis imperfecta (Fig. 2.1) arises from a mutation in the dentin sialophosphoprotein gene. Second, when the malformation is a result of autosomal recessive inheritance, the abnormal gene can express itself only when it is received from both parents. Examples include chondroectodermal dysplasia, some cases of microcephaly, and cystic fibrosis.

All of these conditions are examples of abnormalities in the genetic makeup or genotype of the individual and are classified as genetic defects. The expression of the genotype is affected by the environment in which the embryo develops, and the outcome of development is termed the *phenotype.* Adverse factors in the environment can result in excessive deviation from a functional and accepted norm; the outcome is described as a congenital defect. Teratology is the study of such developmental defects.

PRENATAL DEVELOPMENT

Prenatal development is divided into three successive phases (Fig. 2.2). The first two, when combined, constitute the embryonic stage, and the third is the fetal stage. The forming individual is described as an embryo or fetus depending on its developmental stage.

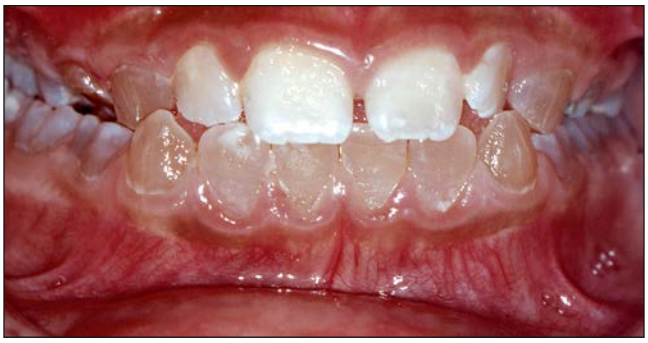

Fig. 2.1 Intraoral view of the dentition of a child with dentinogenesis imperfecta, an autosomal dominant genetic defect. (Courtesy A. Kauzman.)

The first phase begins at fertilization and spans the first 4 weeks or so of development. This phase involves largely cellular proliferation and migration, with some differentiation of cell populations. Few congenital defects result from this period of development because, if the perturbation is severe, the embryo is lost.

The second phase spans the next 4 weeks of development and is characterized largely by the differentiation of all major external and internal structures *(morphogenesis)*. The second phase is a particularly vulnerable period for the embryo because it involves many intricate embryologic processes; during this period, many recognized congenital defects develop.

From the end of the second phase to term, further development is largely a matter of growth and maturation, and the embryo now is called a fetus.

INDUCTION, COMPETENCE, AND DIFFERENTIATION

Patterning is key in development from the initial axial (head-to-tail) specification of the embryo through its segmentation. It is a spatial and temporal event that implicates the classical processes of induction, competence, and differentiation. These concepts also apply to the

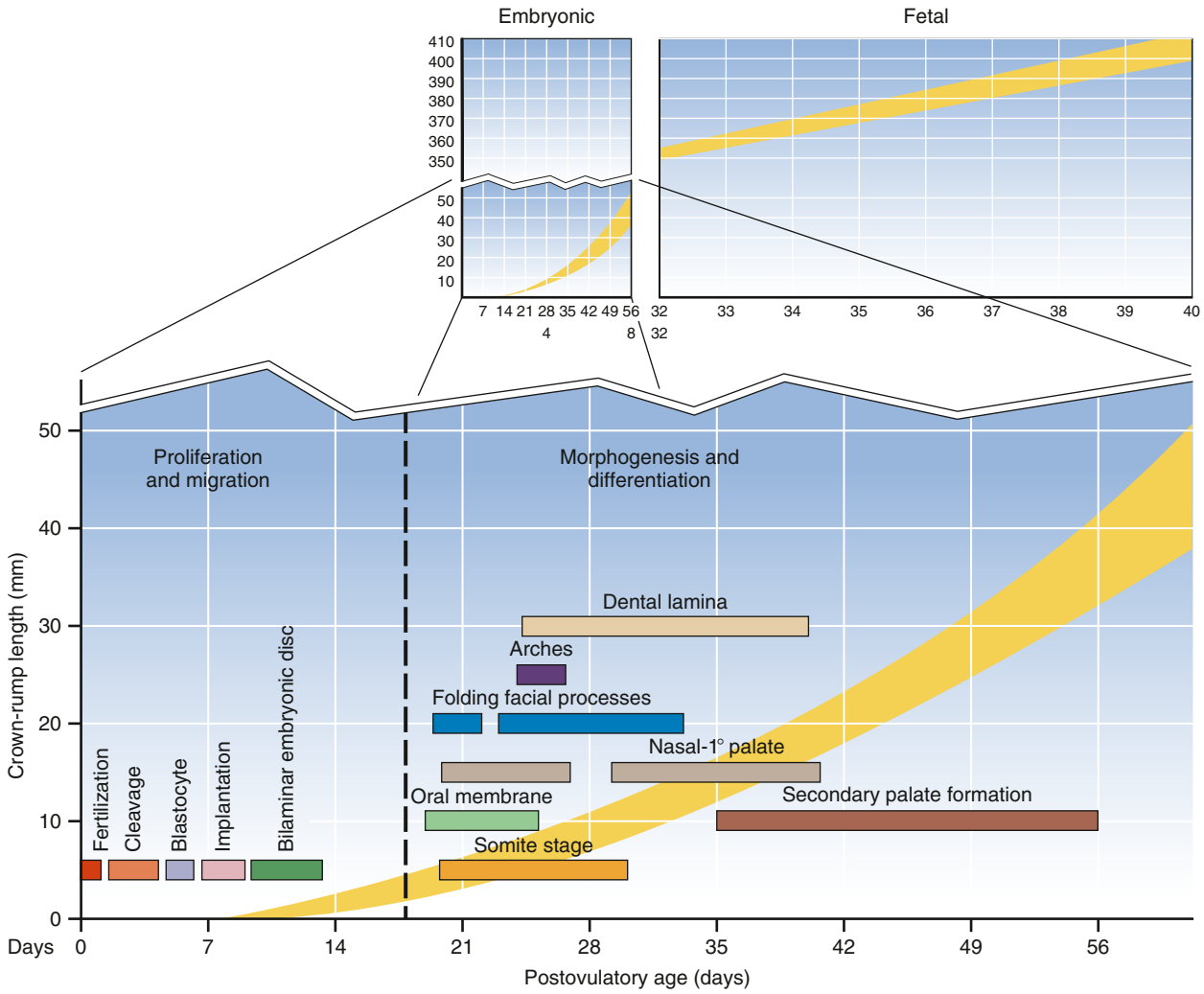

Fig. 2.2 Sequences of prenatal development. The upper diagram shows the distinction between embryonic and fetal stages. The lower part of the embryonic diagram is expanded in the bottom diagram, which distinguishes the stages of proliferation and migration and morphogenesis and differentiation. The timing of key events also is indicated. (From Waterman RE, Meller SM: Congenital craniofacial abnormalities. In Shaw JH, et al., editors: *Textbook of oral biology*, Philadelphia, 1978, WB Saunders Co, pp. 863–896.)

development of the tooth and its supporting tissues, as exemplified by regional development of incisors, canines, premolars, and molars.

Every cell of an individual stems from the zygote. Clearly, they have differentiated somehow into populations that have assumed particular functions, shapes, and rates of turnover. The process that initiates differentiation is induction; an inducer is the agent that provides cells with the signal to enter this process. Furthermore, each compartment of cells must be competent to respond to the induction process. Windows of competence of varying duration exist for different populations of cells.

Homeobox genes and growth factors play crucial roles in development. All homeobox genes contain a similar region of 180 nucleotide base pairs (the homeobox) and function by producing proteins (transcription factors) that bind to the DNA of other downstream genes, thereby regulating their expression. By knocking out such genes or by switching them on, it has been shown that they play a fundamental role in patterning. Furthermore, combinations of differing homeobox genes provide codes or sets of assembly rules to regulate development; one such code is involved in dental development (see Chapter 5).

Homeobox genes act in concert with other groups of regulatory molecules (i.e., growth factors and retinoic acids). Growth factors are polypeptides that belong to a number of families. For them to have an effect, cells must express cell-surface receptors to bind them. When bound by the receptors, there is transfer of information across the plasma membrane and activation of cytoplasmic signaling pathways to cause alteration in the gene expression. Thus a growth factor is an inductive agent, and the appropriate expression of cell-surface receptors bestows competency on a cell. A growth factor produced by one cell and acting on another is described as *paracrine regulation,* whereas the process of a cell that recaptures its own product is known as *autocrine regulation* (Fig. 2.3). The extensive and diverse effects of a relatively few growth factors during embryogenesis can be achieved by cells expressing combinations of cell-surface receptors requiring simultaneous capture of different growth factors to respond in a given way (Fig. 2.4). Such combinations represent another example of a developmental code. By contrast, the retinoic acid family freely enters a cell to form a complex with intracellular receptors, which eventually affects gene expression. Growth factors and retinoids regulate the expression of homeobox genes, which, in turn, regulate the expression of growth factors, an example of the role of regulatory loops in development.

FORMATION OF THE THREE-LAYERED EMBRYO

After fertilization, mammalian development involves a phase of rapid proliferation and migration of cells with little or no differentiation. This proliferative phase lasts until three germ layers have formed.

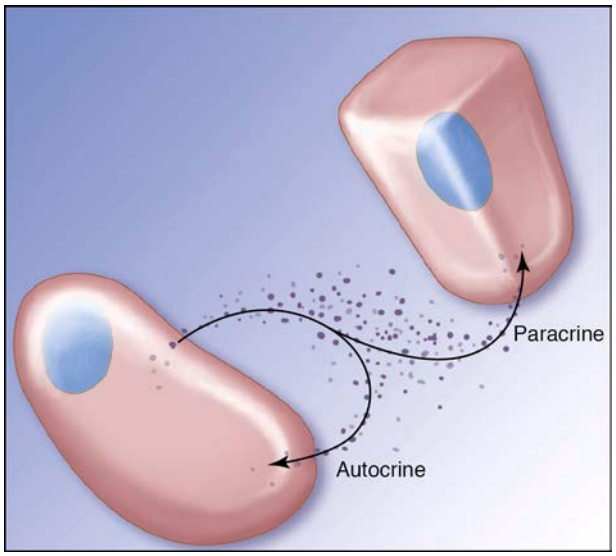

Fig. 2.3 Autocrine and paracrine regulation. On the left, the cell captures its own cytokine (autocrine); on the right, the cytokine is captured by a nearby target cell (paracrine).

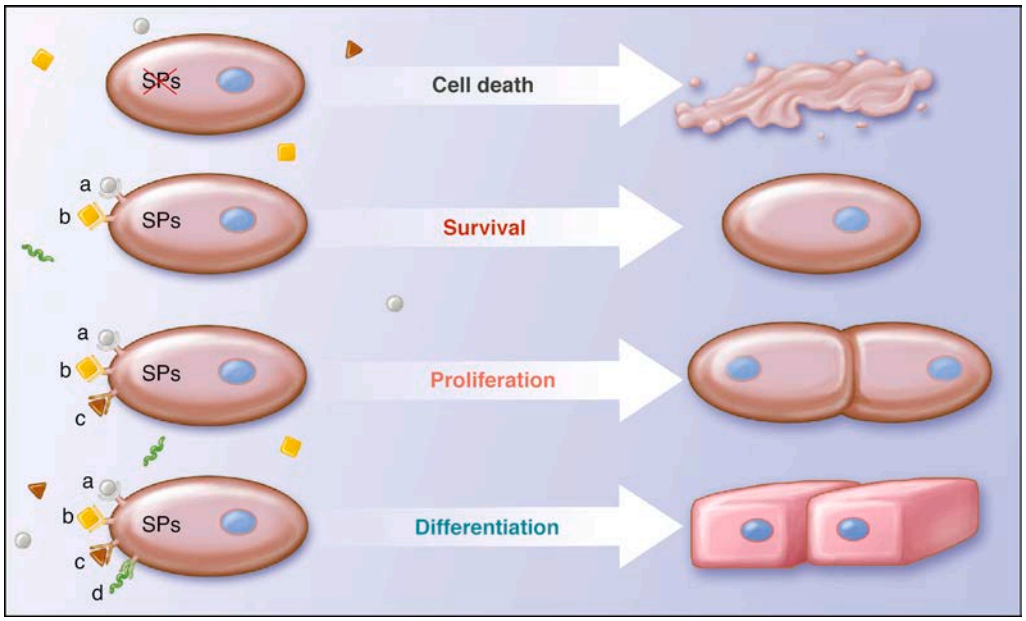

Fig. 2.4 Modulation of the expression of cell-surface receptors *(colored membrane-bound forms)* results in the binding of different combinations of growth factors *(a-d, colored geometric forms)* that influence cellular outcome. When binding occurs, there is transfer of information across the plasma membrane and activation of cytoplasmic signaling pathways *(SPs)* to cause alteration in gene expression. In the absence of receptors, binding with grown factors cannot take place, and cell death occurs.

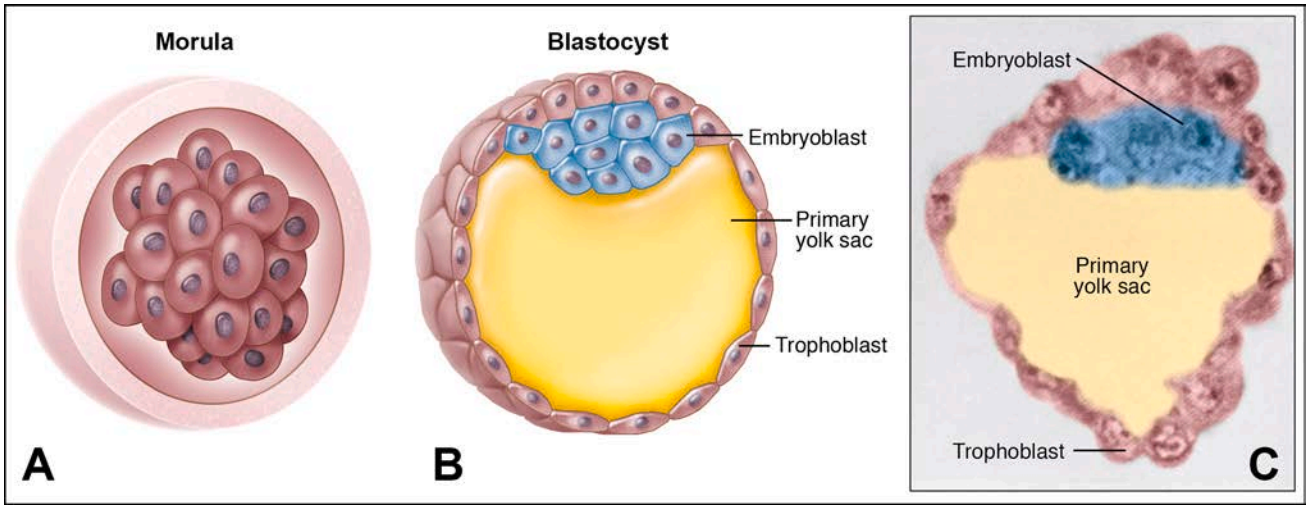

Fig. 2.5 Drawing of the transformation of the (A) morula into a (B) blastocyst. (C) Colorized histologic section illustrates the differentiation at this time of the blastocyst into trophoblast cells that line the cavity of the primary yolk sac and are involved in maintenance of the embryo and of embryoblast cells that form a small cluster within the cavity that are involved in the development of the embryo. Adapted from Hertig AT, et al.: *Contrib Embryol* 35:199–220, 1954, Used with permission from Carnegie Institution for Science.

In summary, the fertilized egg initially undergoes a series of rapid divisions that lead to the formation of a ball of cells called the *morula.* Fluid accumulates in the morula, and its cells realign themselves to form a fluid-filled hollow ball, called the *blastocyst.* Two cell populations now can be distinguished within the blastocyst: (1) those lining the cavity (the primary yolk sac), called *trophoblast cells;* and (2) a small cluster within the cavity, called the *inner cell mass* or *embryoblast* (Fig. 2.5). The embryoblast cells form the embryo proper, whereas the trophoblast cells are associated with implantation of the embryo and formation of the placenta (they are not described further here).

At about day 8 of gestation, the cells of the embryoblast differentiate into a two-layered disk called the *bilaminar germ disk.* The cells on the dorsal aspect, the ectodermal layer, are columnar and reorganize to form the amniotic cavity. Those on the ventral aspect, the endodermal layer, are cuboidal and form the roof of a second cavity (the secondary yolk sac), which develops from the migration of peripheral cells of the extraembryonic endodermal layer. This configuration is completed after 2 weeks of development (Fig. 2.6). During this time the axis of the embryo is established and is represented by a slight enlargement of the ectodermal and endodermal cells at the head (cephalic or rostral) end of the embryo in a region known as the *prochordal* (or *prechordal*) plate where ectoderm and endoderm are in contact (Fig. 2.7A; see also Fig. 2.6A).

During the third week of development, the embryo enters the period of gastrulation during which the germ layers forming the bilaminar embryonic disk are converted to a trilaminar disk (see Fig. 2.7). As previously described, the floor of the amniotic cavity is formed by ectoderm, and, within it, a structure called the *primitive streak* develops along the midline by cellular convergence (see Fig. 2.7A). This structure is a narrow groove with slightly bulging areas on each side. The rostral end of the streak finishes in a small depression called the *primitive node,* or *pit.* Cells of the ectodermal layer migrate through the streak and between the ectoderm and endoderm. The cells that pass through the streak change shape and migrate away from the streak in lateral and cephalic directions. The cells from the cephalic regions form the notochord process, which pushes forward in the midline as far as the prochordal plate. Through canalization of this process, the notochord is formed to support the primitive embryo.

Elsewhere alongside the primitive streak, cells of the ectodermal layer divide and migrate toward the streak where they invaginate and spread laterally between the ectoderm and endoderm. These cells, sometimes called the *mesoblast,* infiltrate and push away the extraembryonic endodermal cells of the hypoblast, except for the prochordal plate, to form the true embryonic endoderm. They also pack the space between the newly formed embryonic endoderm and the ectoderm to form a third layer of cells called the *mesoderm* (see Fig. 2.7B–D). In addition to spreading laterally, cells spread progressively forward, passing on each side of the notochord and prochordal plate. The cells that accumulate anterior to the prochordal plate because of this migration give rise to the cardiac plate, the structure in which the heart forms (see Fig. 2.7A). As a result of these cell migrations, the notochord and mesoderm now separate the ectoderm from the endoderm (see Fig. 2.7C), except in the region of the prochordal plate and in a similar area of fusion at the tail (caudal) end of the embryo called the *cecal plate.*

FORMATION OF THE NEURAL TUBE AND FATE OF THE GERM LAYERS

The series of events leading to the formation of the three-layered, or triploblastic, embryo during the first 3 weeks of development now has been sketched. These initial events involve cell proliferation and migration. During the next 3 to 4 weeks of development, major tissues and organs differentiate from the triploblastic embryo; these include the head, face, and tissues contributing to development of the teeth. Key events are the differentiation of the nervous system and neural crest tissues from the ectoderm, the differentiation of mesoderm, and the folding of the embryo in two planes along the rostrocaudal (head-to-tail) and lateral axes.

The nervous system develops as a thickening within the ectodermal layer at the rostral end of the embryo. This thickening constitutes the neural plate, which rapidly forms raised margins (neural folds). These folds, in turn, encompass and delineate a deepening midline depression—the neural groove (Fig. 2.8). The neural folds eventually fuse so

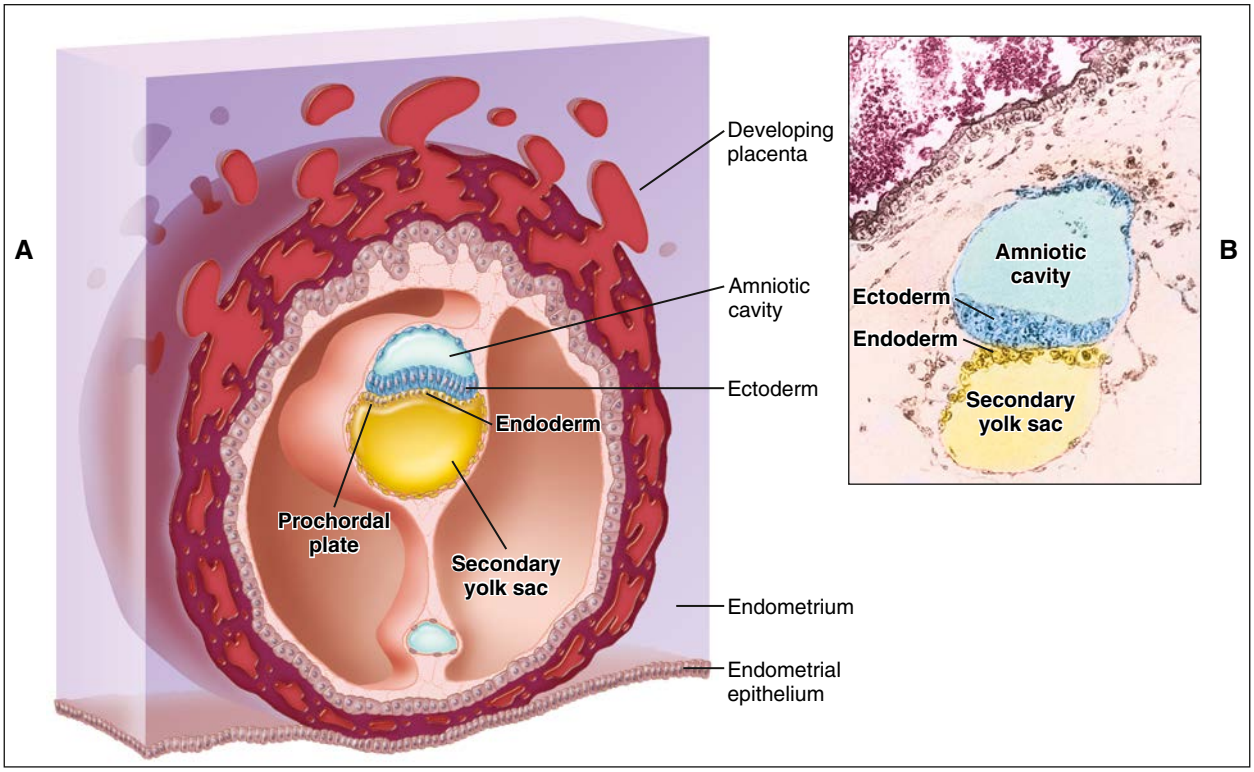

Fig. 2.6 (A) Schematic representation and (B) histologic section of a human blastocyst at 13 days of gestation. An amniotic cavity has formed within the ectodermal layer. Proliferation of endodermal cells forms a secondary yolk sac. The bilaminar embryo is well established. (B, From Brewer JI: A human embryo in the bilaminar blastodisc stage [the Edwards-Jones-Brewer ovum], *Contrib Embryol Carnegie Instn* 27:85–93, 1938.)

that a neural tube separates from the ectoderm to form the floor of the amniotic cavity with mesoderm intervening.

As the neural tube forms, changes occur in the mesoderm adjacent to the tube and the notochord. The mesoderm first thickens on each side of the midline to form paraxial mesoderm. Along the trunk of the embryo, this paraxial mesoderm breaks into segmented blocks called *somites.* Each somite has three components: (1) the sclerotome, which eventually contributes to two adjacent vertebrae and their disks; (2) the myotome, which gives origin to a segmented mass of muscle; and (3) the dermatome, which gives rise to the connective tissue of the skin overlying the somite. In the head region, the mesoderm only partially segments to form a series of numbered somitomeres, which contribute in part to the head musculature. At the periphery of the paraxial mesoderm, the mesoderm remains as a thin layer (intermediate mesoderm), which becomes the urogenital system. Further laterally the mesoderm thickens again to form the lateral plate mesoderm, which gives rise to (1) the connective tissue associated with muscle and viscera; (2) the serous membranes of the pleura, pericardium, and peritoneum; (3) the blood and lymphatic cells; (4) the cardiovascular and lymphatic systems; and (5) the spleen and adrenal cortex.

A different series of events takes place in the head region. First, the neural tube undergoes massive expansion to form the forebrain, midbrain, and hindbrain. The hindbrain exhibits segmentation by forming a series of eight bulges, known as *rhombomeres,* which play an important role in the development of the head (see Chapter 3).

Folding of the Embryo

A crucial developmental event is the folding of the embryo in two planes along the rostrocaudal axis and along the lateral axis (Fig. 2.9). The head fold is critical to the formation of a primitive stomatodeum or oral cavity; ectoderm comes through this fold to line the primitive stomatodeum, with the stomatodeum separated from the gut by the buccopharyngeal membrane (Fig. 2.10).

Fig. 2.11 illustrates how the lateral folding of the embryo determines this disposition of mesoderm. As another result, the ectoderm of the floor of the amniotic cavity encapsulates the embryo and forms the surface epithelium. The paraxial mesoderm remains adjacent to the neural tube and notochord. The lateral plate mesoderm cavitates to form a space (coelom), and the mesoderm bounding the cavity lines the body wall and gut. Intermediate mesoderm is relocated to a position on the dorsal wall of the coelom. The endoderm forms the gut. Fig. 2.12 indicates the final disposition of the mesoderm and the derivatives of the ectoderm, endoderm, and cranial neural crest.

The Neural Crest

As the neural tube forms during neurulation, a group of cells along the dorsal-lateral margins of the closing neural folds become distinct from the neuroectoderm. These so-called *neural crest cells (NCCs)* receive inductive signals to undergo an epithelial-mesenchymal transition (EMT), a process whereby their cell adhesive properties and cytoskeletal organization change, allowing them to delaminate and migrate extensively away from the neural tube to multiple locations in the embryo, where they give rise to a myriad of cell types throughout the body (Figs. 2.13 and 2.14; see also Fig. 2.12). NCCs exhibit the exceptional capacity of stem and progenitor cells, and advances in the NCC field continue to uncover the genes, proteins, and regulatory networks that endow them with such capacity (Box 2.1). Pathway signaling molecules belonging to the bone morphogenetic proteins, Wnt (wingless homologue in vertebrates), fibroblast growth factor and secreted by the surrounding nonneural ectoderm and underlying mesoderm, play a critical role in inducing the NCC cascade. At the molecular level, NCC competence is indicated by the expression

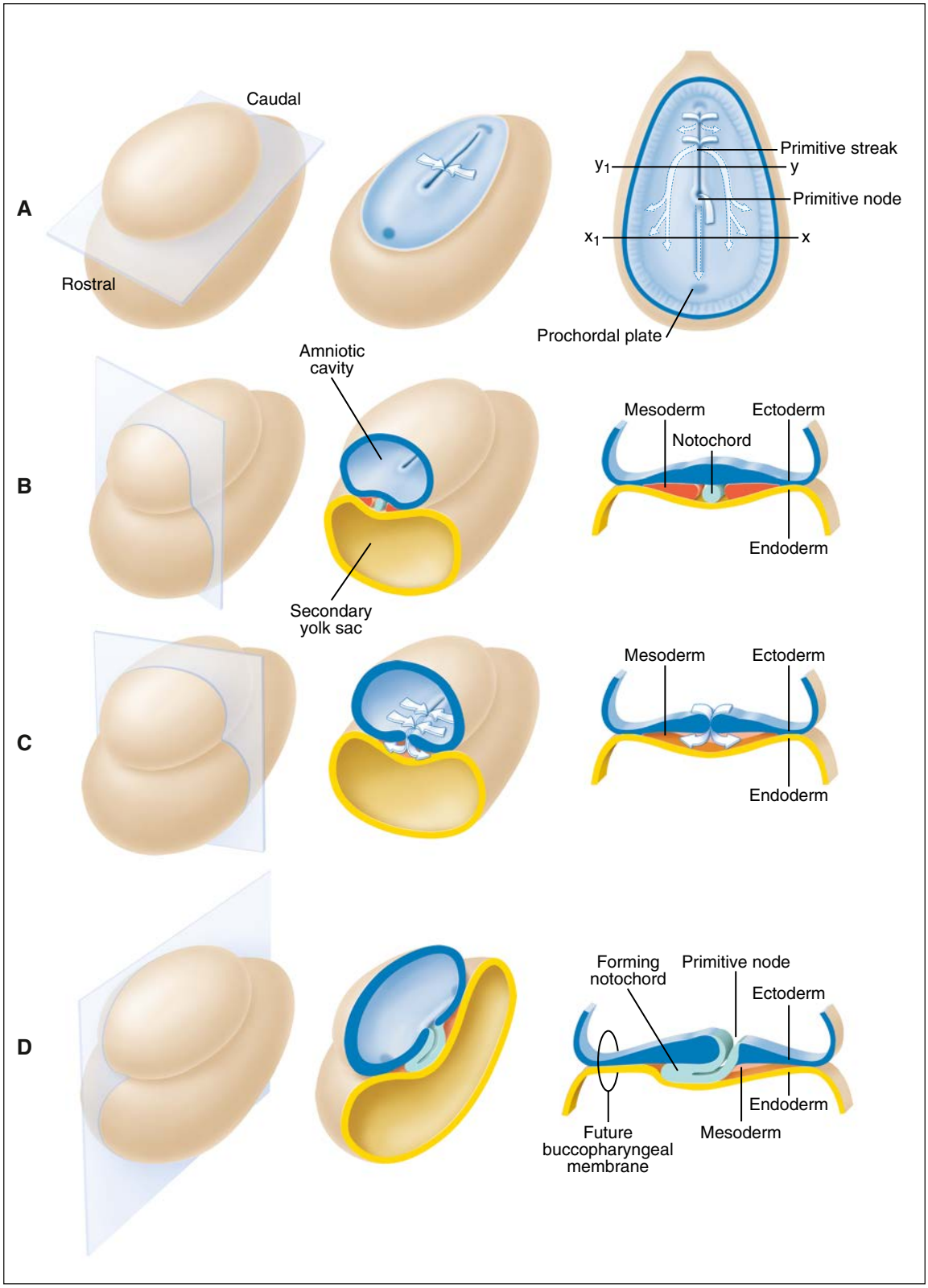

Fig. 2.7 Gastrulation conversion of the bilaminar embryo into a trilaminar embryo. *(Left column)* The plane of section for the middle and right columns. *(Middle column)* A three-dimensional view. *(Right column)* A two-dimensional representation. (A) The floor of the amniotic cavity, formed by the ectodermal layer of the bilaminar embryo. Ectodermal cells converge toward the midline to form the primitive streak, a narrow groove terminating in a circular depression called the *primitive node*. Ectodermal cells then migrate through the streak and between the ectodermal and endodermal layers in lateral and cephalic directions *(arrows)*. A notochord process extends forward from the primitive node. (B) A transverse section through x-x_1, showing the notochord flanked by mesoderm. (C) A section through y-y_1. (D) Notochord pushing rostrally as seen in longitudinal section.

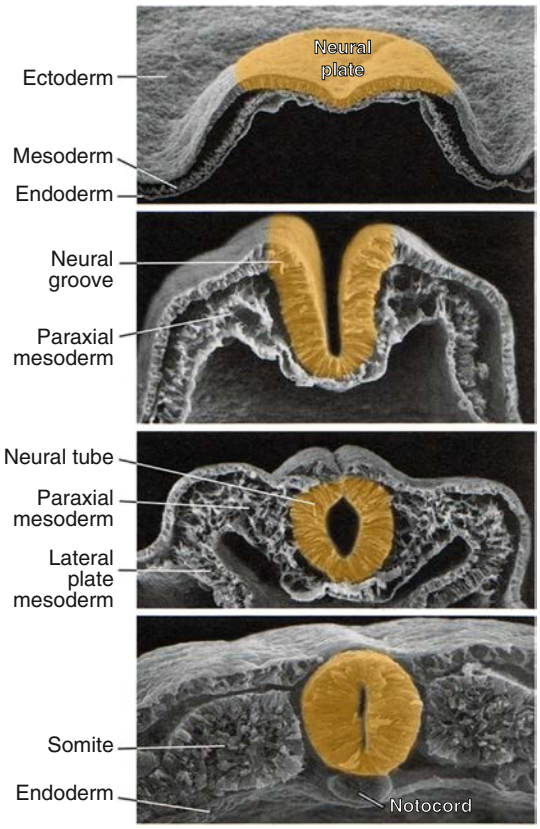

Fig. 2.8 Scanning electron micrograph views of formation and closure of the neural fold elevations. (Courtesy G. Schoenwolf.)

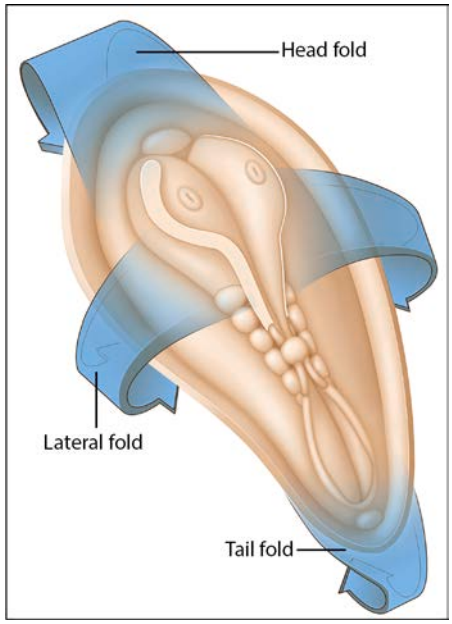

Fig. 2.9 Embryo at 21 days of gestation, before folding. The arrows indicate where folding occurs.

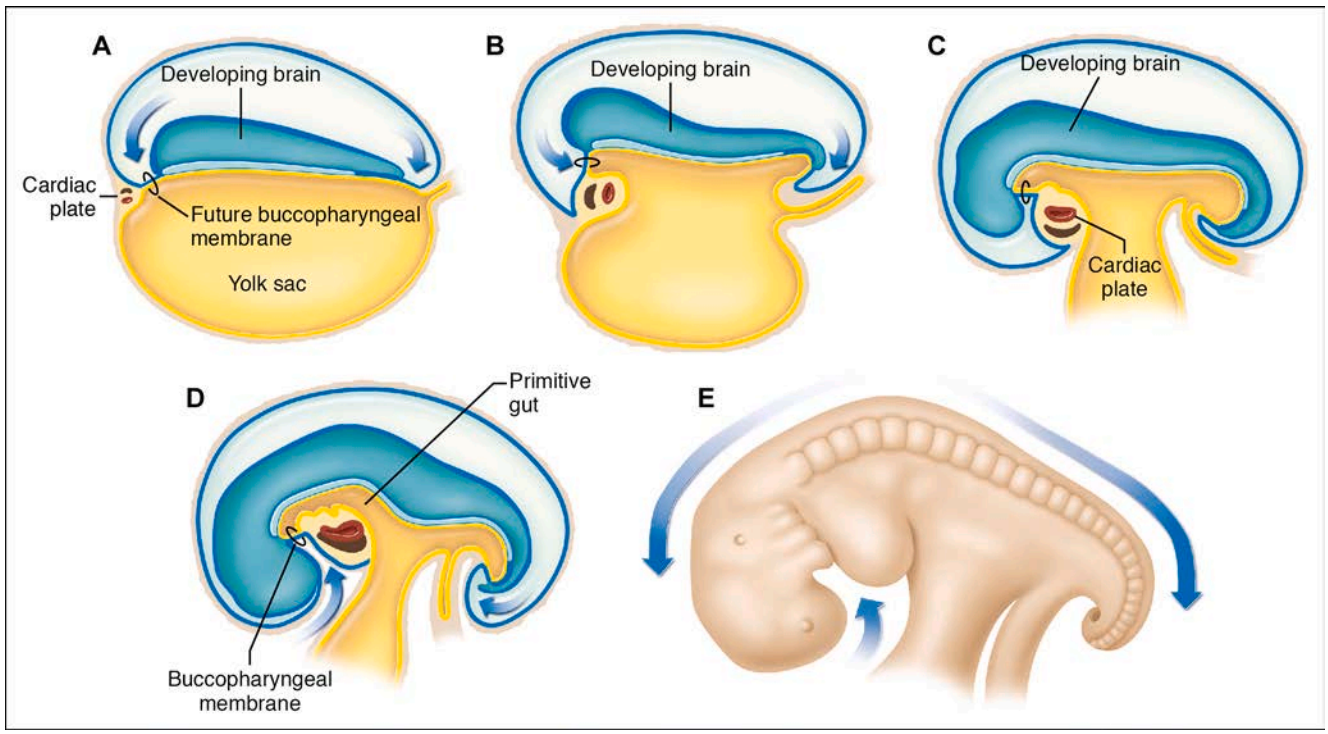

Fig. 2.10 Sagittal sections of embryos illustrate the effects of the caudocephalic foldings. (A) Where folding begins; (B) the onset of folding at 24 days of gestation. (C, D) Days 26 and 28, respectively, show how the head fold establishes the primitive stomatodeum, or oral cavity *(arrow),* bounded by the developing brain and cardiac plate. It is separated from the foregut by the buccopharyngeal membrane. (E) The embryo at completion of folding.

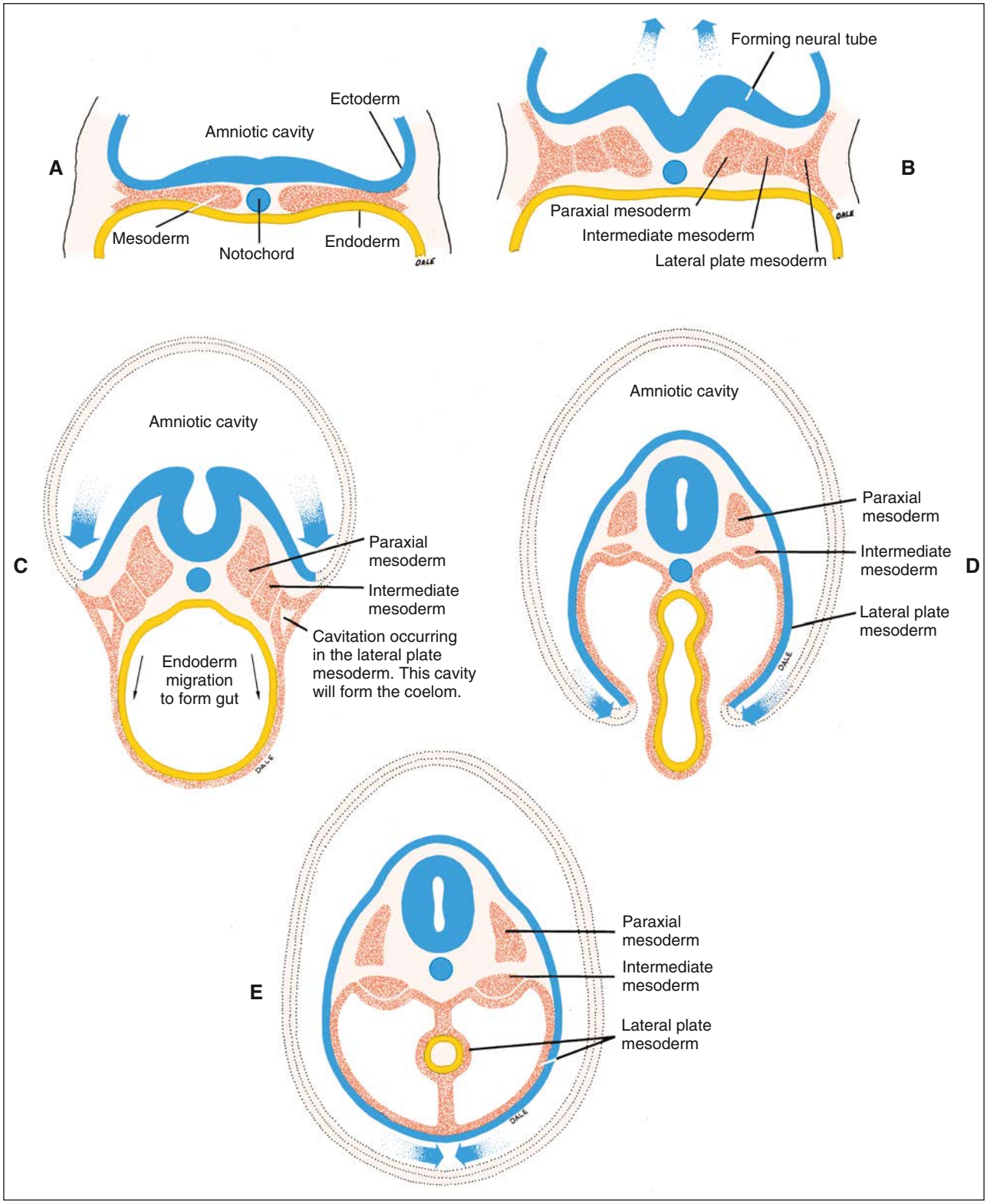

Fig. 2.11 Cross-sectional profiles. (A) The mesoderm, situated between the ectoderm and endoderm in the trilaminar disk. (B) Differentiation of the mesoderm into three masses: the paraxial, intermediate, and lateral plate mesoderm. (C–E) With lateral folding of the embryo, the amniotic cavity encompasses the embryo, and the ectoderm, constituting its floor, forms the surface epithelium. Paraxial mesoderm remains adjacent to the neural tube. Intermediate mesoderm is relocated and forms urogenital tissue. Lateral plate mesoderm cavitates, forming the coelom and its lining the serous membranes of the gut and abdominal cavity.

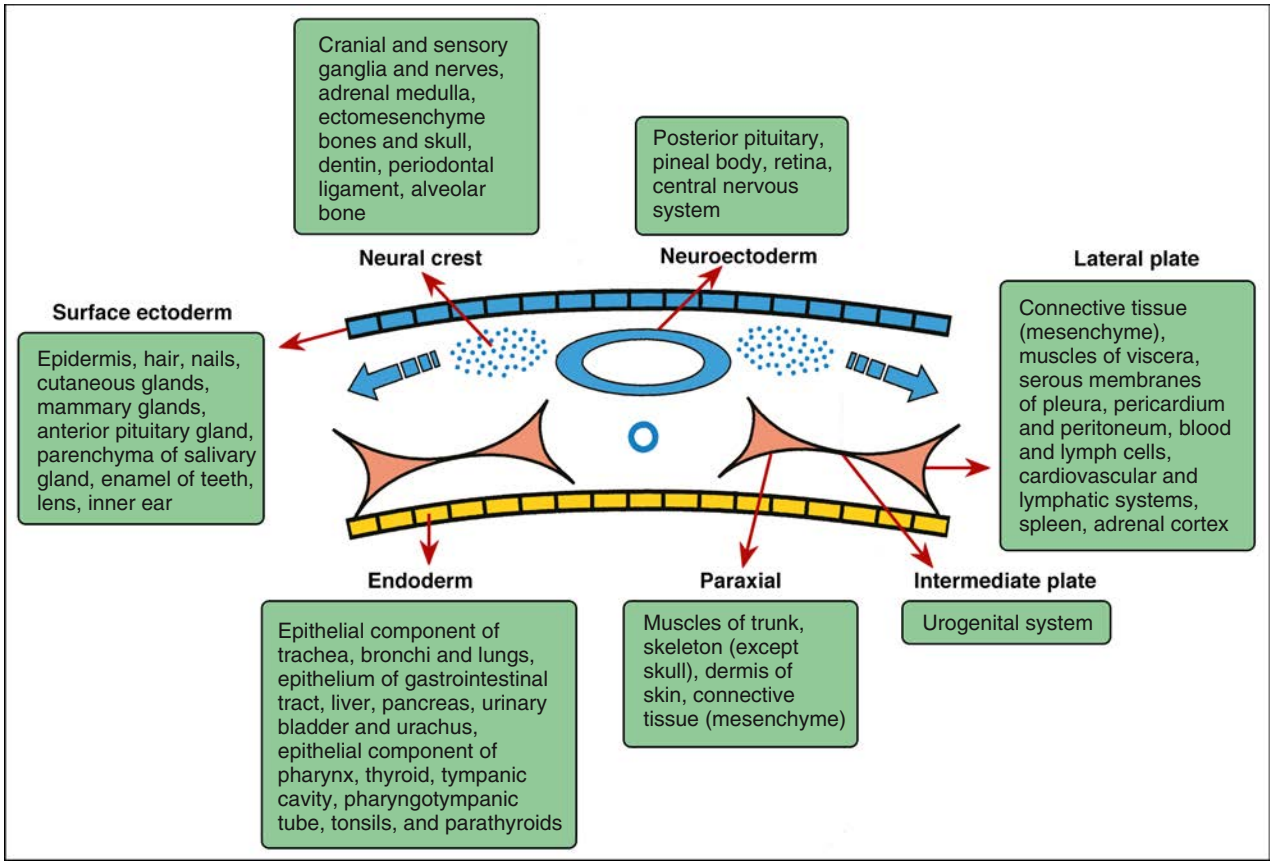

Fig. 2.12 Derivatives of the germ layers and cranial neural crest.

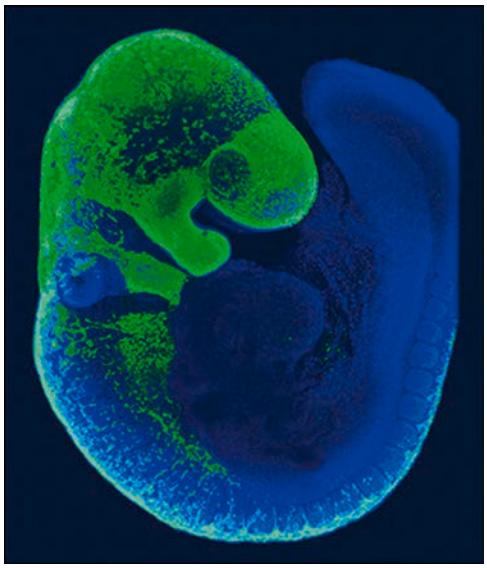

Fig. 2.13 Migration of neural crest cells throughout the embryo traced in a knock-in Pax3-GFP *(green)* transgenic mouse model. (Courtesy A. Barlow and P. Trainor, Stowers Institute for Medical Research.)

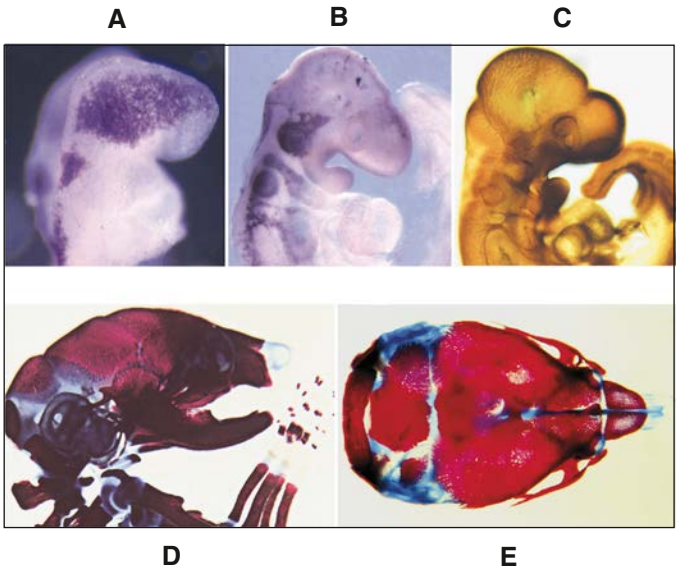

Fig. 2.14 Migration and differentiation of cranial neural crest cells (NCCs). (A) Migrating NCC. (B, C) Neuronal differentiation of NCC. (D) Skeletal differentiation of NCC. (E) Neurocranium (bone, *red;* cartilage, *blue*). (From Trainor P: Specification of neural crest cell formation and migration in mouse embryos, *Semin Cell Dev Biol* 16(6):683–693, 2005.)

BOX 2.1 Neural Crest Cells and Their Application in Regenerative Medicine

Neural crest cells (NCCs) comprise a migratory stem and progenitor cell population that forms during the third to fourth weeks of human embryonic development. Derived from the ectoderm during the period of neurulation, NCCs are essential both for embryo development and throughout adult life. NCCs are formed along almost the entire length of the embryo and can be subdivided into distinct axial populations: cranial, cardiac, trunk, and sacral. Cranial NCCs give rise to the precursors of most of the cranial cartilage, bone and connective tissue of the craniofacial skeleton, the meninges surrounding the brain, and odontoblasts of the teeth. Cranial and trunk NCCs generate neurons and glia within the peripheral and enteric nervous systems, and they differentiate into melanoblasts (pigment cells of the skin). Cardiac NCCs generate smooth muscle cells of the cardiovascular system and form the septum of the heart. Vagal and sacral NCCs give rise to neurons and glia in the gastrointestinal tract. Trunk NCCs also differentiate into hormone-secreting cells of the adrenal gland. In fact, there is barely a tissue or organ throughout the human body that does not receive a contribution from NCCs.

NCCs are considered a vertebrate-specific cell type that has played a central role in the evolution of novel morphologic structures and their variation and adaptation; however, the evolutionary origins of NCCs remain an enigma. One hypothesis suggests that NCCs may have initially emerged in tunicates. Advances in the NCC field continue to uncover the genes, proteins, and regulatory networks that endow NCCs with their stem and progenitor cell–like properties and astonishing array of lineage descendant cell fates. Much of the focus on NCCs therefore currently revolves around their contributions to congenital disorders and diseases, which are collectively termed *neurocristopathies.* This includes disorders of craniofacial development such as cleft palate and craniosynostosis; anomalies of cardiac development, including persistent truncus arteriosus; malformation of gastrointestinal development as occurs in Hirschsprung disease; and cancers such as neuroblastoma and melanoma, which affect the peripheral nervous system and skin, respectively. Understanding the genetic etiology and cell and tissue pathogenesis of individual neurocristopathies offers the potential for developing reparative, regenerative, or preventive therapies for treating neurocristopathies.

Stem cell transplantations have been touted as a therapeutic strategy in the treatment of neurocristopathy disorders and diseases. Although embryonic stem cells were once considered ideal for this purpose because of their extraordinary pluripotency, their derivation is still ethically controversial, and the potential for host rejection remains high. In contrast, adult stem cells are available from numerous tissue sources and can be derived from an affected individual without ethical concern or fear of transplant rejection. The identification of multipotent NCC progenitors in adults has therefore facilitated their therapeutic application in tissue engineering and repair. However, in contrast to stem cells, less than 5% of NCCs exhibit true multipotency. Most NCCs exhibit a limited capacity for producing identical daughter cells, and they are typically unipotent or bipotent, with their fate determined by a combination or intrinsic and extrinsic genetic and environmental cues. Furthermore, despite their persistence in adults, NCCs are generated only transiently during embryo development. Therefore NCCs are more akin to progenitor cells than stem cells, with the true stem cell being the neural stem cell in the neuroepithelium from which NCCs are derived. Nonetheless, studies of NCC contribution to the sciatic nerve in rats revealed that pure populations of NCCs can be isolated through flow cytometry and (more important) that these isolated NCCs retain the capacity to form neurons and glia after transplantation into host avian embryos. Similar populations of NCCs also persist in the gut, epidermis dental pulp, heart, bone marrow, cornea, hard palate, and oral mucosa of adult organisms, providing multiple accessible sources of cells for replacement therapy.

The developmental potential of neural crest stem and progenitor cells may, however, decrease with age. Whereas mouse embryo–derived gut neural crest progenitor cells migrate great distances away from a transplantation site in avian embryos and differentiate into neurons, adult gut–derived neural crest progenitor cells only engraft structures in the proximity of their site of transplantation. Nonetheless, gut-derived neural crest progenitor cells transplanted into the aganglionic gut of a rat model of the Hirschsprung disease engrafted and differentiated into neurons. Furthermore, NCCs isolated from fetal human gut tissue remained viable, engrafted, and established functional connections after transplantation into the bowel of immunodeficient mice.

NCCs derived from the epidermis of the skin also appear to hold considerable therapeutic promise. Not only are they readily accessible for isolation, but the hair follicle contains a mixed population of epidermal, keratinocyte, and melanocyte stem cells, each of which exhibits a high degree of plasticity. Within the hair follicle is a multilayered region of the outer root sheath called the *bulge*. The bulge is where new hair growth occurs and, interestingly, the inner layers are derived from NCCs. Neural crest–derived cells, harvested from the bulge region, can undergo self-renewal, indicating these cells are stem cells. Furthermore, these cells are multipotent, and under differentiation conditions they produce colonies of neurons, smooth muscle cells, rare Schwann cells, melanocytes, and even chondrocytes. These cells have therefore been called *epidermal neural crest stem cells,* and the bulge in which they are found represents their niche.

Recently, neural crest–derived cells isolated from hair follicles were shown to repair sciatic nerve function in vivo in mice. Isolated stem cells from the hair follicle were used in transplants to treat two different injured nerves, the sciatic and tibial nerve. After transplantation, the follicle stem cells incorporated into the nerve, precipitating the recovery of proper nerve function. Functional studies of the gastrocnemius revealed consistent contractions upon stimulation. Furthermore, tibial nerve function was recovered in mice that received a follicle stem cell transplant, as demonstrated by normal walking ability. In contrast, control mice with a severed sciatic nerve but without transplantation displayed no muscle contraction upon stimulation. Taken together, these results determined that transplantation of follicular neural crest stem cells promotes regenerative axonal growth, resulting in the recovery of peripheral nerve function. Consistent with this model, the neuroprotective properties and therapeutic potential of epidermis-derived neural crest stem cells were also demonstrated in the rescue of long-term potentiation and cognitive disability in a rat model of vascular dementia. These experiments elegantly demonstrate the potential of follicle stem cells (of which epidermal neural crest stem cells are a component) as a potential source of cells to be used in stem cell therapies.

Tooth-derived NCCs, such as those from the dental pulp, are also a promising cell source for regeneration because, similar to the epidermis, they are easy to isolate, maintain in culture, and manipulate. Furthermore, their application in peripheral nerve injuries in animal models has revealed their regeneration capacity through glial differentiation and neuroprotection function.

In addition to autologous transplantation without immune rejection, the isolation of adult neural crest progenitor cells, or their induced pluripotent stem cell (iPSC) derivation from patients affected with a neurocristopathy, provides a powerful platform for modeling disease and informing its pathogenesis, as well as drug screening for therapeutics. For example, enteric NCC progenitors derived from human iPSC can migrate, engraft, and differentiate into neurons, rescuing disease-related mortality in mice with Hirschsprung disease. This raises the possibility of generating neural crest progenitor cells via iPSC or isolating them directly from the ganglionic region of the gut of a patient with Hirschsprung disease and then transplanting these cells into the aganglionic region of the same individual. This type of approach may provide a treatment option for Hirschsprung disease and, similarly, familial dysautonomia, a neurodegenerative disorder of the peripheral nervous system that is characterized by autonomic dysfunction without incurring problems with histocompatibility and immunosuppression, which are typical of transplantation surgery.

BOX 2.1 Neural Crest Cells and Their Application in Regenerative Medicine—cont'd

In other studies, human iPSC–derived NCCs were shown to be capable of producing erythropoietin such that when transplanted subcutaneously into anemic mice, they induce erythropoiesis, illustrating their potential clinical use in treating renal and nonrenal anemia. Furthermore, transplanted human iPSC–derived NCCs can rapidly restore corneal thickness and clarity in a rabbit model of corneal endothelial regeneration in rabbits. Lastly, human iPSC–derived NCCs can be differentiated into brain pericyte–like cells that have the molecular and functional characteristics of pericytes.

Human iPSC–derived NCCs exhibit low immunogenicity, which is encouraging for their use in cellular therapy. However, there is still much to learn because not all endogenous NCCs that can be derived from adult tissues or human iPSC are equivalent. This is born out in tests of their capacity in craniofacial regeneration, which revealed the importance of NCC origin for optimal success and surgical outcomes.

Conclusions

Although NCCs are a discrete population, generated only transiently in the embryo, numerous populations of neural crest stem and progenitor cells have been isolated from embryonic and adult tissues. Neural crest–derived stem cells are extremely useful for disease modeling, for drug screening, and in stem cell therapy. They are easily accessible, are relatively easy to maintain in culture, and provide an autologous source of tissue for replacement therapies, thereby bypassing immunorejection. These approaches, when used in combination with advances in genome engineering, make it possible to isolate neural crest progenitor cells from an affected individual, correct a genetic defect in those cells, then transplant those cells back into the same individual, possibly preventing or correcting the disorder. As proof of principle, a similar type of combinatorial stem cell and gene editing approach has recently been successful in the treatment of sickle cell anemia. The application of modern genomic and proteomic techniques, including single-cell RNA sequencing, assay for transposase-accessible chromatin sequencing, identification of posttranslational modifications of proteins, and protein-protein interaction networks will continue to deepen our understanding of the regulation of NCC development and their capacity for tissue-specific regeneration.

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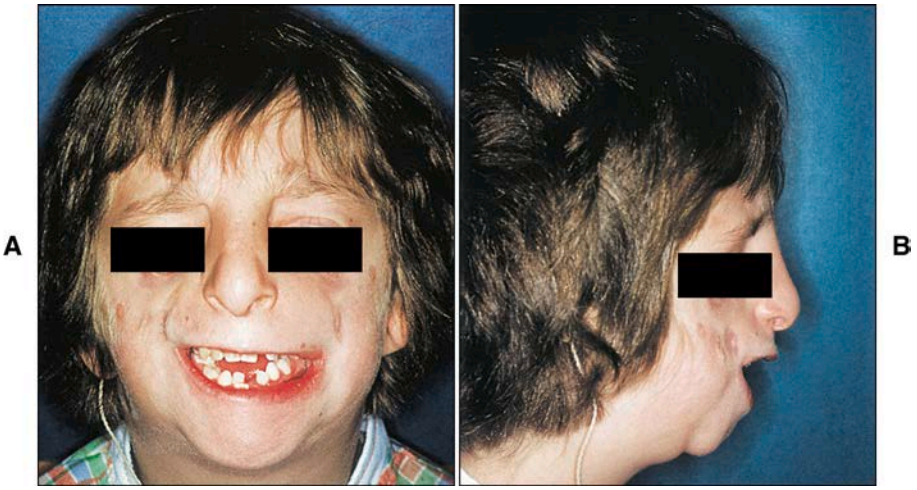

Fig. 2.15 (A, B) A child with mandibulofacial dysostosis (Treacher Collins syndrome). The underdevelopment results from a failure of the neural crest cells to migrate to the facial region. (Photograph courtesy Dr. L.B. Kaban.)

of members of the Snail (Snail and Slug) zinc-finger transcription factor family, who, as master regulators of EMT, repress the expression of the cell adhesion molecule E-cadherin in concert with upregulation of N-cadherin as part of the cadherin switch.

NCCs are formed along almost the entire length of the embryo and can be subdivided into distinct axial populations: cranial, cardiac, trunk, and sacral. Cranial NCCs play a particularly important role in head and facial development. In addition to contributing to formation of the cranial sensory ganglia, cranial NCCs also differentiate to form most of the connective tissue of the head. Embryonic connective tissue elsewhere is derived from mesoderm, known as *mesenchyme*, whereas in the head it is known as *ectomesenchyme*, reflecting its origin from neuroectoderm. Proper migration of NCCs is essential for the development of the craniofacial skeleton and the teeth, and many craniofacial anomalies are therefore considered disorders of NCC development, termed *neurocristopathies*. In Treacher Collins syndrome (Fig. 2.15), for example, full facial development does not occur because of the insufficient generation, proliferation, and survival of NCCs in the facial region. With respect to the teeth, all the tissues (except enamel and perhaps some cementum) and their supporting apparatus are derived directly from NCCs, and their depletion prevents proper dental development.