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Part I

**Molecular and cellular
environment of bone**

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Endochondral bone formation and development in the axial and appendicular skeleton

A. Robin Poole, Sheila Lavery and Fackson Mwale

Introduction

With the exception of the craniofacial skeleton and the clavicle, bone formation during development occurs through a process called endochondral ossification, whereby cartilage is formed as a skeletal tissue, calcified and replaced by bone. Much of the craniofacial skeleton consists of ‘membrane’ bones that form as a result of intramembranous ossification and without a cartilaginous intermediate. The clavicle is the only ‘membrane’ bone in mammals outside the craniofacial skeleton. The axial and appendicular skeletons and portions of the cranial skeleton (calvaria, otic capsule) arise from mesoderm.

In the embryo, bone formation occurs following an orderly and carefully orchestrated differentiation of mesenchymal cells into chondroblasts, perichondrium, periosteum and osteoblasts (Hall, 1987) (Fig. 1.1). Then growth plates are established, first to lengthen bones and then, in the case of long bones, to shape the forming epiphyses.

As part of this process, there is a complex series of events that involves the formation of chondroblasts and then their maturation into chondrocytes. Only mature hypertrophic chondrocytes establish a calcified extracellular matrix, which is then partly resorbed through a process involving angiogenesis. This first occurs early in development within the diaphysis and then later in the growth plates (Fig. 1.1). The calcified cartilage then acts as a template on which osteoblasts form woven bone, which is eventually resorbed and replaced with a mature trabecular bone within the epiphyses and the diaphysis. At the same time, these events are initially preceded by, but later always accompanied by, cortical bone formation.

The purpose of this chapter is to outline some aspects of these endochondral developmental processes and then review some of the key events and regulatory mechanisms which result in the formation of the majority of the skeleton. In line with the guidelines for these chapters, only selected references will be provided.

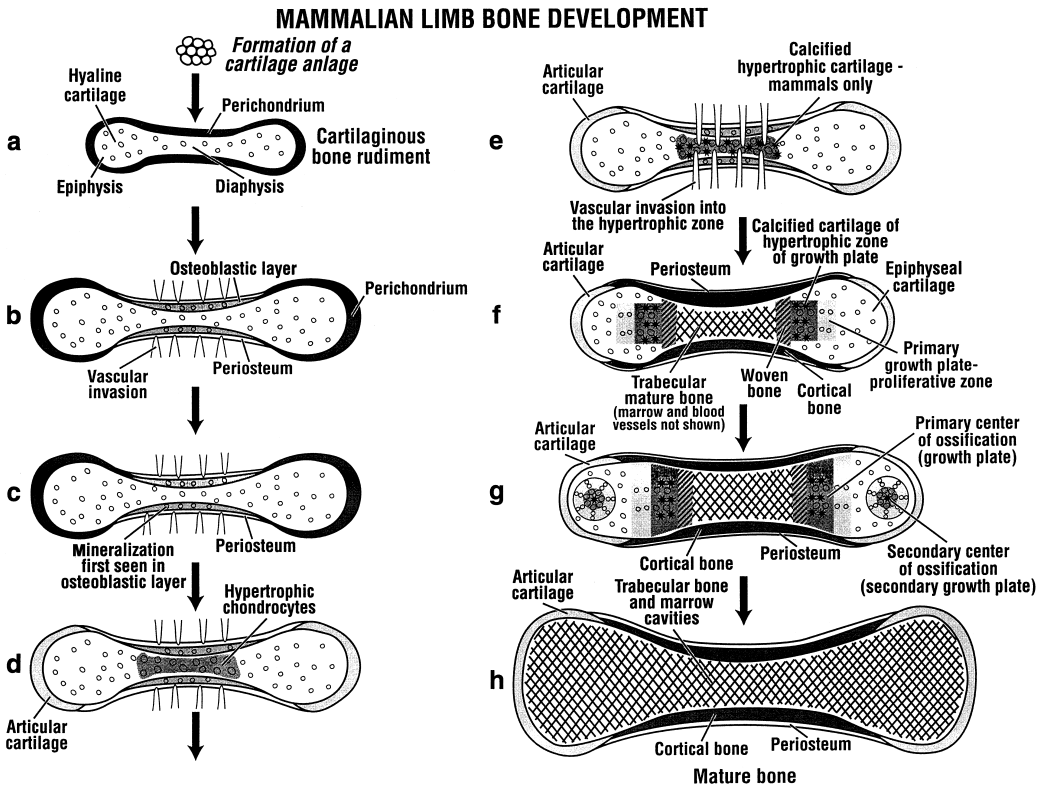


Fig. 1.1 The development of mammalian long bones from the cartilage anlage to the mature bone to show principal steps in cell and tissue differentiation, angiogenesis, growth plate formations and calcification.

The formation of the cartilage anlage in the embryo

Mesenchymal cells first form condensations, whereby cells in close proximity to each other and lacking vascularity, differentiate into chondroblasts (Fig. 1.2). Suppression of the proto-oncogene *c-myc* (which is upregulated in dividing cells) favors chondrogenesis. Around these sites where cartilage forms, blood vessels are present. Here mesenchymal cells differentiate into myogenic elements that form muscle. Distinctive alterations in fibroblast growth factor receptor (FGFR) gene expression in these mesenchymal cells and chondroblasts can be recognized prior to and during differentiation (Fig. 1.2; Szebenyi et al., 1995). These condensing cells transiently express the *Osf2/Cbfa1* runt family transcription factor which is otherwise only subsequently expressed in osteoblasts (Ducy et al., 1997). The synthesis of type II procollagen, which defines the chondroblast/chondrocyte phenotype more than any other molecule, initially involves expression of type IIA procollagen

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in these condensing mesenchymal cells. As they differentiate, so the expression switches to the mature type IIB procollagen, characteristic of the chondroblast/chondrocyte phenotype.

When cells synthesize type IIB collagen, exon II is not expressed and the amino-propeptide is consequently shorter. The functional significance of this remains to be determined.

Abbreviations: GDF, growth differentiation factor; CDMP, cartilage-derived morphogenetic factor; SHH, Sonic hedgehog; IHH, Indian hedgehog; Ptc, patched; FGFR, fibroblast growth factor receptor; PTH, PTHrP, parathyroid hormone and parathyroid hormone-related peptide; GH, growth hormone; IGF-I, insulin-like growth factor-I; TGF, transforming growth factor; BMP, bone morphogenetic protein; T4, thyroxine; T3, triiodothyronine.

First bone formation

A recognizable perichondrium is formed (Fig. 1.1(a)) which serves as the principal site of chondroblast generation and differentiation. This process is called appositional growth. The perichondrium initially surrounds each forming epiphysis and newly formed cartilaginous diaphysis. These chondroblasts mature to chondrocytes and synthesize not only type IIB procollagen but also the large proteoglycan aggrecan link protein and many other specialized matrix molecules, which constitute the extensive extracellular matrix of mature hyaline cartilage.

With continuing development the most peripheral perichondrial tissue enveloping the diaphysis (the shaft of the forming bone) differentiates into the periosteum from which osteoblasts develop and form cortical bone (Fig. 1.1(b)). In mammals, capillaries must first invade the perichondrium before it transforms into the periosteum. These osteoblastic cells mature, establish and calcify an osteoid matrix which contains type I collagen and bone specific molecules, such as bone sialoprotein. Alkaline phosphatase, that is always present in increased amounts in mineralizing tissue, and is required for this process, is first found in the periosteal collar where mineralization, following angiogenesis, is first initiated (Fig. 1.1(c)). The absence of a functional alkaline phosphatase results in hypophosphatasia (see below). There are clear demarcations between the periosteal cellular layer and its underlying osteoid (which together constitute the periosteum) and between the osteoid and the cartilaginous diaphysis.

With maturation, the chondrocytes in the central diaphysis mature and enlarge in size to become 'hypertrophic' (Fig. 1.1(d)): they start to synthesize type X collagen and then calcify their matrix. This maturational change is dependent upon molecule(s) released from the more peripheral osteoblasts. Deletion of the osteoblast

transcription factor *Osf2/Cbfa1* (a runt family member) by homologous recombination results in impaired maturation of chondrocytes as well as of osteoblasts. This results in a lack of periosteal ossification and a very restricted and patchy cartilage calcification following chondrocyte hypertrophy (Fig. 1.1(e); Komori et al., 1997; Otto et al., 1997). Moreover, the calcified cartilaginous diaphysis is not vascularized (a feature characteristic of complete hypertrophic maturation) and fails to develop bone marrow cavities.

In birds, the cartilaginous diaphysis becomes hypertrophic but does not mineralize, but in mammals the cartilage starts to calcify following hypertrophy. The hypertrophy is ordinarily associated with the development of angiogenesis; blood vessels invade the hypertrophic cartilage from the periosteal collar, which has already been penetrated by capillaries. This vascular invasion is seen both at this stage in development and also in the primary and secondary centers of ossification within the growth plates. Hypertrophic chondrocytes produce angiogenic molecules (Alini et al., 1996b; Carlavaro et al., 1997) which probably also includes vascular–endothelial cell growth factor. These therefore serve to induce vascular invasion into this tissue.

With increasing osteoblastic activity, a collar of periosteal bone is formed which expands along the length of the diaphysis, fusing in the mid-diaphysis with the calcified core of the diaphysis. The primary growth plates are then established (at the ends of the long and vertebral bones (Fig. 1.1(f))) to provide accelerated growth in length. It is within these cartilaginous primary growth centers that this enhanced growth continues, both in the embryo and postnatally.

The growth plates

The proliferative zone

When the primary growth plates are first established, they abut the uncalcified epiphyseal cartilage and the calcified diaphysis. In these growth centers there is continuous formation of chondroblasts from a growth center abutting what is usually called the ‘resting zone’ of the epiphyseal cartilage. Cells become organized in an axial manner, giving rise to columns of proliferating cells, expressing *c-myc*. These cells actively establish an extensive extracellular cartilage matrix (Fig. 1.2). Both *c-jun* and *c-fos* proto-oncogene expression are then elevated later in the proliferative phase. These proliferating cells have receptors for growth hormone that generates insulin-like growth factor-I (IGF-I) synthesis that potently drives matrix synthesis as well as cell division (Poole, 1997). Thyroxine (T₄), and more potently, triiodothyronine (T₃), ensure the rapid maturation of these immature chondrocytes to the hypertrophic phenotype as well as regulating cell division. Vitamin D, both 1,25-dihydroxycholecalciferol and/or 24,25-dihydroxycholecalciferol, are required to ensure complete chondrocyte maturation, matrix calcification and vascular

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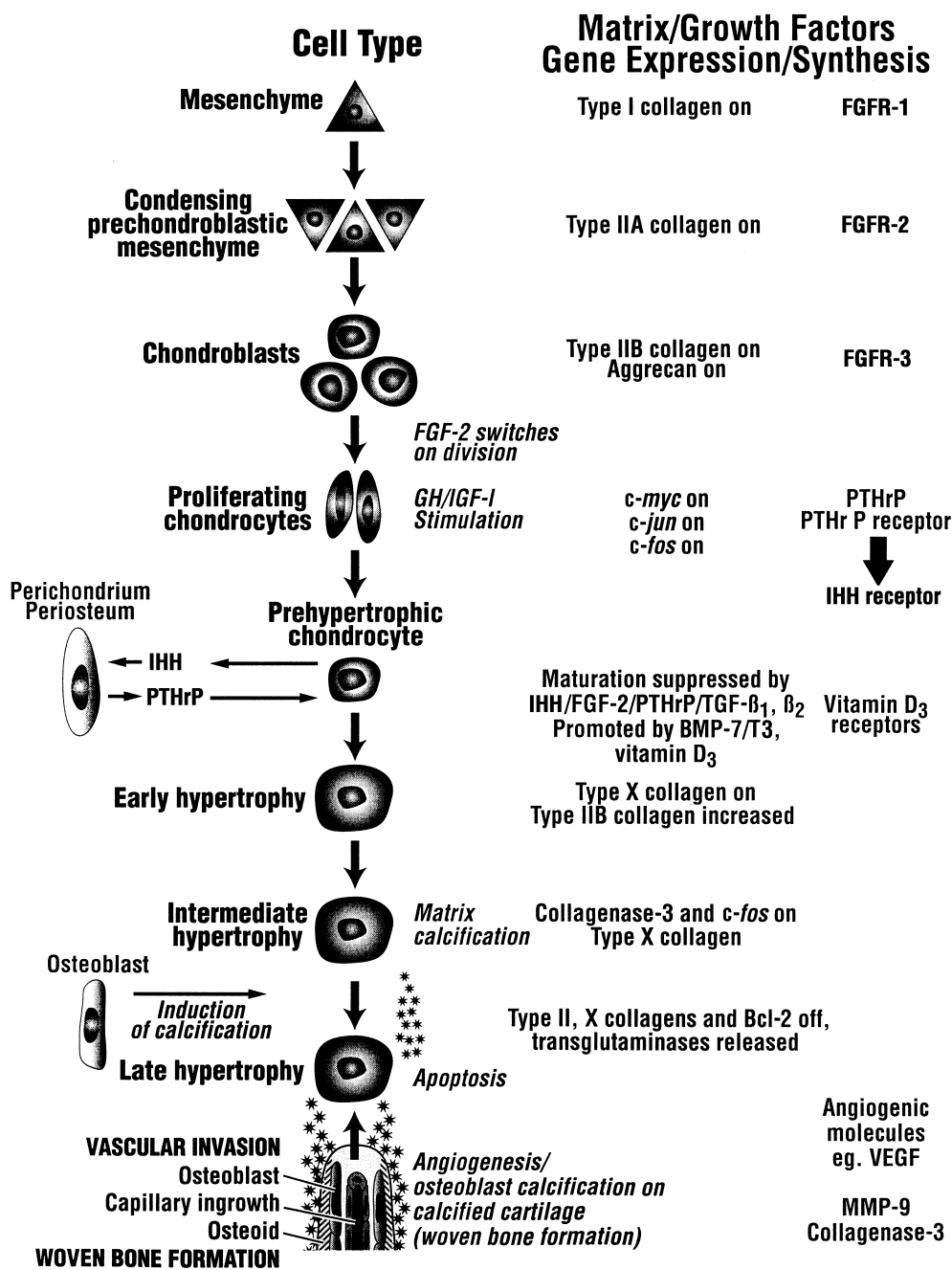


Fig. 1.2 A brief summary of the differentiation and maturation of mammalian chondrocytes, the synthesis of extracellular matrix, its remodeling and calcification and the regulation of these processes. The stages from chondroblasts to hypertrophic chondrocytes and vascular invasion of cartilage are represented in early development of the mammalian diaphysis as well as in the growth plates.

invasion. In the absence of vitamin D, maturation and mineralization is incomplete, the hypertrophic zone is extended and angiogenesis is impaired. The condition resembles hypophosphatasia. Each of these vitamin D metabolites can stimulate production of an angiogenic molecule from the maturing chondrocytes (Alini et al., 1996b) explaining the lack of angiogenesis in vitamin D deficiency.

The hypertrophic zone: early changes

In the proliferative zone, chondrocytes continue to synthesize the extracellular matrix. But then they suddenly upregulate synthesis of type IIB procollagen and start to synthesize type X collagen. The latter characterizes them as hypertrophic chondrocytes (Fig. 1.2). Type X collagen is found in both pericellular sites and in close association with type II collagen fibrils. Although its function is unclear, mutations that alter the structure of this molecule, such as those that give rise to Schmid-type metaphyseal dysplasia, reduce the rate of growth when patients are ambulatory, as compared with those undergoing bed rest. Thus type X collagen may play a structural role in maintaining collagen fibril organization and the mechanical properties of the matrix at a time when there is considerable resorption and remodeling of these type II collagen fibres and a reduction in fibril diameter (see below). Mutations in type II collagen also create a very marked disorganization of the growth plate with a loss of column formation, an irregular hypertrophic zone and disorganized mineralization. These chondrodysplasias are characterized by Kniest dysplasia and spondyloepiphyseal dysplasia (Poole, 1997). Mutations in cartilage oligomeric protein, that produce pseudoachondroplasia, also result in similar abnormalities in growth plates.

When these chondrocytes synthesize type X collagen, they exhibit a number of other fundamental changes. They lose functional receptors for basic fibroblast growth factor-2, which can suppress hypertrophy. Mutations in these receptors, leading to constitutive activity, produce growth abnormalities characterized by the most common skeletal dysplasia called achondroplasia. Ordinarily these hypertrophic chondrocytes also lose receptors for parathyroid hormone-related peptide (PTHrP), which is synthesized by the less mature chondrocytes of the proliferative zone. The loss of this gene, in knockout mice, or the gene for the PTH or PTH/PTHrP receptor, creates premature hypertrophy and disorganization in the growth plate (Amizuka et al., 1994). Thus PTHrP also serves to suppress the onset of hypertrophy.

At the time of early hypertrophy and type X collagen synthesis collagenase-3, a metalloproteinase (MMP-13) that potently degrades these type II collagen molecules, is also upregulated (Gack et al., 1995). The activity of this proteinase leads to a rapid and selective degradation of much of the extracellular type II collagen, resulting in an extensive resorption and remodeling of the extracellular matrix. This permits cellular enlargement or hypertrophy. Collagenase upregulation occurs

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in very close association with enhanced *c-fos* expression (Gack et al., 1994). *C-fos* is a component of the AP-1 transcription factor complex that regulates collagenase expression. This is a heterodimeric complex that also contains *C-jun*. Overexpression of the *c-fos* transgene causes formation of osteosarcomas and chondrosarcomas but suppresses hypertrophy. However, during natural development *c-fos* and *c-jun* expression are required. Increased expression of the latter is seen in prehypertrophic chondrocytes, and expression is enhanced by PTH which inhibits expression of the hypertrophic phenotype. Consistent with this, in *c-fos*^{-/-} mice, there is premature hypertrophy (Wang et al., 1992). Thus both *c-jun* and *c-fos* are involved in the regulation of maturation. An imbalance in the activities of these oncogenes clearly impacts negatively on normal chondrocyte maturation.

Transforming growth factor β_1 can also upregulate collagenase-3 (but not collagenase-1) expression in fibroblasts and chondrocytes (Uría et al., 1998) and may also be involved in upregulation of collagenase-3 in the growth plate during hypertrophy when increased amounts of this growth factor are generated. In contrast, osteoblast collagenase-3 is upregulated by PTH (Selvamuragan et al., 1998) which suppresses hypertrophy in chondrocytes (Amizuka et al., 1994). Moreover, overexpression of *c-fos* and *c-jun* genes is observed in response to PTH activation of these genes in osteoblasts. It is likely that tissue specificity of collagenase regulation is achieved via the presence of a runt binding domain as well as an AP-1 site in bone cells.

Calcification of the hypertrophic zone

Following resorption and remodeling, the residual matrix, which is by now of minimal volume, then starts to calcify (Fig. 1.2). It is enriched in content in the proteoglycan aggrecan (Poole, 1997). Calcification occurs in focal sites in the longitudinal septa where these proteoglycan molecules are concentrated. The C-propeptide of type II collagen, a calcium-binding protein, accumulates in these mineralizing sites. Prior to calcification, matrix vesicles are produced by chondrocytes, starting in the proliferative zone. These apparently serve to generate local elevated concentrations of calcium and phosphate and ensure nucleation of mineral formation, that really increases later following hypertrophy. Mice, in which the gene for matrix Gla protein has been eliminated by homologous recombination, exhibit premature calcification in the proliferative zone, suggesting that this molecule restricts mineralization to the hypertrophic zone where expression of this gene is normally minimal (Luo et al., 1997).

Apoptosis as the end stage of hypertrophic phenotype

The mature hypertrophic cells represent terminally differentiated chondrocytes. Apoptosis occurs in advanced hypertrophy, when mineralization is maximal. This leads to the death of these cells in association with reduced expression of Bcl-2 a

protein which negatively regulates programmed cell death (Amling et al., 1997). Apoptosis leads to the extracellular release of two transglutaminases, a tissue type and a plasma (avian factor XIIIa) form (Nurminskaya et al., 1998). The latter is markedly upregulated in hypertrophic chondrocytes, appears to cross-link matrix molecules and may contribute, like type X collagen, to matrix stabilization. It could alternatively enhance degradation since such cross-linked molecules (e.g., fibronectin) are more susceptible to proteolysis.

Vascular and chondroclastic erosion of hypertrophic zone

A striking feature of the hypertrophic zone, as in earlier development, is its vascular invasion. At this stage it is from the primary spongiosa and occurs in close association with apoptosis of hypertrophic chondrocytes. Following and accompanying the activity of the eroding multinucleate chondroclasts, capillary sprouts penetrate the largely uncalcified transverse septa (Fig. 1.2), accompanied by mononuclear cells rich in cathepsin B called septoclasts. This erosive angiogenic process leads to the progressive destruction of not only the transverse septa but also of many of the calcified septa. It involves the activity of extracellular proteases at the capillary invasion front where there is cleavage of type II collagen by collagenase cleavage of the proteoglycan aggrecan. Gelatinase B (MMP-9), a metalloproteinase present in these sites (Sakiyama et al., 1994; Vu et al., 1998) and produced by chondroclasts and osteoclasts, is involved in this process, since its removal by homologous recombination results in elongation of the hypertrophic zone. This is a consequence of impairment of apoptosis and of angiogenesis which is presumably dependent upon matrix degradation produced by this proteinase (Vu et al., 1998).

The formation of woven bone and its replacement with trabecular bone

As a consequence of this resorptive process in the lower hypertrophic zone, calcified cartilaginous trabeculae remain which extend into the diaphysis. Osteoblasts settle on these trabeculae, synthesize an extracellular matrix (osteoid) and then calcify it (Fig. 1.2). Initially bone sialoprotein is produced which acts as a nucleator for mineral formation. Eventually osteoclasts resorb this woven bone, and it is replaced by mature trabecular bone. Meanwhile, the periosteal bone collar gives rise to the mature cortical bone with its characteristic lamellae and Haversian canals. As osteoblasts mature, osteocalcin is also generated. This molecule somehow regulates bone formation since deletion of this gene results in an increase in bone formation without affecting bone resorption (Ducy et al., 1996). The calcified trabeculae on which woven bone forms are eventually replaced by a process of active osteoclastic resorption. This requires the presence of the osteoprotegerin ligand, without which osteoclasts fail to develop and the woven bone and trabeculae are not resorbed, causing osteopetrosis (Kong et al., 1999). The absence of the osteoclast proteinase

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cathepsin K also leads to osteopetrosis, revealing its essential role in osteoclast mediated bone resorption (Saftig et al., 1998).

The secondary center of ossification

The development of the epiphyses involves changes in shape and size. This is made possible by the formation of a secondary growth centre (Fig. 1.1(g)). The events that lead to its formation and function are essentially the same as those described for the primary centers of ossification or growth plates. At the outer edge there are germinal cells that give rise to proliferative cells which mature and form hypertrophic chondrocytes which calcify their extracellular matrix forming calcified trabeculae on which woven bone is formed. Subsequent events lead to trabecular bone formation. This secondary center appears following the development of hypertrophic chondrocytes in the uncalcified epiphyses: blood vessels invade towards these cells from the periosteum as in early development.

The regulation of chondrocyte development and chondrocyte interactions with the perichondrium and periosteum

Vitamin D

Vitamin D deficient (rachitic) animals exhibit abnormal development. The most pronounced changes are characterized by a lengthened hypertrophic zone and impaired angiogenesis. Nuclear receptors for 1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D₃) have been detected in both hypertrophic and proliferative chondrocytes. Chondrocyte hypertrophy occurs in vitamin D deficiency but calcification of the extracellular matrix is restricted. Treatment of chondrocytes with either 1,25-dihydroxyvitamin D₃, or the metabolite 24, 25 dihydroxy vitamin D₃ causes increased production of an angiogenic molecule produced by the chondrocytes (Alini et al., 1996b). Both metabolites stimulate extracellular matrix synthesis (type II procollagen) and calcification.

Thyroid hormones

Thyroid hormones also play a key role in development. Untreated hypoparathyroidism in children results in marked retardation of growth because of incomplete skeletal maturation. Growth can be rapidly restored by thyroid hormone administration. Both T₄ and T₃ accelerate chondrocyte maturation and hypertrophy in vitro: T₃ is much more potent than T₄ and is considered to be the active metabolite (Alini et al., 1996) acting on the prehypertrophic chondrocytes that express receptors for thyroid hormones. Thyroid hormones also act directly on bone cells although this has received relatively little attention. Both osteoblast and osteoclast activity are stimulated by T₃ and receptors have been reported to be present on both cell types, although this requires confirmation.