Birth Defects Research (Part C) 69:123–143 (2003)

REVIEW

Physiology and Pathophysiology of the Growth Plate

R. Tracy Ballock* and Regis J. O'Keefe

Longitudinal growth of the skeleton is a result of endochondral ossification that occurs at the growth plate. Through a sequential process of cell proliferation, extracellular matrix synthesis, cellular hypertrophy, matrix mineralization, vascular invasion, and eventually apoptosis, the cartilage model is continually replaced by bone as length increases. The regulation of longitudinal growth at the growth plate occurs generally through the intimate interaction of circulating systemic hormones and locally produced peptide growth factors, the net result of which is to trigger changes in gene expression by growth plate chondrocytes. This review highlights recent advances in genetics and cell biology that are illuminating the important regulatory mechanisms governing the structure and biology of the growth plate, and provides selected examples of how studies of human mutations have yielded a wealth of new knowledge regarding the normal biology and pathophysiology of growth plate cartilage. **Birth Defects Research (Part C) 69:123–143, 2003.** © 2003 Wiley-Liss, Inc.

INTRODUCTION

In order for paired human limbs to reach the same adult length and proportions, the longitudinal growth of the skeleton must be tightly regulated. The regulation of longitudinal growth at the growth plate occurs generally through the intimate interaction of circulating systemic hormones and locally produced peptide growth factors, the net result of which is to trigger changes in gene expression by growth plate chondrocytes. These molecular events lead to carefully orchestrated alterations in chondrocyte size, extracellular matrix components, secreted enzymes and growth factors, and receptor expression. The culmination of these events is calcification of the matrix, chondrocyte apoptosis, and the completion of endochondral bone formation.

Over the past few years, the powerful tools of molecular genetics have been unleashed on the inherited group of cartilage diseases referred to collectively as the human chondrodysplasias (Horton and Hecht, 1993). The consequence of this molecular approach has been the elucidation of the underlying cause of nearly all of the human forms of chondrodysplasia at the molecular level (Table 1). In many cases, the identity or normal function of the protein encoded by the mutated gene was unknown prior to the discovery of the gene mutation. This review highlights recent advances in genetics and cell biology that are illuminating the important regulatory mechanisms governing the structure and biology of the growth plate, and provides selected examples of how studies of human mutations have yielded a wealth of new knowledge regarding the normal biology and pathophysiology of growth plate cartilage.

Growth Plate Structure and Function

The growth plate can be divided into a series of anatomic zones that distinguish unique morphological and biochemical stages during the process of chondrocyte differentiation. In the resting zone, the ratio of extracellular matrix to cell volume is quite high, and the cells are in a relatively quiescent state. In the proliferative zone, chondrocytes assume a flattened appearance, begin to divide, and organize into columns. In the zone of maturation, the synthesis of extracellular matrix allows the recently divided cells to separate from each other. This extracellular matrix consists predominantly of collagens and proteoglycans, as well as other noncollagenous proteins. Type II collagen is the primary collagen species in the growth plate; however, type IX and type XI collagen are also highly expressed and have important functions. Collagen IX molecules decorate the surface of the type II collagen fibrils to which they are covalently cross-linked. It is postulated that type IX collagen mediates the interaction of type II collagen with other extracellular matrix components in cartilage

Aggrecan, the large aggregating proteoglycan of cartilage, is the principal proteoglycan molecule in

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bdrc.10014

TABLE 1. Inheritance Patterns and Gene Mutations in Chondrodysplasias

Skeletal dysplasia	Inheritance	Gene mutation
Achondroplasia	Autosomal Dominant	FGFR3
Hypochondroplasia	Autosomal Dominant	FGFR3
Thanatophoric dysplasia	Autosomal Dominant	FGFR3
SADDAN dysplasia	Autosomal Dominant	FGFR3
Pseudoachondroplasia	Autosomal Dominant	COMP
Multiple epiphyseal dysplasia	Autosomal Dominant	COMP COL9A2 COL9A3
Rhizomelic chondrodysplasia punctata	Autosomal Recessive	PEX7
Dyschondrosteosis (Leri-Weill)	Autosomal Recessive	SHOX
Multiple hereditary exostosis	Autosomal Dominant	EXT1 EXT2
Ellis von Creveld syndrome	Autosomal Recessive	EVC
Diastrophic dysplasia	Autosomal Recessive	DTDST
Spondyloepiphyseal dysplasia	Autosomal Dominant	COL2A1
Stickler syndrome	Autosomal Dominant	COL2A1 COL11A1 COL11A2
Kniest dysplasia	Autosomal Dominant	COL2A1
Schmid metaphyseal chondrodysplasia	Autosomal Dominant	COL10A1
Jansen metaphyseal chondrodysplasia	Autosomal Dominant	PTHrP

the cartilage matrix, and provides the osmotic resistance necessary for cartilage to resist compressive loads. Decorin and biglycan, two smaller proteoglycan molecules, also may serve important functions. Decorin, for example, coats the outside of the collagen fibrils and may play a role in regulating collagen fibrillogenesis.

Cartilage oligomeric protein (COMP) is a critical noncollagenous protein found in the extracellular matrix. COMP is an extracellular calcium-binding glycoprotein belonging to the thrombospondin family. The COMP molecule is composed of five flexible arms. At the end of each of these arms is a large, globular domain that resembles a bouquet of flowers. Mutations in *COMP* have been linked to pseudoachondroplasia, as well as to some forms of multiple epiphyseal dysplasia (MED) (Deere et al., 1999; Delot et al., 1999; Maddox et al., 2000).

In the hypertrophic zone, cell division ceases and the chondrocytes begin to terminally differentiate. Terminal differentiation is associated with a large increase in cell volume, a marked increase in alkaline phosphatase enzyme activity, and the synthesis and secretion of type X collagen, a unique short-chain collagen found only in the hypertrophic zone of the growth plate. Although the exact function of type X collagen in the growth plate remains unclear, mutations in the *COLX* gene have been found to cause Schmid metaphyseal chondrodysplasia (Warman et al., 1993). Surprisingly, transgenic mice lacking type X collagen show only subtle alterations in hematopoiesis and growth plate architecture, but no obvious skeletal phenotype (Gress and Jacenko, 2000).

Matrix vesicles, which are formed by budding of the chondrocyte plasma membrane, are deposited into the surrounding extracellular matrix in the hypertrophic zone, and serve as a nidus for mineralization. Mineralization of the cartilage extracellular matrix occurs in a somewhat directional pattern, with the longitudinal septa of cartilage matrix between the columns of hypertrophic chondrocytes being the favored sites of mineral deposition. The mineral deposited consists primarily of poorly crystalline hydroxyapatite.

In the zone of vascular invasion, invading capillary loops from the metaphysis break through the last transverse septum of mineralized cartilage to enter the hypertrophic chondrocyte lacuna. Approximately two-thirds of these transverse septa are actively resorbed by chondroclasts, while the remaining one-third serves as a template for deposition of bone matrix by osteoblasts. These "mixed spicules," which contain both mineralized cartilage and bone matrix, are known as primary trabeculae, and are subsequently remodeled in the metaphysis to trabeculae of lamellar bone, or secondary trabeculae (Farnum and Wilsman, 1993; Farnum et al., 1999).

Synthesis of Extracellular Matrix in the Growth Plate

Chondrocytes synthesize and secrete a characteristic matrix into the extracellular space. Therefore, the factors that regulate the synthesis of cartilage matrix are also many of the same factors responsible for regulating the process of chondrogenesis (i.e., the conversion of mesenchymal stem cells into cells that elaborate the cartilage matrix).

As with myogenesis, adipogenesis, and osteogenesis, the differentiation of mesenchymal cells into chondrocytes during chondrogenesis is regulated by the activity of a

DNA transcription factor that controls the expression of the principal genes encoding the extracellular matrix proteins of cartilage. In the case of cartilage, this transcription factor is Sox9, which is required for expression of several chondrocyte-specific matrix proteins, including type II collagen, type IX collagen, type XI collagen, and aggrecan (de Combrugge et al., 2000). Sox9 binds to specific enhancer regions in the promoters of these target genes in conjunction with two other related proteins (L-Sox5 and Sox6) in order to activate gene transcription (Lefebvre et al., 1998). Chimeric mice containing cells with no functional *Sox9* genes fail to differentiate into chondrocytes due to a block at the stage of mesenchymal condensation, indicating that in addition to regulating the synthesis of extracellular matrix proteins, Sox9 may also control the expression of cell surface proteins involved in the condensation process (Bi et al., 1999). Mutations in Sox9 have been linked to the human skeletal malformation syndrome campomelic dysplasia (for review see de Combrugge et al., 2000).

Chondrodysplasias Characterized by Abnormal Extracellular Matrix Synthesis

Pseudoachondroplasia

Pseudoachondroplasia is an autosomal dominant, short-limbed dwarfism. It is usually diagnosed by the age of 2 years, and is characterized by decreased linear growth, distinctive waddling gait, and ligamentous laxity (Deere et al., 1999). Other clinical features include short fingers, scoliosis, and early-onset osteoarthritis. Radiographs demonstrate small irregular epiphyses, with delayed ossification, flared metaphyses, anterior beaking of vertebral bodies, and delayed maturation of the triradiate cartilage and acetabulum.

The etiology of pseudoachondroplasia has been discovered to be mutations in gene encoding cartilage oligomeric protein (COMP). COMP is an extracellular calcium-binding glycoprotein belonging to the thrombospondin family. The COMP molecule is composed of five flexible arms. At the end of each arm is a large globular domain that resembles a bouquet of flowers. Mutations affecting the type III repeat region, or C terminal domain of the protein, result in decreased calcium binding due to a structural change in the protein (Maddox et al., 2000). Approximately 30% of patients have the same in-frame deletion mutation, which results in four aspartic acid residues instead of five at amino acids 469–473 of the protein (Deere et al., 1999; Delot et al., 1999).

It is interesting to note that mutations in the *COMP* gene have also been discovered in patients with MED (Deere et al., 1999; Delot et al., 1999; Newman et al., 2000). This indicates that pseudoachondroplasia and MED, which were originally described as distinct disorders, are now recognized to be part of a disease spectrum.

Multiple epiphyseal dysplasia (MED)

MED is a genetically heterogeneous, autosomal dominant disorder characterized by mild to moderate short stature and early-onset osteoarthritis. The disease is usually diagnosed in middle to late childhood, when patients present with pain and stiffness in the hips, knees, or ankles (Holden et al., 1999; Paassilta et al., 1999). Affected patients have short, stubby fingers, and unfortunately often develop precocious osteoarthritis in late childhood to early adulthood.

Radiographic findings include irregular and flattened epiphyses with delayed ossification (Figure 1). The vertebral bodies and metaphyseal regions are unaffected; however, some irregularity of the vertebral endplates may be seen (Lohiniva et al., 2000). Some patients have only radiographic features, and no symptoms.

The clinical phenotypes of MED vary widely, but have been classified into at least two groups that define a broad phenotypic spectrum. The milder Ribbing form is characterized primarily by involvement of the hips and short stature. The more severe Fairbank form is characterized by dwarfism, stubby fingers, and small epiphyses of several joints, including the hips. Many families remain unclassified because of the absence of typical Ribbing or Fairbank features.

Three separate genetic loci have been linked to MED. As noted previously, mutations causing all clinical forms of MED have been identified in the gene encoding COMP (Deere et al., 1999; Delot et al., 1999). The phenotype of the mild pseudoachondroplasia patients overlaps with those of MED.

Other mutations have been identified in *COL9A2*, which encodes the alpha-2 chain of type IX collagen (Holden et al., 1999). Type IX collagen is a nonfibrillar heterotrimeric molecule with three chains encoded by three different genes. It is a structural component of hyaline cartilage, intervertebral discs, and the vitreous body of the eye. Type IX collagen decorates the surface of type II collagen molecules, to which it is covalently crosslinked. Its function is postulated to involve mediating the interaction of type II collagen with other extracellular matrix components in cartilage.

The phenotype of the *COL9A2* mutants appears to be milder (with no hip involvement) compared to the more severe disease caused by mutations in *COMP*. All mutations in *COL9A2* described to date result in splicing errors that eliminate exon 3, and hence delete 12 amino acids from the amino terminal portion of the molecule (Holden et al., 1999). This may affect the structure and function of the molecule in mediating interactions between type II collagen molecules and other extracellular matrix components.

Recently, an unclassified form of MED was linked to *COL9A3* in a four-generation family with autosomal dominant disease (Paassilta et al., 1999; Lohiniva et al., 2000). This is the first disease-causing mutation to be identified in *COL9A3*, which encodes another of the three alpha chains of type IX collagen. The phenotype of the

COL9A3 mutants overlaps significantly with *COL9A2* mutants, but differs by the presence of hip involvement.

Rhizomelic chondrodyplasia punctata (RCDP)

RCDP is a lethal autosomal recessive disorder characterized by disproportionate short stature, with rhizomelic limb-shortening, mental retardation, craniofacial dysmorphism (including a broad nasal bridge with anteverted nose), disturbed endochondral bone formation, cataracts, and ichthyosis (Purdue et al., 1999; Raymond, 2001). Radiographs demonstrate stippling of the epiphyses of multiple joints (Figure 2). Most patients with RCDP die in infancy; however, several patients with less severe disease have survived into their teens.

The primary biochemical abnormality that causes RDCP is peroxisomal dysfunction resulting in deficiency in plasmalogen biosynthesis. Peroxisomes are subcellular organelles that contain more than 50 enzymes, are present in all eukaryotic cells, and perform a variety of metabolic functions involving lipids and hydrogen peroxide (Moser, 1999).

Patients with RCDP have mutations in the *PEX7* gene, which encodes a protein that is responsible for importing matrix proteins into the peroxisomes from the cytoplasm (Shimozawa et al., 1999; Braverman et al., 2000). The effect of the mutation is to abolish the import of a set of specific PTS2 matrix proteins into the peroxisome, resulting in elevated levels of phytanic acid. The most common mutation described is a nonsense mutation that converts the leucine codon at position 292 to a termination signal (Purdue et al., 1999).

Diastrophic dysplasia

Diastrophic dysplasia is a rare autosomal recessive dysplasia, which has a higher incidence in the Finnish population compared to other populations. "Diastrophic" is a geological term referring to the twisting and buckling movements of the earth's crust, which was thought to resemble the bony deformities of this disease (Everett and Green, 1999). Clinical features include short-limbed dwarfism with kyphoscoliosis; contractures and dislocations of multiple joints; hitch-hiker thumbs resulting from shortened first metacarpal bones; bilateral clubfeet, which often prove resistant to surgical correction; and cleft palate. Although infant mortality is increased due to respiratory insufficiency, subsequent life expectancy is normal.

Mutations in the diastrophic dysplasia sulfate transporter (*DTDST*) gene have been identified as causing diastrophic dysplasia. The *DTDST* gene is ubiquitously expressed, and encodes a protein that facilitates the transport of sulfate across the cell membrane. The disease primarily affects cartilage due to the importance of negatively charged sulfate groups in the function of proteoglycan molecules, which is to maintain the hydration and compressive strength of cartilage. One in 70 Finnish citizens are carriers of a mutant *DTDST* gene.

Cell Proliferation in the Growth Plate

Proliferation occurs in a narrow band of cells located in the proliferating region of the growth plate. Histomorphometric studies in rats show that one layer of hypertrophic cells is eliminated from the growth plate every 3 hr; therefore, proliferation must produce eight new cells in each chondrocyte column per day (Farnum and Wilsman, 1993; Farnum et al., 1999).

Recent experiments suggest that the proliferation of chondrocytes in the growth plate is under the control of a local feedback loop that primarily involves three signaling molecules synthesized by growth plate chondrocytes: parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh), and transforming growth factor-beta (TGF-β). This feedback loop acts to regulate the rate at which the growth plate cells leave the proliferative zone of the physis and irreversibly commit to terminal differentiated hypertrophic cells.

Cells in the periarticular region of the long bone produce PTHrP; however, the PTHrP receptor is found primarily in the prehypertrophic cells and lower proliferating zone cells. PTHrP delays hypertrophic differentiation in these lower proliferative zone cells by maintaining cells in a prehypertrophic phenotype. Growth plate cells that are beginning to undergo hypertrophic differentiation secrete Ihh, which relays a signal back through the perichondrium to the periarticular cells to increase production of PTHrP (Vortkamp et al., 1996). This perichondrial relay involves the receptors for Ihh (patched and gli), which are located primarily in the cells of the perichondrium, as well as for TGF-β, which is produced by perichondrial cells in response to Ihh. TGF-β then acts on the perichondrial and periarticular cells to increase PTHrP synthesis (Serra et al., 1999; Alvarez et al., 2001b), and also can act directly on chondrocytes to inhibit hypertrophy (Ballock et al., 1993). This increase in PTHrP synthesis in the periarticular cells is transmitted to the late proliferating cells expressing the PTHrP receptor, which slow the production of Ihh-producing cells, thereby controlling the pace of hypertrophic differentiation (Kronenberg et al., 1997).

Recent genetic experiments in mice have confirmed this primary role of PTHrP in controlling the transition between chondrocyte proliferation and differentiation. Transgenic mice lacking either PTHrP or the PTH/PTHrP receptor show evidence of dwarfism resulting from accelerated differentiation and premature hypertrophy (Lanske et al., 1996). Conversely, mice in which PTHrP is overexpressed in the growth plate also exhibit dwarfism; however, this dwarfism is due to marked slowing of the rate of differentiation (Schipani et al., 1997). In humans, mutations in the PTH/PTHrP receptor that result in a constitutively active PTHrP signal have been identified as the cause of Jansen's metaphyseal chondrodysplasia, a dwarfing condition associ-

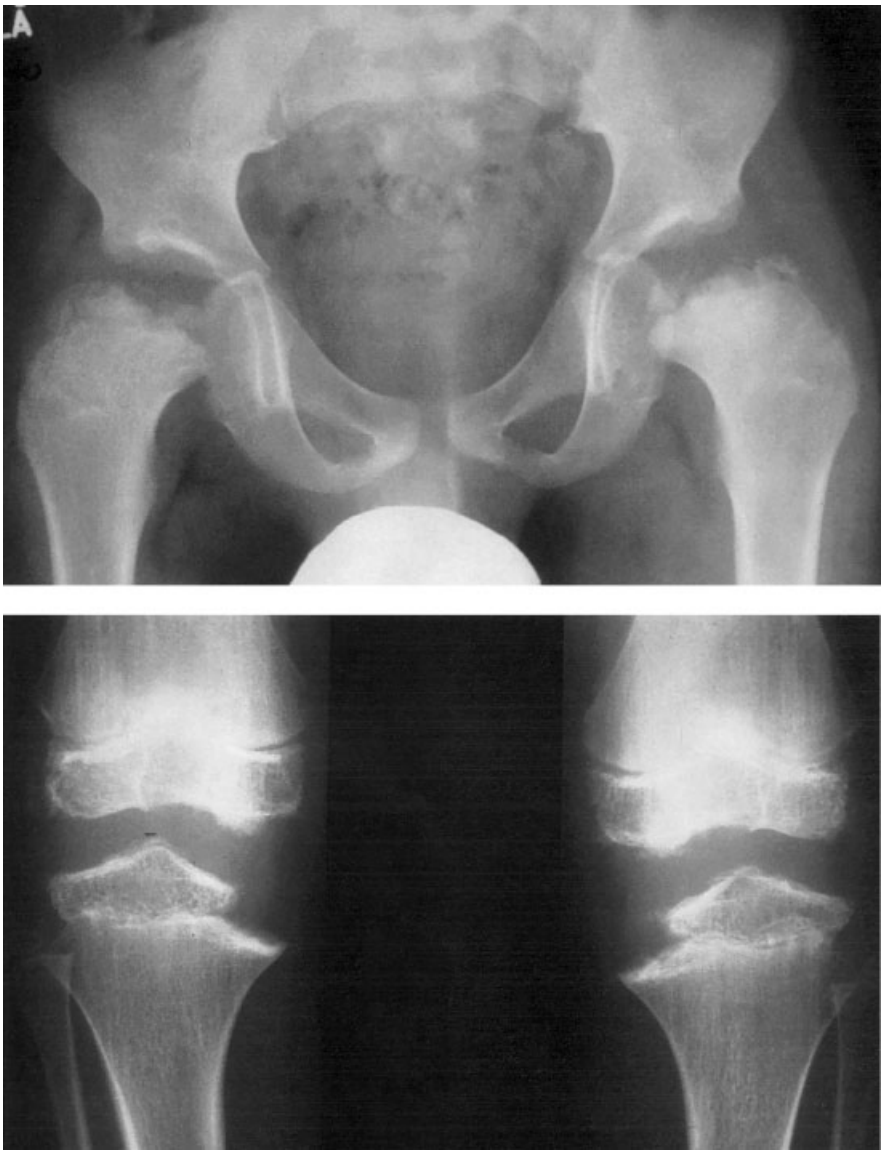

Figure 1. Radiographic features of MED. The ossification centers of the proximal femoral epiphyses (**A**) and the distal femoral and proximal tibial epiphyses (**B**) are flattened and irregular in contour, with delayed ossification.

ated with delays in growth plate mineralization and hypercalcemia (Schipani et al., 1996) (Figure 3).

In the adolescent animal, it is not likely that the periarticular cartilage is a relevant source of PTHrP, considering the large distance between the growth plate and articular surface (Pateder, 2001). Furthermore, recent studies indicate that Ihh is not produced by the growth plates of postnatal animals, which suggests that other signals play a role as regulators of chondrocyte differentiation in adolescent animals (Iwasaki et al., 1997). Evidence is accumulating that points to TGF-β as the key inhibitor of chondrocyte differentiation in adolescence. In vitro, TGF-β is a potent inhibitor of maturation, including cell hypertrophy, *COLX* expression, and alkaline phosphatase activity (Ballock et al., 1993; Bohme et al., 1995; Ferguson et al., 2000; Pateder et al., 2001b). TGF-β actions on the cell are mediated in part by a specific transcription factor, Smad 3 (Ferguson et al., 2000; Pateder et al., 2001b). Mice deficient in Smad 3 have a completely normal skeleton at birth, but by 3 weeks of age they begin to exhibit cartilage abnormalities, including premature hypertrophy of both growth plate and articular chondrocytes, and disorganization of the growth plate columns resulting in decreased longitudinal growth (Yang et al., 2001). The effects on articular cartilage mimic the changes observed during the development of osteoarthritis. Therefore, although TGF-β signaling through Smad 3 is not critical for development of a normal skeleton at birth, it is essential for normal postnatal growth and development.

TGF-β is secreted by chondrocytes in an inactive form bound to a latency molecule, which underlines the importance of defining the factors that lead to TGF-β activation in the growth plate (Pedrozo et al., 1998). To date, these mechanisms include matrix metalloproteinases, other proteases, and acidic conditions, such as those present during osteoclastic bone resorption (Oursler, 1994; Pedrozo et al., 1999; D'Angelo et al., 2001).

Although this PTHrP-Ihh-TGF-β feedback loop currently appears to be the primary regulator of cell proliferation in the growth plate, it is also likely that this regulatory network is modulated by other systemic and local signaling molecules, which previously have been shown to have effects on cell proliferation in the growth plate. For example, genetic disruption of the murine fibroblast growth factor receptor 3 (FGFR-3), which binds at least nine members of the FGF family, results in prolonged endochondral bone growth with expansion of the proliferating and hypertrophic zones of the growth plate (Colvin et al., 1996; Deng et al., 1996). An activating mutation in the FGFR-3 receptor has also been identified as the cause of achondroplasia, a dwarfing condition in which proliferation of growth plate cells is markedly reduced (for review see Vajo et al., 2000). It is therefore likely that FGF signaling is able to modulate the PTHrP-Ihh regulatory feedback loop. In support of this notion, transgenic mice overexpressing the *FGFR-3* gene in growth plate cartilage show markedly reduced proliferation of growth plate cells associated with down-regula-

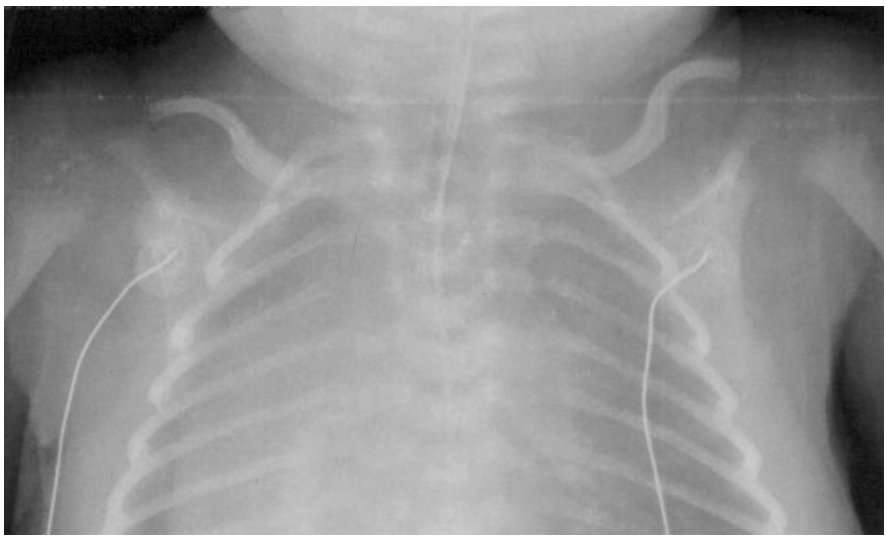

Figure 2. Radiographic features of rhizomelic chondrodysplasia punctata. A stippled ossification pattern is present in the proximal humeral epiphyses bilaterally.

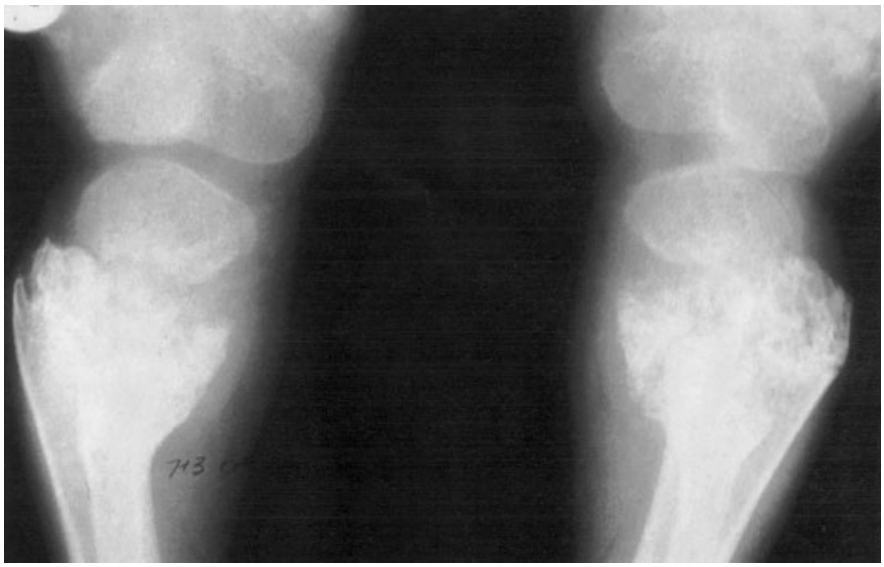

Figure 3. Radiographic features of Jansen metaphyseal chondrodysplasia. The growth plates of the proximal tibias are widened, with deformity and irregular ossification of the metaphyseal region.

tion of Ihh expression (Naski et al., 1998).

Another important growth factor is IGF-I, which is an autocrine factor that stimulates increased rates of cell division (Ohlsson et al., 1992). In addition to its effects on circulating levels of IGF-I, growth hormone increases the local synthesis of IGF-I in growth plate cells, which then leads to increased rates of cell division (Ohlsson et al., 1992). Transgenic mice lacking a functional IGF-I gene demonstrate severe growth retardation and profound defects in the development of major organ systems, including bone, muscle, and reproduction. Many die within 24 hr after birth (Liu et al., 1993). Administration of growth hormone to these IGF-I null mice has no effect on skeletal growth. Likewise, mice in which the growth hormone gene has been deleted show evidence of reduced bone growth that can be rescued by administration of IGF-I (Sims, 2000). In humans, mutations in the growth hormone receptor result in Laron syndrome, a hereditary dwarfism associated with truncal obesity and low serum IGF-I levels (Laron et al., 1966). Recently, a new line of transgenic mice have been created that lack only IGF-I derived from the liver (Liu et al., 2000). These mice have serum levels of IGF-I that are 75% lower than those of normal mice, yet they demonstrate normal longitudinal bone growth. Therefore, IGF-I produced locally by growth plate chondrocytes in response to growth hormone is the critical source for maintenance of normal postnatal skeletal growth. In part, IGF-I may mediate some of its effects through other growth factors, since it has been shown to enhance the effects of TGF-β on proliferation (O'Keefe, 1994b).

One possible mechanism for integrating the effects of signaling molecules, such as PTHrP, TGF-β, IGF-I, and FGF, on proliferation of growth plate chondrocytes is modulation of the cell division cycle. The cell cycle is exquisitely regulated and is characterized by several sequential phases. Cells pass through an initial growth phase (G1) of the cell cycle before entering into S phase, where DNA is replicated in preparation for another obligatory round of cell division. Alternatively, cells may withdraw from the cell cycle and undergo terminal differentiation (Grana and Reddy, 1995; MacLachlan et al., 1995). The passage of cells across this critical G1/S restriction point is controlled by phosphorylation of the retinoblastoma protein (Rb), or the closely related proteins p107 and p130 (Hollingsworth et al., 1993; Zhu et al., 1993; Claudio et al., 1994; Riley et al., 1994; Weinberg, 1995; Chen et al., 1996). Simultaneous deletion of both p107 and p130 genes in mice results in deregulated chondrocyte growth, defective endochondral bone development, shortened limbs, and neonatal lethality (Cobrinik et al., 1996).

The phosphorylation, or activation state, of the Rb proteins is controlled by the action of a family of cyclin-dependent kinases (CDKs) that require binding of cyclin proteins for their kinase activity (Hollingsworth et al., 1993; Zhu et al.,

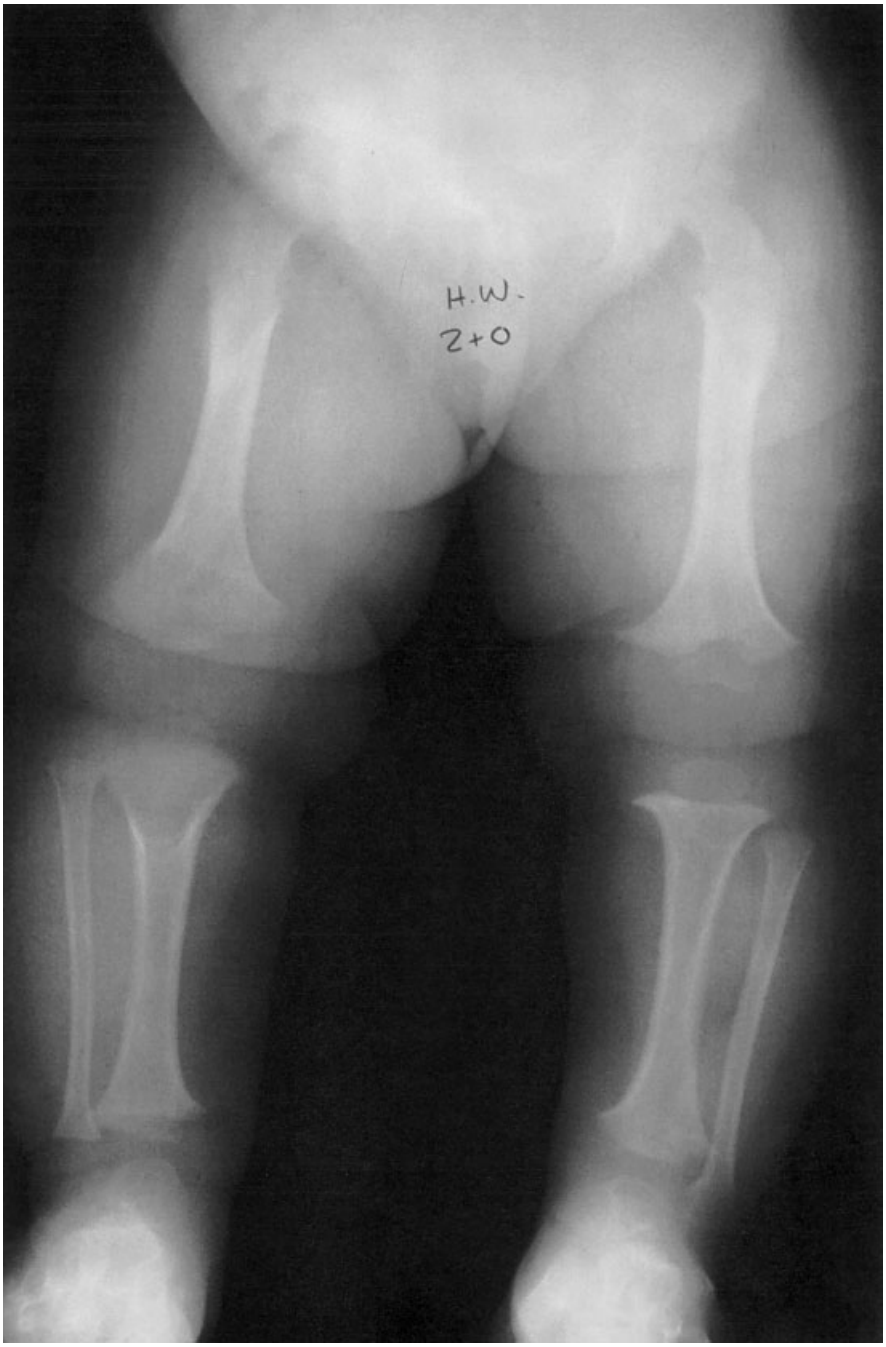

Figure 4. Radiographic features of achondroplasia in the lower extremities. The long bones are short and broad, with flaring of the metaphyses. These findings are consistent with abnormal endochondral ossification affecting longitudinal growth, with normal intramembranous ossification allowing appositional growth to proceed.

1993; Claudio et al., 1994; Riley et al., 1994; Weinberg, 1995; Chen et al., 1996). Several inhibitory proteins have been found that also compete with the cyclin subunits for binding to their respective CDKs, thereby preventing Rb phosphorylation and passage into S phase. For growth plate cells to continue proliferation, the balance between cyclins and CDK inhibitors must be weighted in favor of maintaining Rb (or the related proteins p107 and 130) in a hyperphosphorylated state (for review see Beier et al., 1999).

Elevated levels of cyclin D1, for example, result in stimulation of CDK activity, Rb hyperphosphorylation, and chondrocyte proliferation. It was recently demonstrated that both PTHrP and TGF-β can stimulate transcription of the cyclin D1 gene through specific regulatory sites in the cyclin D1 promoter region (Beier et al., 1999). Conversely, expression of the CDK inhibitors, $p21^{waf-1}$ and $p27^{kip-1}$, has been found to be elevated in growth plate cells undergoing hypertrophy in vivo (Stewart et al., 1997), and in growth plate cells induced to stop proliferating, and terminally differentiate in response to thyroid hormone (Ballock et al., 2000b). In contrast, TGF-β regulates cell cycle progression in other cell types by reducing the expression of the cyclin-dependent kinase inhibitors, $p15^{ink4B}$,and $p27^{kip-1}$ (Hannon and Beach, 1994; Reynisdottir et al., 1995). This important reciprocal relationship between the cell cycle regulators and the events of proliferation and differentiation dictates that when the balance of cyclins and cyclin-dependent kinase inhibitors favor Rb family hyperphosphorylation, proliferation occurs. In contrast, when the balance is tipped toward inhibitors of the cyclin-dependent kinases, Rb family members remain hypophosphorylated, and chondrocytes undergo terminal differentiation. These basic cell cycle events are likely to be coordinately regulated by important signaling events mediated by hormonal and growth factor–receptor interactions.

Chondrodysplasias Characterized by Abnormal Cellular Proliferation

Achondroplasia family

The FGFRs are a group of four tyrosine kinase proteins that span the cell membrane and bind a family of at least 18 FGFs with varying affinities. For example, FGFR3 binds FGF1, -2, -4, -8, and -9 ligands, and is expressed primarily in nervous system and cartilage rudiments of developing bone (Vajo et al., 2000). The binding of the growth factor to the receptor requires the dimerization of two FGF monomers, as well as a heparin-binding step.

Several chondrodysplasia pheno-

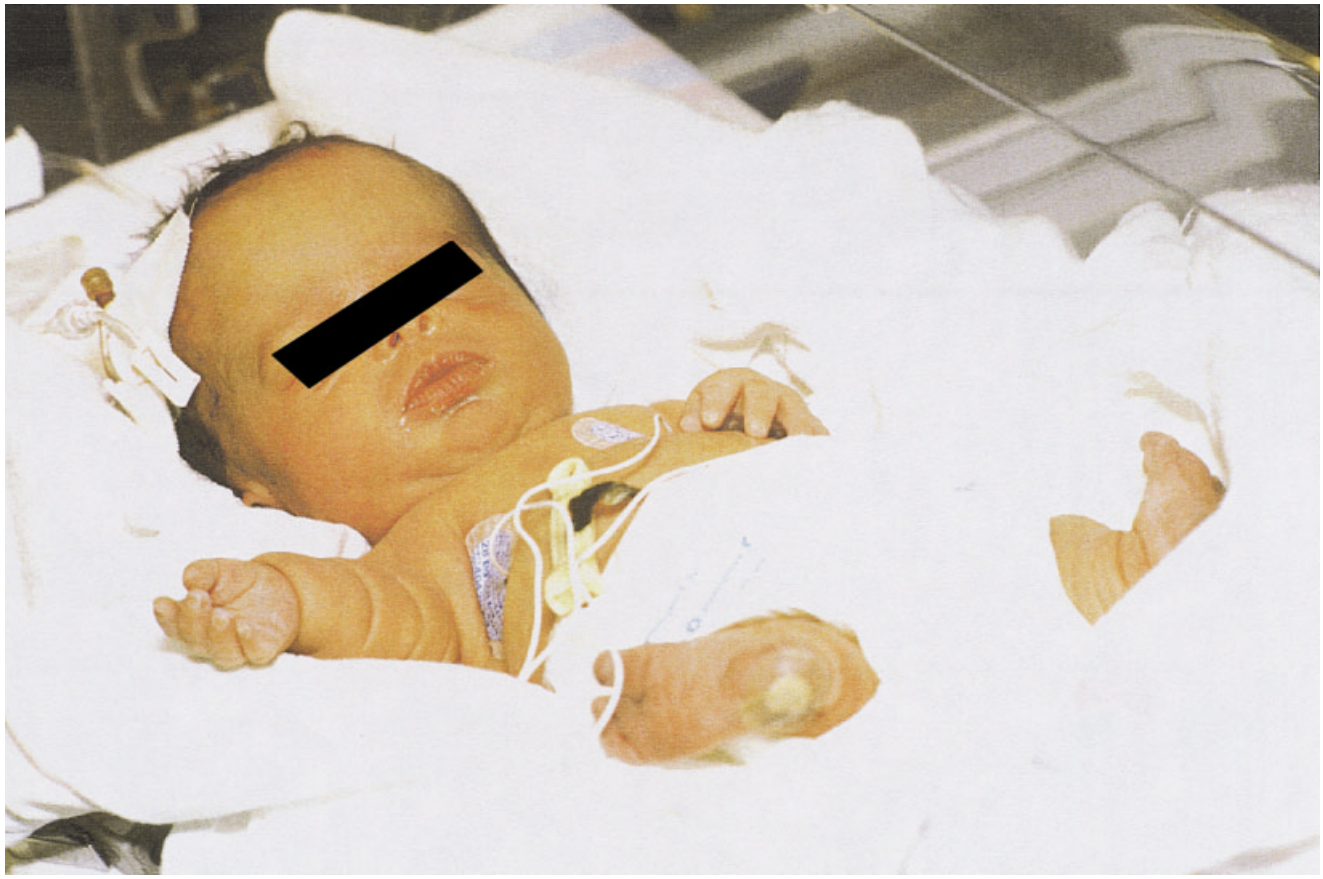

Figure 5. Clinical features of thanatophoric dysplasia include a prominent forehead (frontal bossing) and depressed nasal bridge.

types have been discovered to result from mutations in the *FGFR3* gene encoding the FGFR3, including achondroplasia, hypochondroplasia, thanatophoric dysplasia, and a newly described disorder known as "severe achondroplasia with developmental delay and acanthosis nigricans" (SADDAN) dysplasia. It is now apparent that these diseases can be classified as a family of disorders representing a continuum of severity.

Achondroplasia

Achondroplasia is an autosomal dominant condition with complete penetrance. It is the most common form of dwarfism in humans, affecting 1:15,000–1:40,000 individuals (Vajo et al., 2000). Clinical features of achondroplasia include rhizomelia, macrocephaly, depressed nasal bridge, frontal bossing, and trident hands. Radiographs demonstrate shortening of long bones with squared-off iliac wings, narrow sacrosciatic notches, and narrowing of the interpedicular distance in the lower lumbar spine (Figure 4). Histology of affected growth plate cartilage reveals a failure of chondrocytes in the growth plate to mature into hypertrophic cells.

Achondroplasia arises from a sporadic (as opposed to inherited) mutation in more than 90% of affected individuals, and is caused by a single nucleotide substitution in over 95% of cases. In fact, it is thought that nucleotide 1138 of the *FGFR3* gene may be the most mutable nucleotide in the human genome to date (Vajo et al., 2000). The specific mutation in achondroplasia converts G to A, or G to C at position 380 in the transmembrane domain of the protein, resulting in a glycine-to-arginine substitution. This single amino acid substitution causes not only stabilization of the receptor protein and its accumulation on the cell surface, but also uncontrolled, prolonged ligand-dependent activation of the receptor (Monsonego-Ornan et al., 2000).

Expression of the mutated receptor protein in CFK2 chondrocytic cells in culture results in inhibition of proliferation and maturation of these cells. This effect appears to be associated with altered integrin expression, as well as increased sensitivity to apopotosis in response to serum withdrawal (Henderson et al., 2000).

A transgenic mouse model of achondroplasia has been developed by several laboratories, in which the human *FGFR3* mutant gene is expressed under the transcriptional control of the mouse gene. These mice display many of the same phenotypic features seen in the human disorder, including macrocephaly, midface hypoplasia, and shortened long bones (Chen et al., 1999; Li et al., 1999; Wang et al., 1999; Segev et al., 2000). Studies of transgenic mice expressing a

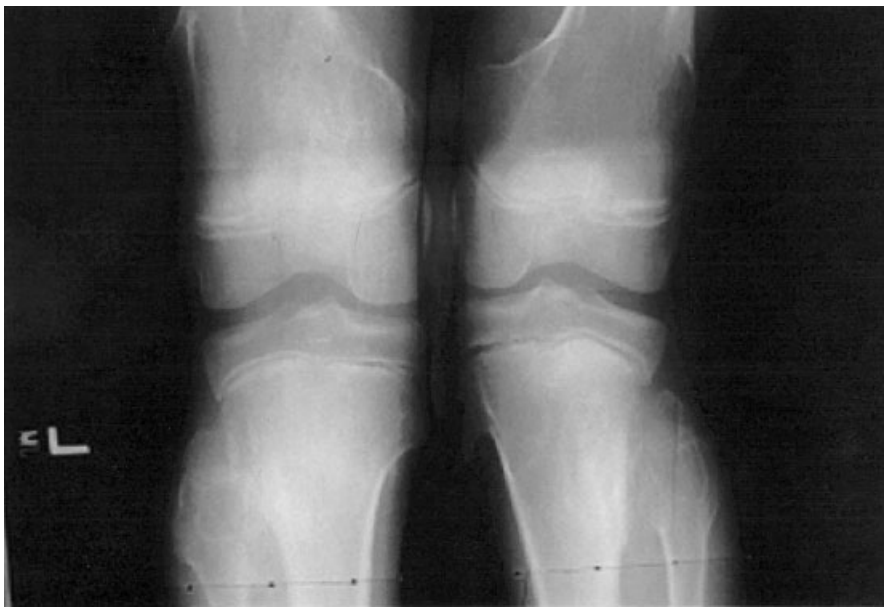

Figure 6. Radiographic features of multiple hereditary exostoses. Bony projections with cartilage caps (osteochondromas) develop near the ends of long bones, and may continue to enlarge until skeletal maturity is reached.

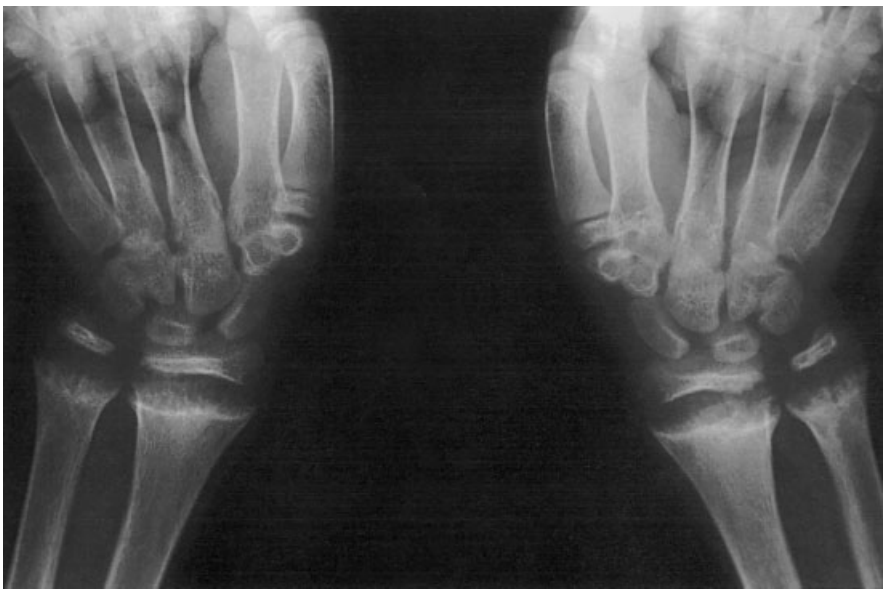

Figure 7. Radiographic features of rickets. The physes of the distal radius and ulna are widened and cup-shaped, with elongation of the zone of mineralization.

Lys644Glu mutation in *FGFR3* reveal activation of several members of the Stat family of DNA transcription factors, as well as cell cycle inhibitors belonging to the ink4 family, including p16, p18, and p19 (Li et al., 1999). This observation indicates there is a mechanism for the abnormal chondrocyte proliferation and maturation that involves dysregulation of cell cycle progression.

A phenotype similar to achondroplasia can also be produced by overexpression of the FGF9 ligand in mice (Garofalo et al., 1999). FGF9 is expressed in a developmental pattern similar to that of FGFR3; however, mice lacking FGF9 have a normal skeletal phenotype.

Hypochondroplasia

Hypochondroplasia is a mild, autosomal dominant, short-limbed dwarfism. It is characterized by short stature, macrocephaly with mild frontal bossing, micromelia, and lumbar lordosis. In general, the clinical features, radiographic findings, and histology are similar to achondroplasia but are more mild. The paucity of specific clinical manifestations, and the absence of pathognomonic radiographic features make it difficult to diagnosis this disorder (Mortier et al., 2000). The symptoms are rarely apparent before 2 years of age, and many cases are initially referred to an endocrinologist for evaluation of short stature, with heights in the low-normal range.

Spinal radiographs of patients with hypochondroplasia demonstrate either no change or a decrease in the interpedicular distance from the first to the fifth lumber vertebra, as well as short pedicles. Pelvic films may reveal short iliac bones with flat acetabular roofs and small sacrosciatic notches. Radiographic evaluation of the extremities often shows short tubular bones, short and broad femoral necks, and relative elongation of the distal or proximal fibula (Mortier et al., 2000).

A common mutation causing hypochondroplasia is a C-to-A or C-to-G change at nucleotide 1620, resulting in an asparagine-to-lysine substitution at amino acid 540 in the proximal tyrosine kinase domain of the FGFR3 protein (Mortier et al., 2000). However, other cases of hypochondroplasia have been described that do not involve mutations at this position, and some of these are not even linked to chromosome 4 on which the FGFR3 gene is located. Therefore, it is clear that hypochondroplasia, unlike achondroplasia, is a genetically heterogeneous condition.

Thanatophoric dysplasia

The severe, often lethal form of the achondroplasia family of disorders is known as thanatophoric dysplasia. Thanatophoric dysplasia is a rhizomelic form of dwarfism with macrocephaly, platyspondyly, and reduced size of the thoracic cavity (Figure 5). Although normally it is

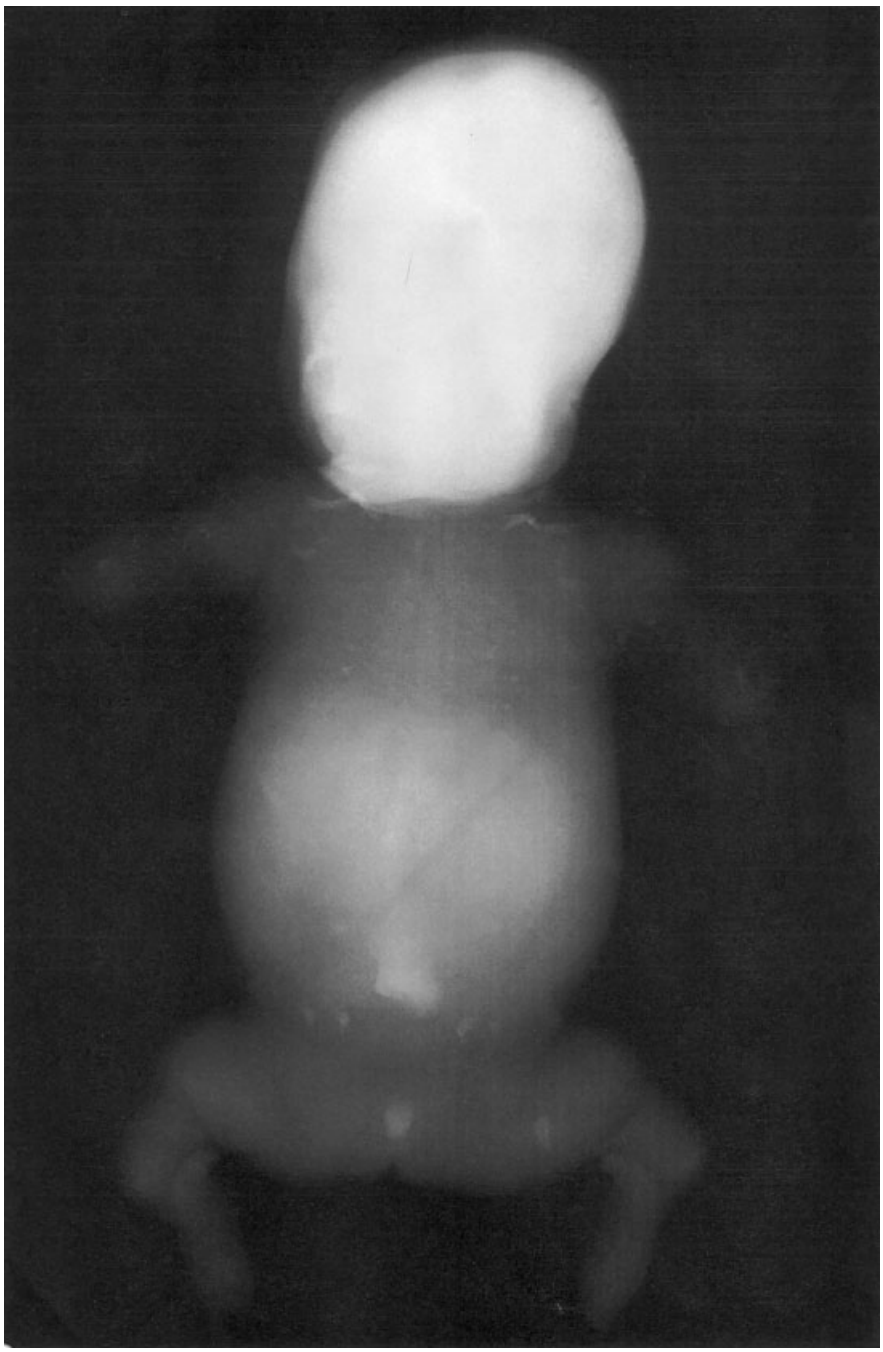

Figure 8. Radiographic features of hypophosphatasia. There is a striking lack of mineralization that can be detected radiographically.

lethal in the neonatal period, some surviving patients with thanatophoric dysplasia have been reported.

Thanatophoric dysplasia was recently classified into two separate disorders (TDI and TDII) based on the morphology of the femurs and the underlying genetic mutation. In TDI, the femurs are curved and the molecular defect consists of a stop codon mutation or missense mutation in the extracellular domain of the FGFR3 protein, resulting in a newly created cysteine residue (Vajo et al., 2000). In TDII, the femurs are straight, and the disease is caused by a single nucleotide substitution resulting in replacement of lysine with glutamine at position 650 in the tyrosine kinase 2 domain of the receptor. The TDII mutation results in an activated FGFR3 receptor, which up-regulates both the activity of the DNA transcription factor Stat1 and the expression of cell cycle inhibitor $p21^{WAF1/CIP1}$ in cultured cells (Su et al., 1997). This finding is similar to the previously noted observation that mice expressing a Lys644Glu mutation in FGFR3 show activation of Stat proteins and cell cycle inhibitors (Li et al., 1999).

SADDAN dysplasia

Recently, a new chondrodyplasia was described that also belongs to the achondroplasia family. Originally named "skeletal, skin, and brain" (SSB) dysplasia, this disorder is also known as "severe achondroplasia with developmental delay and acanthosis nigricans" (SADDAN) dysplasia (Bellus et al., 1999; Tavormina et al., 1999). Affected individuals exhibit extreme short stature, severe tibial bowing, profound developmental delay, and acanthosis nigricans.

The mutations in the *FGFR3* gene that cause SADDAN dysplasia include an A-to-T change at nucleotide 1949, resulting in lysine-to-methionine substitution at amino acid 650 (Bellus et al., 1999; Tavormina et al., 1999).

Hereditary multiple exostosis (HME)

Another relatively common chondrodysplasia that results from abnormalities in proliferation of growth plate cells is HME. HME is an autosomal dominant disorder with a prevalence of 1:50,000, in which bony projections with cartilage caps develop near the ends of multiple long bones (Figure 6). These exostoses continue to grow until skeletal maturity is reached, and may cause partial growth inhibition in the adjacent physis, resulting in limb deformity, leg length discrepancy, and occasionally subluxation of an adjacent joint. Although it is initially a benign lesion, transformation to a malignant chondrosarcoma occurs in 0.5–2% of cases (Bernard et al., 2000).

HME is caused by mutations in *EXT1* or *EXT2*, members of a newly described family of putative tumor suppressor genes that encode glycosyltransferases (Simmons et al., 1999). EXT1 and EXT2 proteins have been localized by immunohistochemical techniques to the Golgi apparatus of the cell, where they form a protein complex that is responsible for the biosynthesis of heparan sulfate glycosaminoglycans (Kobayashi et al., 2000; McCormick et al., 2000). The consequence of the disease-causing mutations is loss of heparan sulfate proteoglycan on the cell surface, where the molecule frequently functions as a coreceptor for peptide growth factors to increase binding affinity.

Although *EXT* genes are ubiquitously expressed, the disease appears to only result in aberrant proliferation of growth plate chondrocytes. Histologically, abnormal cytoskeletal inclusions consisting of actin and α-actinin are found in chondrocytes comprising the cartilage cap (Bernard et al., 2000). However, the relationship between this observation and the aberrant chondrocyte proliferation is unclear. Recently, it was demonstrated that the *Drosophila EXT1* homologue *tout-velu* is necessary for the diffusion of the hedgehog protein in the developing wing tissue of the fly by affecting the transduction of the hedgehog signal across adjacent cells (Bellaiche et al., 1998). Therefore, *EXT* mutations may also affect the function of the IHH in the growth plate, which modulates the rate at which chondrocytes stop proliferating and differentiate into hypertrophic cells.

Current genetic observations indicate that HME may be a neoplastic condition, rather than a true chondrodysplasia (Bovee et al., 1999; Porter and Simpson, 1999; Kivioja et al., 2000). According to the Knudsen "two-hit" model of tumor suppressor gene inactivation in cancer, both copies of the gene must be inactivated to abolish the normal tumor suppressor activity. In hereditary cancer, the first hit usually consists of a germline mutation, while the second hit is the inactivation of the remaining wild type copy through a somatic mutation. This model was recently applied to patients with HME. An inherited haploinsufficiency (malfunction of one of the two working copies of a gene within the cell), combined with a subsequent loss of function in the remaining copy of the gene through a somatic mutation, is required for osteochondroma formation (Bovee et al., 1999). The ensuing growth dysregulation then predisposes the cell to further genetic alterations, resulting in chondrosarcoma in a small percentage of cases.

Maturation and Hypertrophy of Growth Plate Chondrocytes

Chondrocyte maturation is marked by related physical and biochemical changes that occur in a spatial and temporal pattern (Buckwalter et al., 1986; Linsenmyer et al., 1991). The progression of chondrocytes through the resting, proliferating, and hypertrophic stages of differentiation, culminating in matrix calcification and programmed cell death, occurs within 24 hr in rapidly growing animals (Hunziker et al., 1997). During differentiation, chondrocytes undergo a five- to 10-fold increase in intracellular volume (Hunziker et al., 1997) This is not a passive swelling of the cell; rather, it reflects an active process marked by an increase in the number of intracellular organelles, including mitochondria and endoplasmic reticulum (Hunziker et al., 1997). It is not clear what factors stimulate cellular enlargement, but it likely involves alterations in ion channels that lead to an ingress of water.

Chondrocyte hypertrophy has an important role in the longitudinal growth of the skeleton (Hunziker, 1994). It has been shown that the increase in chondrocyte height is responsible for 44–59% percent of long bone growth, with the remainder due to matrix synthesis and chondrocyte proliferation (Wilsman et al., 1996; Farnum and Wilsman, 2001). Furthermore, the differential growth of various bones appears to be related to differences in cell size of hypertrophic chondrocytes. Chondrocytes in bones with rapid growth, such as the femur, undergo a larger increase in size than chondrocytes in growth plates in bones (such as the radius) that grow less rapidly (Wilsman et al., 1996; Farnum and Wilsman, 2001). The factors that control these local differences in hypertrophic cell size have not been defined, but likely involve an interaction of both local and systemic factors (Farnum and Wilsman, 2001).

Hypertrophic chondrocytes also have an important metabolic function, in that they prepare the extracellular matrix for calcification. Terminally differentiated hypertrophic chondrocytes uniquely express type X collagen, as well as high levels of the enzyme alkaline phosphatase (Lewinson et al., 1982; O'Keefe et al., 1994a; Eerola et al., 1996; O'Keefe et al., 1997). Alkaline phosphatase is essential for calcification of the matrix (Anderson et al., 1997) and is present in high concentration in matrix vesicles, which are small membrane vesicles secreted by hypertrophic chondrocytes into the surrounding matrix (Anderson, 1995; Anderson et al., 1997). These matrix vesicles are believed to be the first sites of calcium hydroxyapatite nucleation. Alkaline phosphatase increases the concentration of phosphate ions, which are necessary for this calcification process. Indeed, the absence of alkaline phosphatase, as observed in hypophosphatasia, is associated with decreased mineralization of the matrix and widening of the growth plate, as well as with defective mineralization of bone (Anderson et al., 1997).

Chondrocyte differentiation and hypertrophy are essential steps in longitudinal growth; therefore, intense investigations are under way to identify the regulators of these processes. To date, several paradigms have emerged from this research: 1) chondrocytes in the endochondral ossification pathway are primed to spontaneously complete maturation and undergo hypertrophy; 2) negative regulators of differentiation and hypertrophy

are critically important; and 3) different, or complementary, mechanisms probably control this process during embryonic and adolescent development (Iwasaki et al., 1997; Grimsrud et al., 1999; Volk and Leboy, 1999).

Both in vitro and in vivo models of chondrocyte differentiation show that chondrocytes spontaneously undergo hypertrophy in the absence of inhibitory factors. There is substantial evidence that the bone morphogenetic proteins (BMPs) and their receptors are responsible for the spontaneous completion of maturation (Enomoto-Iwamoto et al., 1998; Volk et al., 1998; Grimsrud et al., 1999; Volk and Leboy, 1999; Grimsrud et al., 2001). Isolated chondrocytes in culture are induced to undergo hypertrophy and increase the expression of hypertrophic markers, including type X collagen and alkaline phosphatase activity in the presence of BMPs (Leboy et al., 1997; Enomoto-Iwamoto et al., 1998; Grimsrud et al., 1999; Grimsrud et al., 2001). In contrast, inhibition of BMP signaling prevents chondrocyte maturation (Volk et al., 2000; Grimsrud et al., 2001). In vivo studies demonstrate that expression of either of the BMP antagonists, noggin or chordin, in the developing chick limb bud prevents chondrocyte hypertrophy and expression of associated genes associated with maturation (Pathi et al., 1999; Zhang et al., 2002).

Other factors that induce chondrocyte maturation also appear to act through BMP signaling. Thyroxine induces type X collagen synthesis and other maturational characteristics in growth plate chondrocytes in culture through induction of BMP2, an effect that can be blocked by addition of the BMP antagaonist noggin (Ballock et al., 2000a). Similarly, the induction of chondrocyte differentiation by retinoic acid appears to be related to effects on BMP signaling. Retinoic acid was recently shown to induce the expression of the BMP signaling molecules Smad1 and Smad5 in chondrocytes, making retinoic acid-treated chondrocytes more sensitive to BMP-mediated signaling events.

Similar to the role of Sox9 in chondrogenesis, the transcription factor CBFA-1 appears to have a critical role during the process of chondrocyte hypertrophy and terminal differentiation (Karsenty, 2001). Although CBFA-1 is able to induce terminal differentiation in chondrocytes, mice without CBFA-1 lack hypertrophic chondrocytes in some growth plates, while in other growth plates hypertrophy proceeds normally, suggesting that other transcription factors are involved in this process (Inada et al., 1999; Kim et al., 1999). The transcription factors in the BMP signaling pathway (Smad1, -5, and -8) also appear to mediate hypertrophy (Leboy et al., 2001; Li et al., 2001), and have recently been shown to interact with CBFA1 at the type X collagen promoter to induce the expression of this gene (Leboy et al., 2001).

Diseases of Abnormal Chondrocyte Maturation and Hypertrophy

Hypothyroidism

The actions of thyroid hormone in enhancing longitudinal bone growth and maturation are among the most sensitive effects of this hormone. In children with hypothyroidism, deficiency of thyroid hormone results in delayed bone age, reduced thickness of the growth plates in long bones, disorganization of the normal cartilage columns of the growth plates, and impaired differentiation of growth plate chondrocytes into hypertrophic cells (Ray et al., 1954; Hall, 1973). These abnormalities are often clinically manifested as severe growth retardation and mechanical failure of the growth plates of the hips (slipped capital femoral epiphysis) (Wells et al., 1993).

In vivo studies have suggested that thyroid hormone stimulates longitudinal bone growth by increasing the secretion of growth hormone (Hervas et al., 1975); however, growth hormone alone is unable to stimulate cartilage maturation (Ballock and Reddi, 1994). There are also indications that thyroid hormone can act directly on growth plate chondrocytes through growth hormone-independent mechanisms (Thorngren and Hansson, 1973). The strongest evidence for this direct mechanism of action is the observation that thyroid hormone is able to stimulate longitudinal bone growth in animals in which growth hormone secretion has been ablated by hypophysectomy (Lewinson et al., 1994). Thyroid hormone also facilitates chondrocyte differentiation in both thyroidectomized and hypophysectomized rats, which growth hormone cannot facilitate (Ohlsson et al., 1993).

Many investigators have observed similar effects of thyroid hormone on markers of chondrocyte differentiation in vitro. In several chondrocyte culture systems, administration of thyroid hormone increases in a dose-responsive manner the synthesis of type X collagen mRNA and protein, alkaline phosphatase activity, and cellular hypertrophy, all markers of the terminally differentiated phenotype of the growth plate chondrocyte (Burch and Lebovitz, 1982; Burch and Van Wyk, 1987; Bohme et al., 1992; Carrascosa et al., 1992; Ohlsson et al., 1992a; Quarto et al., 1992; Ballock and Reddi, 1994). Similarly, thyroid hormone suppresses DNA synthesis by chondrocytes, and inhibits cell proliferation even in the presence of mitogens (Bohme et al., 1992; Ohlsson et al., 1992a; Quarto et al., 1992).

The molecular cloning of the thyroid hormone receptor, and its surprising identification as a proto-oncogene (c-erbA) and a member of the steroid hormone family of intracellular receptors (Thompson et al., 1987; Lazar et al., 1988; Murray et al., 1988; Hodin et al., 1989) provide clues about the molecular mechanisms through which thyroid hormone exerts its profound control over skeletal growth and maturation. The thyroid hormone-receptor complex has been shown to bind to specific sequences in the promoter region of the DNA to directly regulate gene transcription. This consensus sequence consists of a direct or inverted repeat (palindrome) of AGGTCA, separated by four nucleotides (Umesono et al.,

1991; Kliewer et al., 1992; Perlmann et al., 1993). It is interesting to note in this regard that the receptors for 1,25 $(OH)_2$ vitamin D3 and retinoic acid, two other members of the steroid hormone family that are known to influence chondrocyte differentiation (Iwamoto et al., 1989, 1991), can bind to the same consensus sequence if it is separated by three or five bases, respectively (Umesono et al., 1991). Furthermore, the receptors for 9-cis retinoic acid and vitamin D are able to form heterodimers with thyroid hormone receptors to act as coregulators of gene transcription (Yu et al., 1991; Kliewer et al., 1992; Hallenbeck et al., 1992; Cheskis and Freedman, 1994; Lee et al., 1994; Liu and Freedman, 1994; Mano et al., 1994; Schrader et al., 1994).

These observations suggest that thyroid hormone may control chondrocyte differentiation by directly controlling the transcription of a number of target genes associated with chondrocyte maturation. Obvious candidate genes for direct transcriptional regulation by thyroid hormone would include the genes encoding type X collagen and alkaline phosphatase, two widely accepted markers of the terminally differentiated growth plate chondrocyte. However, analyses of the 5′ untranslated regions of these two genes have thus far failed to demonstrate the presence of the consensus DNA binding sequences for thyroid hormone. It is therefore likely that the key regulatory step mediated by thyroid hormone occurs earlier in the differentiation cascade than the transcriptional activation of genes normally associated with chondrocyte hypertrophy and terminal differentiation.

Lead toxicity

Clinical data from human populations suggest that the growth plate is an important target tissue. Both the Second and Third National Health and Nutrition Examination Surveys (NHANES II and NHANES III) demonstrated decreased skeletal growth in lead-exposed children (Schwartz et al., 1986; Ballew et al., 1999). The reduction in stature was similar in the two data bases. In the more recent study, the reduction in height was approximately 1.57 cm in stature for each 0.48 μmol/liter (10 μg/dl) in serum lead concentration, and was highly significantly independent of all other covariates on a multiple regression analysis (Ballew et al., 1999). The negative association between stature and lead levels is present even at concentrations as low as 4 μg/dl (Schwartz et al., 1986). This concentration of lead is typically not associated with measurable pathological effects in other tissues, and demonstrates the sensitivity of the developing skeleton to lead.

Recent work has shown that the inhibition of skeletal growth in children exposed to lead, along with associated growth plate morphological abnormalities, may be due to an alteration in chondrocyte responses to PTHrP and TGF-β (Schwartz et al., 1986; Gonzalez-Riola et al., 1997; Zuscik et al., 2002). Lead interferes with the inhibitory effect of PTHrP and TGF-β on chondrocyte differentiation, and thus alters the normal regulatory events that control the rate of chondrocyte hypertrophy (Zuscik et al., 2002).

Growth Plate Mineralization

Chondrocytes are metabolically active cells that secrete and maintain a highly specialized matrix. The function of this tissue is to promote calcification of cartilage that serves as a template for bone formation by osteoblasts. This mineralization of cartilage occurs primarily in the matrix located between distinct hypertrophic chondrocyte columns, and not in the interzone between hypertrophic chondrocytes in the same column (Johnstone et al., 2000). Matrix vesicles are the initial sites of mineralization in the hypertrophic region of the growth plate, and are critical components of the calcification process (Anderson, 1995; Kirsch et al., 1997). Matrix vesicles are 100-nm-diameter, extracellular membrane-bound particles that are released by budding from the surfaces of chondrocytes, osteoblasts, and odontoblasts (Anderson, 1995). Matrix vesicle accumulation of calcium appears to be dependent upon a family of calcium channel molecules referred to as annexins (Kirsch et al., 2000a, b). Annexin II, V, and VI are present with the lipid bilayer of matrix vesicles and are required for accumulation of calcium in these structures. Ca(2+) channel blockers specific for annexins block their uptake of calcium (Kirsch et al., 2000a,b).

Type II and type X collagen bind to matrix vesicles due to interactions with annexin V. In the absence of annexin V, these collagens do not interact with matrix vesicles (Kirsch et al., 2000a). Furthermore, type II and type X collagen both stimulate the activity of the annexin V calcium channel (Kirsch et al., 2000a). Thus, one of the roles of type X collagen, which is present only in hypertrophic cartilage, may be to facilitate the deposition of calcium in the matrix. Recent work in animals demonstrates that uremia, which results in retarded growth in children, is associated with an increase in the width of hypertrophic cartilage, as well as a decrease in the deposition of both type II and type X collagen, alterations in collagen fibril architecture, and defective mineralization (Alvarez et al., 2001a).

Matrix vesicles also contain enzymes, such as alkaline phosphatase and matrix metalloproteinases. The role of alkaline phosphatase in matrix mineralization is not certain, but likely involves the important step of metabolism of pyrophosphophosphate to yield two molecules of orthophosphate (Anderson, 1995). Whereas pyrophosphate is a known inhibitor of hydroxyapatite crystal formation, pyrophosphate stimulates mineralization (Anderson, 1995).

Matrix metalloproteinases (MMPs) are responsible for catabolism and turnover of the matrix, and are induced during the process of chondrocyte hypertrophy (D'Angelo et al., 2000, 2001). MMP2, -9 and -13 are present in matrix vesicles, and mineralization of the matrix is associated

with a marked increase in the cleavage of type II collagen by collagenase (Mwale et al., 2000; D'Angelo et al., 2001). Matrix vesicles also contain TGF-β, which is present in a latent form, but is activated by MMP13. The increased levels of active TGF-β present in the growth plate at the onset of mineralization are believed to be due, in part, to the presence of MMP13 in matrix vesicles (D'Angelo et al., 2001). Additionally, MMPs are critical for angiogenesis in the growth plate, and thus are necessary for normal calcification and bone formation (Zhou et al., 2000). Mice without MMP9 (gelatinase B) have defective angiogenesis, reduced chondrocyte apoptosis, widening of the hypertrophic zone, and decreased mineralization of the matrix (Vu et al., 1998). The effects of the MMPs on angiogenesis are as yet unclear, but they may be related to a decrease in catabolism of the matrix, release of important growth factors, or other factors.

Proteoglycans are major components of the extracellular matrix in cartilage. Like articular cartilage, the growth plate contains large aggregating proteoglycans, and is thus characterized as hyaline cartilage. Although it was initially thought that the content of aggregating proteoglycans is reduced in the hypertrophic region, it has been established that increased concentrations of aggregating proteoglycans are present at the onset of calcification (Matsui et al., 1991; Byers et al., 1997). However, there are changes in the relative content of the proteoglycan monomers, their degree of sulfation, and their size during maturation that are probably important (Byers et al., 1997). In contrast, there is an increase in the concentrations of some of the smaller, nonaggregating proteoglycans during chondrocyte hypertrophy. The mRNA and protein for the proteoglycan biglycan are detected in hypertrophic chondrocytes and the surrounding matrix, but not in other areas of the epiphysis (Takagi et al., 2000), which suggests that this proteoglycan is associated with mineralization.

Cartilage also contains components that inhibit calcification of the extracellular matrix. The best characterized is matrix Gla protein (MGP), a 14-kD extracellular matrix protein of the mineral-binding Gla protein family. MGP is expressed by proliferative and late hypertrophic chondrocytes, but not by the intervening chondrocytes (Newman et al., 2001). MGP inhibits calcification both in vitro and in vivo (Luo et al., 1997; Newman et al., 2001). MGP-deficient mice have inappropriate calcification of the growth plate that leads to short stature, osteopenia, and fractures (Luo et al., 1997).

Disorders of Matrix Mineralization in the Growth Plate

Rickets and hypophosphatasia

It is well known that vitamin D deficiency also results in defective mineralization and widening of the growth plate, and that these effects are mediated through classic vitamin D receptor (VDR)-dependent mechanisms, as well as through a membrane-mediated signaling mechanism (Boyan et al., 1999) (Figure 7). Vitamin D increases alkaline phosphatase and MMP activity in chondrocytes (Boyan et al., 1999). However, some of the effects of vitamin D on the growth plate are secondary to hormonal and metabolic effects. Mice lacking the classic VDR develop rickets, with thickening of the growth plate and decreased mineralization (Amling et al., 1999). However, in the VDR-ablated mice with preservation of normal mineral ion homeostasis, growth plate morphology and width is normal (Amling et al., 1999). Therefore, a principal action of vitamin D on the growth plate is its role in intestinal calcium absorption. The skeletal consequences of absence of the vitamin D receptor appear to be related to impaired intestinal calcium absorption and/or the resultant secondary hyperparathyroidism and hypophosphatemia (Amling et al., 1999).

Hypophosphatasia is a heritable disease characterized by deficient activity of the tissue nonspecific isoenzyme of alkaline phosphatase (Figure 8). It results in rickets, due to decreased calcification of the matrix (Anderson et al., 1997). Electron microscopy studies have recently shown that matrix vesicles in patients with rickets maintain their ability to concentrate calcium and phosphate internally, and to initiate mineral formation (Anderson et al., 1997). However, in the absence of alkaline phosphatase, there is retarded extravesicular calcium-hydroxyapatite crystal propagation (Anderson et al., 1997).

Apoptosis of Growth Plate Chondrocytes

In all organisms, growth and development require the proliferation, differentiation, and removal of cells. Apoptosis is the mechanism by which cells undergo programmed cell death, a process that is necessary for the homeostasis of most organs, including the growth plate (Nagata, 1997; Green, 1998; Vaux and Korsmeyer, 1999). The cells in the growth plate that undergo apoptosis are terminally differentiated chondrocytes (Fujita et al., 1995; Shapiro et al., 1995; Mello and Tuan, 1997; Roque and Gibson, 1997; Mansfield et al., 1999). The role of terminally differentiated chondrocytes is to prepare the matrix for calcification, which then acts as a template for osteoblastic bone formation. Once the cartilage matrix calcifies, the death and removal of terminally differentiated hypertrophic chondrocytes provides space for the ingress of vascular channels and bone stromal cells (Skawina et al., 1994; Aharinejad et al., 1995; Shapiro et al., 1995; Roach et al., 1998).

While it was initially thought that hypertrophic chondrocytes died by a passive process due to a depletion of nutrients and oxygen tension in the hypertrophic region of the growth plate, it is now recognized that the process is an active and regulated event. Thus, chondrocytes in the hypertrophic region of the growth plate share morphological features similar to those of other cells undergoing apoptosis (Shapiro et al., 1995). The morphological events result from activation

of a set of enzymes that target and metabolize important intracellular structures (Salvesen and Dixit, 1997; Adams and Cory, 1998). Morphologic changes in cells undergoing programmed cell death include condensation of the nuclear chromatin, cell shrinkage, and plasma membrane blebbing (Fraser and Evan, 1996; Vaux and Korsmeyer, 1999). In contrast to necrotic cells, which lyse and release degradative enzymes into the local environment, apoptotic cells are rapidly recognized by and taken up by neighboring or phagocytic cells, but, unlike necrotic cells, they do not induce inflammation (Fraser and Evan, 1996).

The enzymes that initiate apoptosis are called caspases (Nagata, 1997; Salvesen and Dixit, 1997; Adams and Cory, 1998). Caspases are a family of cysteine proteases that cleave target proteins with high specificity. All cells contain caspases in their cytoplasm in an inactive, or zymogen, form, and are therefore primed to undergo apoptosis. In addition to the caspases, there are a series of inhibitor molecules that can block caspase activation. Recently, several protein families with this activity have been identified, including the bcl-2 family of proteins (Adams and Cory, 1998; Green, 1998). Bcl-2 stabilizes the mitochondria and prevents the release of cytochrome c, while another member, BAX, stimulates the release of cytochrome c and leads to apoptosis (Adams and Cory, 1998; Green and Reed, 1998). The relative concentration of the inhibitory protein, bcl-2 and the stimulator, BAX, appears to be a critical determinant of whether a cell undergoes apoptosis or continues to survive. The relative levels of these proteins have been shown to be important not only in the pathogenesis and progression of cancer, but also in the homeostasis of normal tissue (Green and Reed, 1998).

The mechanisms regulating physiologic cell death in the growth plate are not been well defined, but they likely involve cell interactions with extracellular matrix, growth factors, and cytokines. Mineralization of the matrix is associated with the release of phosphate ions. In vitro work has demonstrated that chondrocytes have increased apoptosis in the presence of elevated phosphate concentrations, and that the effect is dependent upon the maturational state of the cells. Furthermore, differentiated chondrocytes are more sensitive to increased phosphate levels than less differentiated cells (Mansfield et al., 1999). The increase in phosphate concentration is associated with abnormalities in mitochondrial function. Chondrocytes have loss of mitochondrial membrane potential, and greater reliance on glycolysis with progression through hypertrophy. It is hypothesized that Pi triggers apoptosis in these energy-compromised cells by promoting a mitochondrial membrane transition, leading to the release of cytochrome c and other pro-apoptotic factors, thereby inducing the death process (Rajpurohit et al., 1999).

It is also clear that local growth factors regulate programmed cell death, and that abnormalities in these factors or their signaling molecules are associated with some of the developmental diseases involving the growth plate. Achondroplasia, the most common cause of human dwarfism, results from an activating mutation of the FGF3 receptor. Normally, the growth factor FGF2 binds to the FGF3 receptor and leads to a reduction in proliferation and an increase in apoptosis of growth plate chondrocytes (Sahni et al., 2001). In transgenic mice, both the FGFR3 activating mutation and overexpression of FGF2 in chondrocytes results in growth abnormalities that mimic the human condition of achondroplasia (Sahni et al., 2001). Recently, it was shown that the transcription factor STAT1 is responsible for the FGF effects. In mice without STAT1, the growth-plate abnormalities and early apoptosis associated with FGF2 overexpression are corrected (Sahni et al., 2001), indicating that this specific signal downstream of the FGF2 receptor is a regulator of apoptosis.

PTHrP is a potent inhibitor of apoptosis, and likely mediates this action by up-regulation of the apoptosis inhibitor, bcl-2 (Lee et al., 1996; Amling et al., 1997). In contrast, the adverse effects of glucocorticoids and radiation on skeletal growth are mediated in part by an increase in apoptosis (Silvestrini et al., 2000; Pateder et al., 2001a). Animals treated with a 10-day course of glucocorticoids have increased rates of apoptosis in hypertrophic chondrocytes in vivo, and reduced width of the growth plate (Silvestrini et al., 2000). Similarly, radiation stimulates apoptosis and inhibits the expression of bcl-2, while stimulating a fivefold increase in caspase 3 levels (Pateder et al., 2001). Thus, radiation favors the expression of factors that positively regulate apoptosis. Understanding the normal and pathological events involved in growth-plate chondrocyte apoptosis will lead to novel therapeutic strategies to protect the growing skeleton from the detrimental effects of radiotherapy and other toxic agents (Pateder et al., 2001).

Vascular Invasion From the Metaphysis

The growth plate is essentially an avascular structure that relies on diffusion of both oxygen and nutrients for cell metabolism from vascular arcades located on the metaphyseal side of the growth plate (Skawina et al., 1994). The metaphyseal vascular channels are found in compartments bounded by calcified cartilage beneath the last row of hypertrophic chondrocytes. These vascular channels are aligned along the longitudinal axis of the bone, and contain an ascending and descending capillary system (Aharinejad et al., 1995). It has been recognized in recent years that vascular invasion is a pivotal event in the regulation of endochondral ossification and is necessary for normal bone formation (Bittner et al., 1998).

Vascular endothelial growth factor (VEGF) appears to be the factor responsible and necessary for vascular ingrowth into the growth plate (Wedge et al., 2000). VEGF is a 44-kDA protein that targets vascular endothelial cells and stimulates

their proliferation and migration, and ultimately the formation of blood vessels (Ferrara, 1999; Carlevaro et al., 2000; Wedge et al., 2000). *VEGF* is expressed by hypertrophic chondrocytes in the growth plate, but is absent in resting and proliferating chondrocytes (Gerber et al., 1999; Horner et al., 1999; Carlevaro et al., 2000; Garcia-Ramirez et al., 2000). In animals, inhibition of VEGF function (by the use of an oral inhibiting agent or an injected genetically engineered protein that blocks activation of the receptor for VEGF) leads to loss of vascular invasion (Gerber et al., 1999; Wedge et al., 2000).

This inhibition of vascular invasion in the absence of VEGF leads to profound disturbances in the architecture of the growth plate and affects longitudinal growth. Calcified cartilage persists due to a decrease in the recruitment and differentiation of osteoclasts/chondroclasts (Gerber et al., 1999), with widening of the hypertrophic region and diminution of trabecular bone formation. Although chondrocyte proliferation, differentiation, and maturation apparently remain normal, resorption of terminal chondrocytes is diminished. In contrast, upon cessation of anti-VEGF treatment, there is a return of normal growth-plate structure and function with resumption of capillary invasion, restoration of bone growth, resorption of the hypertrophic cartilage, and normalization of growth plate architecture. These findings indicate that VEGF-mediated capillary invasion is an essential signal that regulates growth-plate morphogenesis and triggers cartilage remodeling. Hypertrophic chondrocytes also express the receptor for VEGF (VEGF receptor 2/Flk1), suggesting that this factor may have an autocrine role in these cells; however, the nature of the direct effect on chondrocytes is not known (Carlevaro et al., 2000).

Other factors have also been found to have a role in angiogenesis, although their effects may be related to modulation of *VEGF* expression (Horner et al., 1999). Studies have shown that growth factors that inhibit maturation (such as PTHrP) prevent angiogenesis, whereas factors that accelerate chondrocyte hypertrophy (such as the transcription factor CBFA1) induce angiogenesis (Schipani et al., 1997; Takeda et al., 2000). Basic fibroblast growth factor (bFGF) is produced by chondrocytes and has known angiogenic properties. Infusion of bFGF into the rabbit proximal tibial growth plate accelerates vascular invasion and ossification of growth-plate cartilage. (Baron et al., 1994).

Finally, there is evidence to suggest that endothelial cells may also influence the terminal differentiation of chondrocytes. In cell culture, chondrocytes can be induced to undergo terminal differentiation when cocultured with vascular endothelial cells. This activity is due to a secreted factor by these cells, and is unique to endothelial cells, as smooth muscle cells, fibroblasts, or hypertrophic chondrocytes do not secrete this activity (Bittner et al., 1998). Similarly, in a model of cartilage explants cultured on a chick embryonic chorioallantoic membrane, chondrocyte hypertrophy was found to occur in regions adjacent to several blood vessels. These findings suggest that cumulative release of diffusible factors from more than one vessel triggers chondrocyte hypertrophy (Roach et al., 1998). However, the nature of the diffusible factors from endothelial tissues that contain this activity remains to be determined.

Closure of the Growth Plate

As skeletal maturity approaches, the rate of longitudinal bone growth diminishes as growth plate chondrocytes decrease their proliferation. This decreased growth rate is associated with structural changes in the physis, including a gradual decline in growth plate width due to the reduced height of the proliferative and hypertrophic zones, as well as reduced hypertrophic cell size and column density (Weise et al., 2001). In man and some other mammals, the growth plate is completely resorbed following puberty, resulting in fusion of the epiphysis to the metaphysis.

Recently, it has become evident that this process of physeal closure is primarily under the control of estrogen in both sexes. Patients with genetic mutations in either the gene encoding the aromatase enzyme that converts androgen to estrogen, or in the gene encoding the estrogen receptor-α fail to close their physes at the time of sexual maturation, and show evidence of increased height due to longitudinal bone growth well into adulthood (Smith et al., 1994; Carani et al., 1997; Bilezikian et al., 1998). Conversely, patients with precocious puberty who are exposed to estrogen prematurely close their physes earlier than predicted (Sigurjonsdottir and Hayles, 1968).

The molecular mechanisms involved in estrogen-mediated physeal closure remain incompletely characterized. Experiments in rabbits, which like humans resorb their growth plates following sexual maturation, suggest that estrogen may exert its effect by promoting a process of programmed replicative senescence in growth-plate chondrocytes, rather than by accelerating vascular invasion or ossification (Weise et al., 2001). Once the proliferative potential of the growth-plate cells is exhausted, epiphyseal fusion may occur spontaneously.

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