Available online at www.sciencedirect.com
ScienceDirect
The International Journal of Biochemistry & Cell Biology 40 (2008) 46–62
IJBCB
www.elsevier.com/locate/biocel

Review

Endochondral ossification: How cartilage is converted into bone in the developing skeleton

E.J. Mackie *, Y.A. Ahmed, L. Tatarczuch, K.-S. Chen, M. Mirams

School of Veterinary Science, University of Melbourne, Parkville, Victoria 3010, Australia

Received 27 April 2007; received in revised form 5 June 2007; accepted 8 June 2007
Available online 29 June 2007

Abstract

Endochondral ossification is the process by which the embryonic cartilaginous model of most bones contributes to longitudinal growth and is gradually replaced by bone. During endochondral ossification, chondrocytes proliferate, undergo hypertrophy and die; the cartilage extracellular matrix they construct is then invaded by blood vessels, osteoclasts, bone marrow cells and osteoblasts, the last of which deposit bone on remnants of cartilage matrix. The sequential changes in chondrocyte behaviour are tightly regulated by both systemic factors and locally secreted factors, which act on receptors to effect intracellular signalling and activation of chondrocyte-selective transcription factors. Systemic factors that regulate the behaviour of chondrocytes in growth cartilage include growth hormone and thyroid hormone, and the local secreted factors include Indian hedgehog, parathyroid hormone-related peptide, fibroblast growth factors and components of the cartilage extracellular matrix. Transcription factors that play critical roles in regulation of chondrocyte gene expression under the control of these extracellular factors include Runx2, Sox9 and MEF2C. The invasion of cartilage matrix by the ossification front is dependent on its resorption by members of the matrix metalloproteinase family, as well as the presence of blood vessels and bone-resorbing osteoclasts. This review, which places an emphasis on recent advances and current areas of debate, discusses the complex interactions between cell types and signalling pathways that govern endochondral ossification.

Keywords: Chondrocyte; Hypertrophy; Cell death; Extracellular matrix; Bone development

Contents

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BMP, bone morphogenetic protein; DDR2, discoidin domain receptor-2; FGF, fibroblast growth factor; FGFR3, fibroblast growth factor receptor-3; GH, growth hormone; HDAC4, histone deacetylase-4; IGF, insulin-like growth factor; IGFR1, insulin-like growth factor receptor-1; Ihh, Indian hedgehog; MEF2C, myocyte enhancer factor-2C; MMP, matrix metalloproteinase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; STAT, signal transducer and activator of transcription; T3, triiodothyronine; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor

* Corresponding author. Tel.: +61 3 8344 7360; fax: +61 3 8344 7374.
E-mail address: ejmackie@unimelb.edu.au (E.J. Mackie).

1357-2725/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2007.06.009

1. Introduction

Most bones develop through a process known as endochondral ossification, the initial stage of which is the formation of a cartilage model. During foetal development and postnatal growth, this model is gradually replaced by bone. Cartilage models are formed through condensation of mesenchymal cells, followed by their differentiation into chondrocytes and secretion of typical cartilage extracellular matrix components (Fig. 1A). The cartilage model once formed is invaded first at its centre and later at each end by a mixture of cells that establish the primary and secondary (respectively) centres of ossification (Fig. 1B–D). These centres of ossification gradually encroach on the remaining cartilage, ultimately replacing it completely (except at the articular surfaces) by the time skeletal maturity is achieved (Fig. 1E). The importance of the cartilage model lies not only in its provision of a mechanically stable template for bone formation, but also in its role as the source of longitudinal bone growth. The dynamic events that occur in growth cartilage leading to its replacement by bone will be the subject of this review. A number of recent reviews have described the roles of specific groups of molecules in endochondral ossification; the aim of this review is to provide an overview of the complex molecular and cellular interactions underlying the process of endochondral ossification, with an emphasis on recent advances and current areas of debate.

2. Morphology of growth cartilage

Cartilage that participates in endochondral ossification within developing and growing bones will be referred to in this review as growth cartilage. Growth cartilage is found in two locations at each end of a developing long bone: the growth plate and the articular-epiphyseal growth cartilage, which drive expansion of the primary and secondary centres of ossification, respectively (Fig. 1D). There are differences in the spatial organization of chondrocytes between the two locations, but these will not be discussed here (Byers & Brown, 2006).

No matter what the location or stage of development, chondrocytes in growth cartilage are arranged in morphologically distinct zones, which reflect changes in the functional state of the cells (Figs. 1 and 2A). The zone furthest from the ossification front is the zone of resting chondrocytes. Adjacent to this is the zone of proliferation; round proliferating chondrocytes become flattened as they are packed into multicellular clusters. Following proliferation, chondrocytes pass through a transition stage in which they are known as 'pre-hypertrophic' chondrocytes. These cells then undergo hypertrophy, increasing their volume dramatically, at the same time secreting extracellular matrix, which eventually becomes mineralised; chondrocyte proliferation and matrix secretion between them cause the elongation of the bone. Hypertrophic chondrocytes then die, and as they do so, the transverse septa of cartilage matrix surrounding them are broken down, leaving vertical septa largely intact, but allowing entry of the invading cells of the ossification front: blood vessels, osteoclasts (multinucleate bone-resorbing cells), and precursors of osteoblasts (bone-forming cells) and bone marrow cells. The osteoclasts assist in the removal of cartilage matrix, and the differentiating osteoblasts use the remnants of cartilage matrix as a scaffold for the deposition of bone matrix.

Chondrocytes in growth cartilage are usually considered to be a uniform population, but ultrastructural studies demonstrated many years ago that two types of cells, 'light' and 'dark', can be observed in growth cartilage (Fig. 2B–D; Hwang, 1978; Wilsman, Farnum,

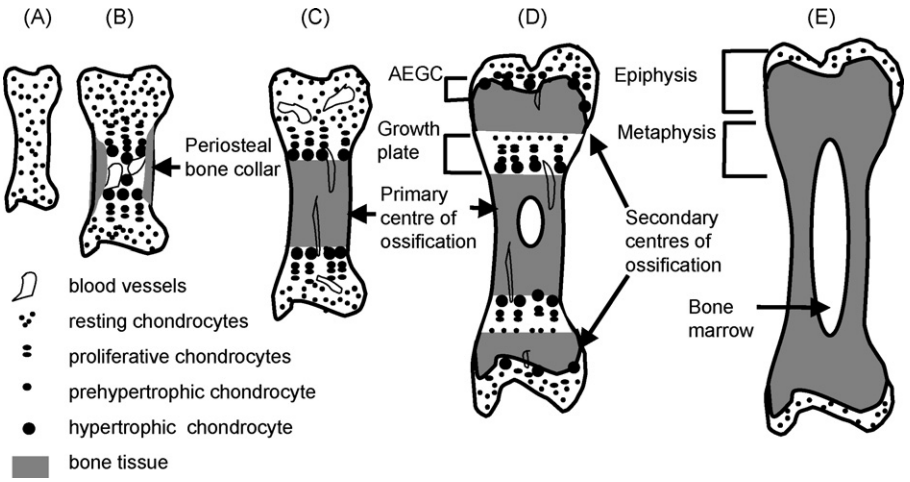
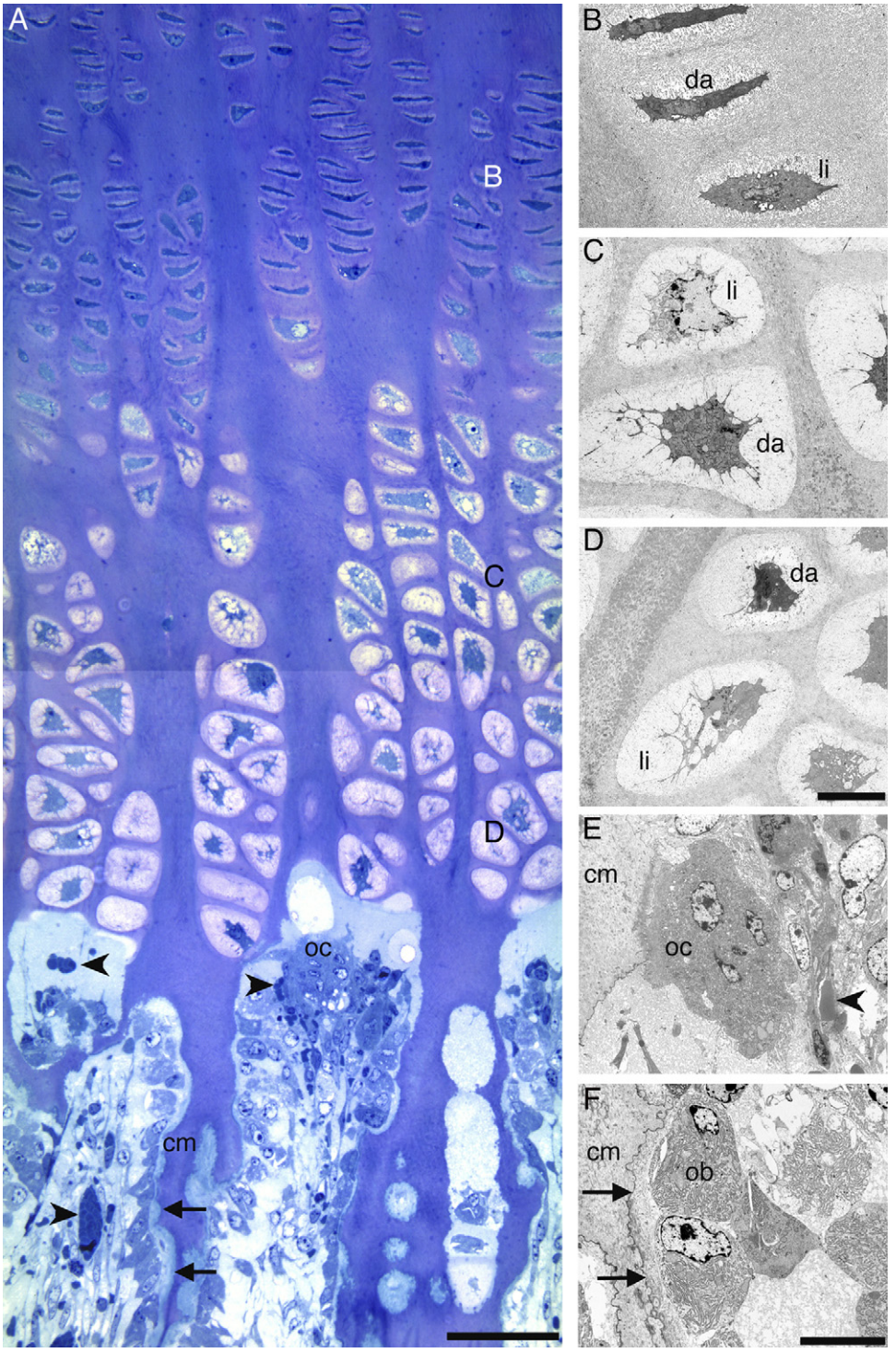


Fig. 1. Development of endochondral bones. Schematic diagram showing the events leading to replacement of an embryonic cartilage model by bone. (A) Cartilage model. (B) Initiation of formation of the primary centre of ossification in the centre of the cartilage model, with chondrocyte hypertrophy and vascular invasion. (C) Well established primary centre of ossification; blood vessels are present in cartilage canals in the remaining cartilage. (D) The secondary centres of ossification have formed and are separated from the primary centre of ossification by the growth plate, which is responsible for longitudinal growth. Articular-epiphyseal growth cartilage (AEGC) remains under the permanent articular cartilage, and is responsible for growth and shaping of the epiphysis. (E) In the adult bone, the metaphyseal and epiphyseal bone have fused to each other, leading to the disappearance of the growth plate. The AEGC has been replaced by bone, and the only remaining cartilage is the permanent articular cartilage at each end of the bone.

Hilley, & Carlson, 1981). Light chondrocytes have sparse endoplasmic reticulum and an inconspicuous Golgi region. Dark chondrocytes, in contrast, have well developed endoplasmic reticulum and a prominent Golgi zone; they possess numerous cytoplasmic processes, with vesicles budding from the cell surface. We have recently provided evidence that light and dark chondrocytes represent two distinct post-proliferative chondrocyte populations, with different patterns of gene expression (Ahmed et al., 2007).

The process by which hypertrophic chondrocytes die is currently the subject of debate. Many authors have described these cells as dying by apoptosis (as reviewed by Adams & Shapiro, 2002; Gibson, 1998). Apoptosis is a form of physiological cell death characterised by distinctive morphological features, including intense condensation of chromatin into geometric shapes, and fragmentation of the nucleus and cytoplasm into membrane-bound apoptotic bodies (Kerr, Wyllie, & Currie, 1972). Many of the papers describing apoptosis of hypertrophic chondrocytes have done so on the basis of detection of DNA strand breaks or other molecular features known to be associated with apoptosis, rather than on the basis of the definitive morphology (Adams & Shapiro, 2002; Gibson, 1998). On the basis of early ultrastructural studies and recent observations, however, in a number of publications it has been argued strongly that the process by which hypertrophic chondrocytes die is morphologically distinct from apoptosis (Colnot, Sidhu, Balmain, and Poirier (2001); Roach, Aigner, and Kouri (2004); Roach & Clarke, 1999, 2000). Shapiro, Adams, Freeman, and Srinivas (2006) have put forward the hypothesis that hypertrophic chondrocytes die by autophagic cell death. We have recently demonstrated that light and dark hypertrophic chondrocytes die by cell type-specific (non-apoptotic) processes (Ahmed et al., 2007). Light cells appear to disintegrate within their cell membrane, whereas dark cells undergo progressive

Fig. 2. Morphology of tissue undergoing endochondral ossification. (A) Light micrograph of the growth plate of an equine foetus. Labels (B)–(D) indicate the regions corresponding to electron micrographs of chondrocytes shown in parts (B)–(D) of this figure. The invading ossification front is seen in the lower part of the image, and includes blood vessels (arrowheads) and (multinucleate) osteoclasts (oc); arrows indicate the bone matrix being deposited on remnants of cartilage matrix (cm) by a row of osteoblasts. (B–F) Electron micrographs of different cell types seen in part (A). Light (li) and dark (da) chondrocytes are seen in the late zone of proliferation (B), the middle of the zone of hypertrophy (C) and the last few lacunae before the ossification front, where the cells are dying (D). (E) An osteoclast adherent to a remnant of cartilage matrix, adjacent to an erythrocyte (arrowhead) in a blood capillary. (F) Two osteoblasts (ob) depositing bone matrix (arrows) on a remnant of cartilage matrix. Bar in (A) = 50 μm. The magnification for parts (B)–(D) is the same, and the magnification for parts (E) and (F) is the same; bar = 10 μm.


A
B
C
D
oc
cm
B
da
li
C
li
da
D
da
li
E
cm
oc
F
cm
ob

extrusion of cytoplasm into the extracellular space; for both cell types, nuclear condensation is late and irregular (Fig. 2B–D).

Another area relating to the morphology of chondrocytes in growth cartilage that has received some attention recently is the role of the cytoskeleton in hypertrophy. The chondrocyte cytoskeleton, composed of an interconnected network of microtubules, actin and vimentin, is essential for maintenance of chondrocyte shape and phenotype, as well as organization of the Golgi apparatus and intracellular trafficking (Benjamin, Archer, & Ralphs, 1994); little is known, however, about its role in hypertrophy. The actin-binding protein adseverin has recently been shown to be expressed selectively by prehypertrophic chondrocytes as well as by hypertrophic chondrocytes (Nurminsky, Magee, Faverman, & Nurminskaya, 2007). Overexpression of adseverin in non-hypertrophic chondrocytes induces changes in the actin cytoskeleton, a dramatic increase in volume and expression of molecular markers of hypertrophy. The primary cilium is an organelle found in most eukaryotic cells, including chondrocytes; it consists of a basal body and a ciliary axoneme that extends several micrometers from the cell surface (Poole, Flint, & Beaumont, 1985; Wilsman, Farnum, & Reed-Aksamit, 1980). The primary cilium of chondrocytes interacts with components of the extracellular matrix as well as with the microtubular component of the cytoskeleton (Jensen et al., 2004; Poole, Zhang, & Ross, 2001; Wilsman et al., 1980). Defects in endochondral ossification have recently been described in two genetically manipulated mouse strains with defects in chondrocyte primary cilium formation. One has partial loss of the *Tg737* gene, which encodes the primary cilium protein polaris, and the other has chondrocytes that do not express Kif3a, a subunit of the kinesin II motor complex which is required for intraflagellar transport and the formation of cilia (McGlashan et al., 2007; Song, Haycraft, Seo, Yoder, & Serra, 2007). Dwarfism of neonatal animals is apparent in both of the mutant strains, although in the former there is evidence of failure of terminal chondrocyte hypertrophy, whereas in the latter hypertrophy appears to be accelerated. These two studies demonstrate that the chondrocyte primary cilium plays an important role in organization of growth cartilage, but precisely what this role may be is not yet clear. It can be concluded that various components of the cytoskeleton play critical roles in chondrocyte hypertrophy.

The matrix surrounding late hypertrophic chondrocytes is mineralised through the deposition of hydroxyapatite (comprised of calcium and phosphate). Membrane-bound matrix vesicles, which are released from the surface of hypertrophic chondrocytes and contain a specific combination of proteins, including annexins, phosphate transporters and phosphatases, are thought to provide the nucleation site for mineralisation (Anderson, 1969; Kirsch, 2006; Kirsch, Nah, Shapiro, & Pacifici, 1997). Mineralisation of matrix vesicles and cartilage matrix is dependent on alkaline phosphatase activity, as demonstrated by results obtained from tissue non-specific alkaline phosphatase (TNAP)-deficient mice (Anderson et al., 2004; Fedde et al., 1999). Alkaline phosphatase hydrolyses organic phospho compounds, and it is thought that its role in mineralisation may be to remove extracellular pyrophosphate, a putative inhibitor of mineralisation, rather than to generate inorganic phosphate (Kirsch, 2006). The specific role of mineralisation of cartilage matrix in endochondral ossification is not entirely clear. In alkaline phosphatase-deficient mice as well as in humans with mutations in the *TNAP* gene (i.e. patients with hypophosphatasia), chondrocytes in growth cartilage fail to undergo terminal hypertrophy, however this may be due to roles of TNAP in addition to promotion of mineralisation. Carminerin is a transcriptional inhibitor of nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), which generates pyrophosphate by hydrolysing extracellular adenosine triphosphate analogues (Terkeltaub, 2001; Yamada et al., 2006). Carminerin-null mice show reduced mineralisation of growth cartilage matrix, but no alterations in growth plate morphology, suggesting that mineralisation is not required for normal chondrocyte maturation and death (Yamada et al., 2006). These mice do, however, show a decrease in bone volume in the primary spongiosa (the newly formed bone of the metaphysis), but not in the remodelled bone of the secondary spongiosa, suggesting that cartilage mineralisation may be required for normal deposition of bone on cartilage remnants during endochondral ossification.

3. Regulation of chondrocyte behaviour during endochondral ossification

Chondrocytes in growth cartilage are subject to the influence of a plethora of extracellular factors, including systemic and local soluble factors, as well as components of the cartilage extracellular matrix. A number of transcription factors have been identified as playing critical roles in specific stages of endochondral ossification, and the existence of complex interactions between extracellular factor-activated signalling pathways and transcription factors are starting to be identified. An overview of some of the more important effects and interactions is provided in Fig. 3.

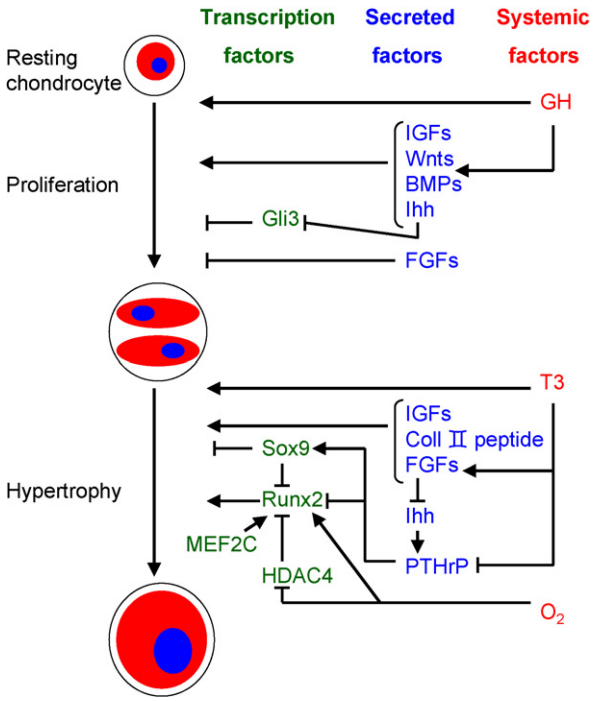


Fig. 3. Schematic diagram providing an overview of the roles of systemic factors, locally secreted factors and transcription factors in the regulation of chondrocyte proliferation and hypertrophy during endochondral ossification. Arrows indicate stimulatory pathways (i.e. stimulation of expression or activation of a factor or of its receptor, or of proliferation or hypertrophy) and crossed lines indicate inhibitory pathways. For the sake of simplicity, not all effects mentioned in the text are included here. Coll II: collagen type II.

3.1. Systemic factors

Growth hormone (GH) has been known for many years to be an important regulator of longitudinal bone growth (reviewed by Nilsson, Marino, De Luca, Phillip, & Baron, 2005). Children with elevated GH levels due to pituitary adenomas exhibit gigantism. Impaired growth is seen in children with GH deficiency or a genetic inability to respond to GH due to mutations in its receptor or components of its signalling pathway. Liver-derived insulin-like growth factor-1 (IGF1) is responsible for much of the effect of GH on skeletal growth. A reduction in circulating IGF1 levels in mice leads to a reduction in longitudinal growth (Yakar et al., 2002), and administration of IGF1 compensates for inactivating mutations in GH receptor in mice and humans (Guevara-Aguirre et al., 1997; Sims et al., 2000). GH can, however, also stimulate longitudinal growth through a local action on growth cartilage, which appears to be dependent on local production of IGF1, but may also be partly IGF1-independent (Nilsson et al., 2005). The main effect of GH on chondrocytes in growth cartilage is to stimulate their proliferation (Fig. 3; Hunziker, Wagner, & Zapf, 1994).

At puberty, the ossification front overruns the growth plate, resulting in fusion of the bone formed in the primary centre of ossification (the metaphysis) to the bone formed in the secondary centre of ossification (the epiphysis; Fig. 1E). This process occurs in human males as well as females under the influence of oestrogen, as demonstrated by the failure of normal epiphyseal fusion in patients with oestrogen deficiency resulting from mutations in the aromatase gene, or with oestrogen resistance resulting from a mutation in the oestrogen receptor-α (Morishima, Grumbach, Simpson, Fisher, & Qin, 1995; Smith et al., 1994). It has been speculated that epiphyseal fusion occurs when growth plate chondrocytes exhaust their proliferative potential, and that this process of senescence is accelerated by oestrogen treatment (Weise et al., 2001). It is likely that these effects of oestrogen are mediated by direct effects on oestrogen receptors expressed by chondrocytes in growth cartilage (discussed in Nilsson et al., 2005).

Thyroid hormone is another systemic regulator of skeletal growth (reviewed by Shao, Wang, & Ballock, 2006). In hypothyroidism in humans, longitudinal bone growth is slowed, growth plates are thinned and chondrocyte hypertrophy is impaired. *In vitro*, thyroid hormone induces cartilage maturation, as demonstrated many years ago by Fell and Mellanby (1955). More recently, in a variety of culture systems, thyroid hormone and triiodothyronine (T3) have been shown to induce morphological hypertrophy, as well as molecular markers of hypertrophy, including collagen type X expression and alkaline phosphatase activity, without inducing proliferation (Fig. 3; Ballock & Reddi, 1994; Bohme, Conscience-Egli, Tschan, Winterhalter, & Bruckner, 1992; Burch & Lebovitz, 1982). T3 stimulates hypertrophy of both light and dark chondrocytes, and also induces death of hypertrophic light and dark cells by non-apoptotic modes of physiological death morphologically identical to those observed *in vivo* (Fig. 4; Ahmed et al., 2007). Receptors for thyroid hormone are nuclear receptors; two thyroid hormone receptor genes (α and β) exist, and are subject to differential splicing, resulting in multiple isoforms. Thyroid hormone receptors-α appear to be those responsible for responses in growth cartilage, on the basis of their expression in bone as well as phenotypic observations in genetically manipulated mice. Mice heterozygous for inactivating mutations in the thyroid hormone receptor-α gene show longitudinal growth retardation and impaired chondrocyte hypertrophy, whereas mice with corresponding manipulation of the thyroid hormone receptor-β gene

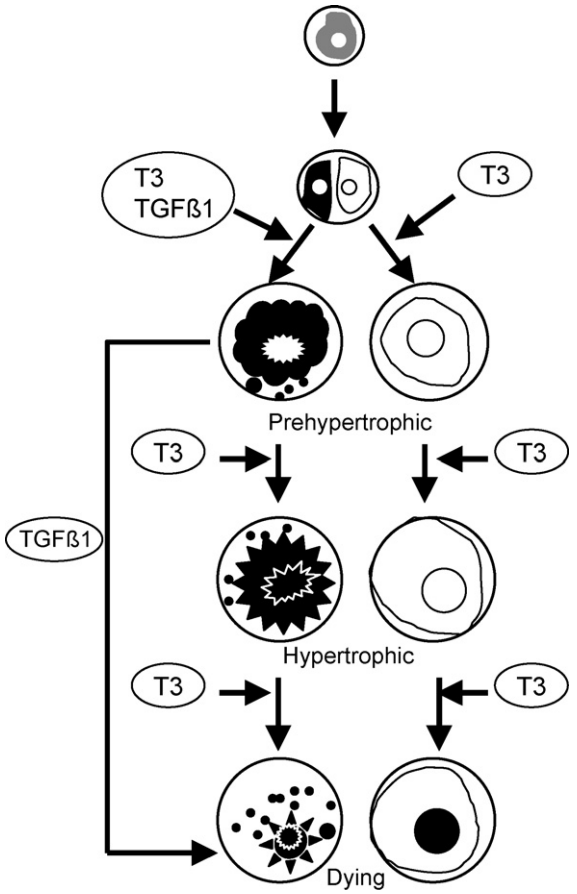


Fig. 4. Schematic diagram explaining regulation of the morphological heterogeneity of chondrocytes in growth cartilage. Following proliferation, chondrocytes are identifiable as either dark or light cells, each of which undergoes a distinctive set of morphological changes during hypertrophy and death. Factors known to influence specific events related to dark and light cell maturation are indicated.

have no growth deficit (Kaneshige et al., 2001). Further studies in these mice have demonstrated that thyroid hormone receptor-α activation is required for normal levels of GH receptor, IGF1 and IGF1 receptor expression, as well as for normal phosphorylation of the GH receptor target, signal transducer and activator of transcription-5 (STAT5), and the IGF1 receptor target, Akt, in growth plate chondrocytes (O'Shea et al., 2005); these observations indicate that responsiveness of growth cartilage to GH is dependent on intact thyroid hormone receptor activity. Since the GH/IGF1 axis appears to be involved primarily with chondrocyte proliferation, whereas thyroid hormone induces hypertrophy *in vitro*, it is unlikely that GH/IGF1 signalling plays a major role in mediating thyroid hormone's distinctive effects on chondrocytes in growth cartilage. Regulation by thyroid hormone of other local regulators of chondrocyte behaviour will be discussed in the relevant sections below.

3.2. Locally produced soluble extracellular factors

3.2.1. Insulin-like growth factors

The role of IGF1 as a local mediator of GH's effects on growth cartilage has already been mentioned. Insulin-like growth factor-2 is also expressed in growth cartilage, and is essential for normal embryonic skeletal growth (DeChiara, Robertson, & Efstratiadis, 1991). The relative importance of locally produced IGF1 and IGF2, and whether their major effects are stimulation of chondrocyte proliferation or hypertrophy (or both) is the subject of some debate (discussed by van der Eerden, Karperien, & Wit, 2003). Mice lacking the type 1 IGF receptor (IGFR1), which is responsible for all of IGF1's effects and most of IGF2's effects on growth, have a more severe growth deficit than either IGF1-null or IGF2-null mice, indicating that the roles of the two IGFs do not completely overlap (Baker, Liu, Robertson, & Efstratiadis, 1993). The importance of IGF1 in regulating growth cartilage function is underlined by the recent observation that a single IGF1 allele is a major determinant of small size in dogs (Sutter et al., 2007).

3.2.2. Indian hedgehog

Indian hedgehog (Ihh), a secreted factor expressed by prehypertrophic chondrocytes, stimulates chondrocyte proliferation, and inhibits chondrocyte hypertrophy (Bitgood & McMahon, 1995; Koyama, Leatherman, Noji, & Pacifici, 1996; St-Jacques, Hammerschmidt, & McMahon, 1999; Vortkamp et al., 1996). The latter but not the former effect is dependent on induction by Ihh of expression of parathyroid hormone-related peptide (PTHrP; Fig. 3). Heterozygous missense mutations in the *Ihh* gene have been shown to cause brachydactyly type A-1 in humans, a condition involving shortening or absence of the middle phalanges (Gao et al., 2001). Binding of Ihh to its cell surface receptor (patched-1) results in inhibition of phosphorylation and proteolytic processing of members of the Gli family of transcription factors, thus inhibiting their activity as transcriptional repressors (reviewed by Ehlen, Buelens, & Vortkamp, 2006). Experiments have been conducted in *Ihh*/*Gli3* double knockout mice to investigate the role of Gli3 in modulation of responses to Ihh (Koziel, Wuelling, Schneider, & Vortkamp, 2005). Loss of *Gli3* in *Ihh*-null mice restores chondrocyte proliferation, allows reactivation of PTHrP expression and delays the accelerated onset of hypertrophic differentiation seen in *Ihh*-null mice.

These observations are interesting, not only because they add to the understanding of the mechanism of Ihh's effects on chondrocyte behaviour, but also because they indicate that in the absence of both Ihh and the transcriptional repressor Gli3, chondrocyte proliferation, PTHrP expression and rate of hypertrophy are normal. Thus there must be at least one other pathway that independently regulates these processes. The IGFR1 signalling pathway appears to be one such pathway. Since Ihh and IGFs exert similar effects on skeletal growth, the possibility that there is an interaction between responses to the two factors was recently examined. Using a variety of genetic manipulation approaches in mice, Long, Joeng, Xuan, Efstratiadis, and McMahon (2006) have demonstrated that disruption of both IGF and Ihh signalling results in an additive reduction in longitudinal bone growth, by comparison with disruption of each individually. Moreover, disruption of one signalling pathway did not interfere with activity of the other. These observations indicate that Ihh and IGF signalling pathways in growth cartilage operate independently of each other.

Ihh exerts effects not only on chondrocytes, but also on the mesenchymal cells surrounding the cartilage model. Ihh stimulates osteoblastic differentiation of mesenchymal cells; it is essential for the formation of the periosteal bone collar that surrounds the zone of hypertrophic chondrocytes prior to formation of the primary centre of ossification and remains throughout its expansion (Fig. 1; Nakamura et al., 1997; St-Jacques et al., 1999).

3.2.3. Parathyroid hormone-related peptide

PTHrP is expressed by perichondrial cells and early proliferating chondrocytes, then diffuses away from its site of production to act on PTH/PTHrP receptor-bearing cells (reviewed by Kronenberg, 2006). The PTH/PTHrP receptor is expressed at low levels by proliferating chondrocytes, but at substantially higher levels once these cells stop proliferating; PTHrP maintains chondrocytes in a proliferative state and prevents hypertrophy (Lee et al., 1996). By limiting the transition from proliferation to hypertrophy, PTHrP limits the number of cells capable of expressing Ihh, and thus participates in a negative feedback loop regulating its own expression and the rate of chondrocyte differentiation. The extent of the zone of proliferative chondrocytes will always be limited by the greatest distance of its cells from the site of PTHrP production; thus the furthest cells will be able to escape from PTHrP control, undergo the early stages of hypertrophy and initiate Ihh expression, which will feed back and maintain PTHrP expression. Mutations leading to defective or absent PTH/PTHrP receptors have been found in human foetuses with Blomstrand chondro-osteodystrophy, a condition characterised by prenatal death and shortened limbs with premature ossification (Karaplis et al., 1998; Zhang, Jobert, Couvineau, & Silve, 1998). Mutations leading to constitutive activation of the PTH/PTHrP receptor are found in patients with Jansen-type metaphyseal dysplasia, which is characterised by short limbs associated with delayed chondrocyte hypertrophy (Schipani, Kruse, & Juppner, 1995).

The PTH/PTHrP receptor is a G protein-coupled receptor, and activation of G_s mediates PTHrP's inhibition of chondrocyte hypertrophy (Bastepe et al., 2004). Activation of G_s leads to production of cyclic AMP, which activates protein kinase A. PTHrP stimulates protein kinase A-dependent phosphorylation of the transcription factor Sox9, and thus activates it; Sox9 is thought to delay chondrocyte hypertrophy, and therefore probably helps to mediate this effect of PTHrP (Fig. 3; Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002; Huang, Chung, Kronenberg, & de Crombrugghe, 2001; Huang, Zhou, Lefebvre, & de Crombrugghe, 2000). PTH (which like PTHrP activates the PTH/PTHrP receptor) also inhibits expression by chondrocytes in growth cartilage of the transcription factor Runx2, which stimulates chondrocyte hypertrophy; this observation, together with observations of Runx2 and PTHrP single and double knockout mice, provide evidence that PTHrP maintains cells in a proliferative state through both Runx2-dependent and -independent mechanisms (Guo et al., 2006). PTHrP expression is elevated in hypothyroid rats and PTH/PTHrP receptor expression is suppressed in rats with excessive circulating levels of thyroid hormone, indicating that thyroid hormone exerts its effects at least in part by suppressing signalling through the PTH/PTHrP receptor (Stevens et al., 2000).

3.2.4. Bone morphogenetic proteins

Another group of factors, in addition to the IGFs, that could induce skeletal growth independently of Ihh are the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β (TGFβ) superfamily. These proteins play diverse roles in skeletogenesis, including important roles in the initiation of cartilage formation (chondrogenesis), which is outside the purview of this review. BMP7 is expressed by proliferative chondrocytes and BMP6 is expressed by pre-hypertrophic and hypertrophic chondrocytes (reviewed by van der Eerden et al., 2003). It has consistently been observed that BMP signalling supports proliferation of chondrocytes in growth cartilage (Fig. 3;

Minina et al., 2001; Yoon et al., 2006). Investigations into the effects of BMP signalling on chondrocyte hypertrophy have, however, led to observations that in some cases appear to be contradictory. Treatment of isolated chondrocytes with BMP6 or expression of constitutively active BMP receptors leads to enhanced expression of collagen type X, suggesting that BMP signalling induces hypertrophy (Grimsrud et al., 1999, 2001). Treatment of embryonic limb explant cultures with the secreted BMP antagonist Noggin leads to a reduction in the domain of collagen type X expression (as assessed by *in situ* hybridisation) and expansion of the domain of expression of osteopontin, an extracellular matrix protein expressed by late hypertrophic chondrocytes, leading to the conclusion that BMP signalling delays terminal hypertrophy (Minina et al., 2001). In contrast, corresponding bones from mice with targeted ablation of the gene for BMP receptor 1A in chondrocytes show an expanded domain of collagen type X expression (as detected by immunohistochemistry), leading the investigators to conclude that BMP signalling is required for completion of terminal hypertrophic differentiation (Yoon et al., 2006). The apparently contradictory observations presumably result from the considerable differences between the experimental approaches used in these studies. It is clear, however, that BMP signalling is required for normal rates of onset and progression of chondrocyte hypertrophy.

BMP signalling induces Ihh expression, but effects of BMP signalling on proliferation and hypertrophy are independent of Ihh; conversely, Ihh induces expression of various BMPs, but effects of Ihh on proliferation are independent of BMP signalling (Grimsrud et al., 2001; Minina et al., 2001).

Transforming growth factors-β inhibit chondrocyte hypertrophy (Ballock et al., 1993), and this effect appears to be partly mediated by induction of PTHrP expression (Serra, Karaplis, & Sohn, 1999). Moreover, activation of Ihh signalling pathways failed to induce PTHrP or inhibit chondrocyte hypertrophy in embryonic metatarsal organ cultures from TGFβ2-null mice, indicating that TGFβ2 is a mediator of these responses to Ihh (Alvarez et al., 2002). Although TGFβs inhibit chondrocyte hypertrophy, TGFβ1 has recently been shown to induce post-proliferative chondrocytes to undergo early dark cell differentiation exclusively, and to induce non-apoptotic physiological death of these cells (Fig. 4; Ahmed et al., 2007).

3.2.5. *Wnt family*

The Wnt family of secreted proteins bind to their receptor Frizzled (Frz), and its co-receptor Lrp5 or Lrp6 to activate the canonical (β-catenin-mediated) signalling pathway; binding to Frz alone can activate the non-canonical (calcium-dependent kinase C-mediated) signalling pathway, which can lead to degradation of β-catenin and thus suppression of canonical Wnt signalling (reviewed by Yates, Shortkroff, & Reish, 2005). A number of members of the Wnt family, including Wnts capable of activating both canonical and non-canonical signalling are expressed in growth cartilage; expression levels are highest in proliferative and hypertrophic zones (Andrade, Nilsson, Barnes, & Baron, 2007). Activation of a combination of canonical and non-canonical Wnt signalling pathways appears to be essential for chondrocyte survival, proliferation and hypertrophy (Akiyama et al., 2004; Mak, Chen, Day, Chuang, & Yang, 2006; Yang, Topol, Lee, & Wu, 2003). Constitutive activation of β-catenin in immature chondrocytes suppresses hypertrophy, but in more mature chondrocytes promotes terminal differentiation (Tamamura et al., 2005). The requirement for β-catenin-mediated Wnt signalling in chondrocyte survival appears to occur downstream from Ihh in the early proliferative zone, but independently of Ihh in the later proliferative zone (Mak et al., 2006). The secreted Wnt pathway antagonist Frzb-1 is expressed by prehypertrophic chondrocytes and is likely to act as an endogenous modulator of Wnt's effects on chondrocyte maturation (Enomoto-Iwamoto et al., 2002).

3.2.6. *Fibroblast growth factors*

Activation of fibroblast growth factor receptor-3 (FGFR3), which is expressed by proliferating and early hypertrophic chondrocytes, leads to an inhibition of chondrocyte proliferation and acceleration of hypertrophy (Fig. 3; Minina, Kreschel, Naski, Ornitz, & Vortkamp, 2002; reviewed by Ornitz, 2005). Mutations in the FGFR3 gene that lead to constitutive activation of the receptor are responsible for a range of different forms of short-limbed dwarfism in humans, including the most common form, achondroplasia (Ornitz, 2005). A number of FGFs are capable of activating FGFR3, but expression patterns and the phenotypes of genetically manipulated mice suggest that the most important one in growth cartilage is likely to be FGF18 (Ornitz, 2005). STAT1 appears to be a critical intracellular mediator of signalling in response to FGFR3 activation in chondrocytes (Ornitz, 2005).

FGF2 treatment of limb explant cultures leads to a reduction in the number of cells expressing Ihh (Minina et al., 2002). The acceleration in the onset of hypertrophy by FGF2 in this system is inhibited by forced expression of Ihh, indicating that FGF signalling acts upstream of Ihh expression. In contrast, inhibition of proliferation by

FGF2 is not counteracted by forced expression of Ihh, indicating that FGF signalling suppresses proliferation independently of Ihh. The effects of FGF signalling on chondrocyte proliferation, Ihh expression and hypertrophy are antagonised by BMP signalling (Minina et al., 2002). Thyroid hormone induces expression of FGFR3 in cultured chondrocyte-like cells, and expression of FGFR3 is markedly suppressed in the growth plates of thyroid hormone receptor-α-null mice, by comparison with those of wildtype animals (Barnard et al., 2005). Thus, in addition to suppression of PTHrP expression, induction of FGFR3 expression provides another potential mechanism by which thyroid hormone stimulates chondrocyte hypertrophy in growth cartilage.

3.3. Components of the extracellular matrix

The major components of cartilage extracellular matrix are the fibrillar cartilage-specific collagen type II, and large aggregates of hyaluronan with the cartilage-specific proteoglycan aggrecan. Synthesis of collagen type II is down-regulated with chondrocyte hypertrophy, at which stage synthesis of the non-fibrillar collagen type X is initiated (van der Eerden et al., 2003). The combination of fibrillar collagen and polyanionic hyaluronan-aggrecan aggregates confers on cartilage its special physical properties. The importance of collagen type II and many other structural components of the extracellular matrix for cartilage integrity and normal skeletal growth has been identified largely through the identification of mutations in the relevant human genes (reviewed by Whyte, 2006). Much of this information has been available for many years, and will not be discussed here. The components of cartilage matrix are not only important for their structural roles, however, but some also exert specific receptor-mediated effects on cell behaviour.

Collagen type II interacts with cells through members of the integrin family and the non-integrin collagen receptor, discoidin domain receptor-2 (DDR2; a receptor tyrosine kinase; reviewed by Leitinger & Hohenester, 2007). Collagen type II is required for chondrocyte survival, as demonstrated by the use of *Col2a1*-null mice (Yang et al., 1997). A cryptic peptide derived from proteolytic degradation of collagen type II induces chondrocyte hypertrophy, as assessed by expression of collagen type X; it also induces expression of matrix metalloproteinase-13 (MMP13), a collagenase expressed by hypertrophic chondrocytes (Tchetina et al., 2007). Mutations in the *COL10A1* gene cause Schmid metaphyseal chondrodysplasia in humans, but it is not clear whether the defects in endochondral ossification in this condition result exclusively from disruption of the extracellular matrix network, or whether collagen type X also exerts essential direct effects on chondrocyte behaviour (Chan & Jacenko, 1998). Collagen type X binds to chondrocytes through integrin $\alpha_2\beta_1$, but also through an interaction with DDR2 that leads to autophosphorylation of the receptor, suggesting that this protein may indeed exert effects on intracellular signalling pathways in chondrocytes (Leitinger & Kwan, 2006; Luckman, Rees, & Kwan, 2003).

The glycosaminoglycan side chains of chondroitin sulphate proteoglycans such as aggrecan are sulphated in the Golgi by sulphotransferases that transfer sulphate groups to specific positions on the carbohydrate moiety. Alterations in chondroitin sulphation can lead to defects in growth cartilage morphology. The *C4st1* gene encodes chondroitin 4-sulphotransferase-1, which catalyses the sulphation at the 4-0 position of chondroitin and dermatan sulphate. Homozygous mice lacking the transmembrane and intra-Golgi catalytic domains of this enzyme display severe dwarfism and neonatal death (Kluppel, Wight, Chan, Hinek, & Wrana, 2005). There is an altered distribution of chondroitin sulphate and aggrecan in the growth cartilage of these mice; the cellular defect appears to be an increased rate of proliferation and maturation of chondrocytes, culminating in premature cell death. The molecular mechanism underlying the pathology is not clearly understood, but it is associated with an increase in the level of TGFβ signalling in the growth cartilage, which may result from interference with the proper sequestration of TGFβ in the extracellular matrix, thus resulting in constitutive TGFβ receptor activation (Kluppel et al., 2005).

3.4. Transcription factors

Sox9 is expressed by proliferative, but not hypertrophic, chondrocytes in growth cartilage, and induces expression of components of cartilage matrix including collagen type II *in vitro* (reviewed by Lefebvre & Smits, 2005). It has been difficult to investigate the role of *Sox9* in regulation of chondrocyte behaviour in growth cartilage *in vivo*, because its deletion in undifferentiated limb bud mesenchymal cells results in a complete failure of chondrogenesis, and if the gene is inactivated following initiation of chondrogenesis (in collagen type II-expressing cells), most cells fail to undergo differentiation into chondrocytes, i.e. they do not reach the stage of differentiation of the cells being considered here (Akiyama et al., 2002). These studies do provide evidence, however, that *Sox9* is required for normal rates of chondrocyte proliferation, and for appropriately

delaying the onset of hypertrophy. As mentioned above, there is evidence for a role for Sox9 as a phosphorylation target downstream from PTH/PTHrP receptor activation in mediating the inhibition of hypertrophy in response to PTHrP. Sox9 is required for expression of the related transcription factors Sox5 and Sox6, which are mutually interchangeable in terms of function in growth cartilage; these factors allow the orderly progression of chondrocyte hypertrophy by delaying the onset of terminal hypertrophic differentiation (Smits, Dy, Mitra, & Lefebvre, 2004).

Runx2 expression by chondrocytes is initiated in the early stages of hypertrophy and maintained until terminal hypertrophic differentiation. Runx2 promotes the full progression of hypertrophic differentiation, and is assisted in this task by Runx3 (Fig. 3; reviewed by Lefebvre & Smits, 2005). Runx2 is a transcriptional activator of hypertrophic chondrocyte markers such as the collagen type X gene *Col10a1*. Runx2 also activates the *Ihh* promoter and thus stimulates Ihh expression (Fig. 3; Yoshida et al., 2004). As mentioned above, PTHrP inhibits Runx2 expression, an effect that appears to contribute to the ability of PTHrP to delay chondrocyte hypertrophy (Guo et al., 2006). These observations in combination suggest that Runx2 contributes to the Ihh/PTHrP negative feedback loop, and thus plays an important role in maintaining an appropriate balance between continued proliferation and progression to hypertrophy in chondrocytes in growth cartilage. In addition to inhibition of Runx2 expression, regulation of Runx2 transcriptional activity has been identified as a mechanism by which hypertrophy is delayed. The activity of Runx2 is repressed by Sox9 through a direct interaction between the two transcription factors (Zhou et al., 2006). The corepressor histone deacetylase-4 (HDAC4) also interacts with and inhibits the activity of Runx2 (Vega et al., 2004).

Nkx3/Bapx1 is a transcriptional repressor selectively expressed by proliferative chondrocytes in growth cartilage, and its expression is dependent on PTHrP signalling (Provot et al., 2006). Nkx3/Bapx1 inhibits Runx2 expression and chondrocyte hypertrophy, and appears to be a mediator of the actions of PTHrP (Provot et al., 2006).

Recently, another transcription factor, myocyte enhancer factor-2 (MEF2C), which is known to regulate muscle and cardiovascular development, has been shown to be required for normal chondrocyte hypertrophy and subsequent ossification. Chondrocytes in developing bones of *Mef2c*-null mice fail to undergo hypertrophy; moreover, they fail to express *Runx2*, indicating that MEF2C acts upstream of Runx2 in the induction of chondrocyte hypertrophy (Fig. 3; Arnold et al., 2007). Chondrocytes from *Mef2c*-null mice also fail to express *Col10a1*, which is a direct transcriptional target of MEF2C. MEF2C and HDAC4 exert antagonistic effects on chondrocyte maturation. The developing bones of *Hdac4*-null mice display premature chondrocyte hypertrophy and ossification, and *Hdac4* overexpression in proliferating chondrocytes *in vivo* results in defective chondrocyte hypertrophy (Vega et al., 2004). Failure of endochondral ossification in heterozygous *Mef2c*-null mice can be reversed by deletion of *Hdac4* alleles, and the premature ossification of *Hdac4*-null mice can be reversed by deletion of a *Mef2c* allele (Arnold et al., 2007). These observations indicate that the normal initiation and progression of chondrocyte hypertrophy depends on the balance between transcriptional activation by MEF2C and repression by HDAC4.

Both *Runx2* expression and HDAC4 activity can be further regulated by oxygen tension (Fig. 3). When chondrogenic cells are cultured under hypoxic conditions, expression of *Col10a1* is inhibited as a result of downregulation of *Runx2* expression (Hirao, Tamai, Tsumaki, Yoshikawa, & Myoui, 2006). Hypoxia in these cells also leads to activation of HDAC4 as assessed by its translocation from the cytoplasm to the nucleus; silencing of *Hdac4* expression counteracts hypoxia-induced suppression of both *Runx2* and *Col10a1* expression, indicating that it plays a role in mediating the effects of hypoxia on chondrocyte hypertrophy. The importance of oxygen tension in regulating chondrocyte behaviour lies in the fact that cartilage is largely avascular. In most larger mammals (but not in rodents), blood vessels within cartilage canals provide nutrients to growth cartilage (Fig. 1C; Thorp & Dixon, 1991; Wilsman & Van Sickle, 1972); most chondrocytes are, however, a considerable distance from these blood vessels, and thus function under a lower oxygen tension than is found in other tissues (Schipani et al., 2001). Blood vessels within the invading ossification front lead to an increase in oxygen tension with increasing proximity to the ossification front; it is not surprising, therefore, that oxygen tension appears to play a role in regulating the rate of chondrocyte hypertrophy.

4. Regulation of cartilage matrix degradation during endochondral ossification

The increase in cell volume experienced by chondrocytes as they undergo hypertrophy requires degradation of the extracellular matrix immediately surrounding the cells. Moreover, invasion of the ossification front requires more extensive (but nevertheless selective) degradation of the transverse struts of cartilage surround-

ing late hypertrophic cells. The vertical struts remain as a framework on which bone is deposited by the invading osteoblasts, and which is only degraded later as the metaphyseal bone is modelled during growth to allow for expansion of the marrow cavity. A number of studies have been undertaken in recent years to identify the proteolytic enzymes responsible for these degradative events, as well as the cells responsible for their synthesis. The main emphasis for these studies has been placed on proteinases capable of degrading the two major proteinaceous components of cartilage matrix, collagen type II and aggrecan. Within growth cartilage, MMP13, which degrades both fibrillar collagen and aggrecan, is selectively expressed by hypertrophic chondrocytes (reviewed by Cawston & Wilson, 2006). In *Mmp13*-null mice, chondrocytes undergo apparently normal hypertrophy, but the zone of hypertrophic chondrocytes is expanded, and invasion of the ossification front is delayed (Inada et al., 2004; Stickens et al., 2004). Selective deletion of *Mmp13* expression in chondrocytes results in a similar defect in growth cartilage, indicating that MMP13 release by these cells (rather than by osteoblasts, which also express it) is required for normal removal of transverse septa and invasion of the ossification front (Stickens et al., 2004). A mutation in *MMP13* leading to the absence of active MMP13 results in the defect in human growth known as Missouri type spondyloepimetaphyseal dysplasia (Kennedy et al., 2005). MMP9, in contrast to MMP13 does not cleave native fibrillar collagens, but does cleave denatured collagens and aggrecan (Cawston & Wilson, 2006). A similar phenotype to that of *Mmp13*-null mice is seen in *Mmp9*-null mice, but the source of the MMP9 that is required for normal removal of cartilage matrix appears to be bone marrow-derived cells, rather than the chondrocytes (Vu et al., 1998). Deletion of both *Mmp9* and *Mmp13* in mice results in a more extreme delay in ossification than is seen with deletion of either gene alone (Stickens et al., 2004). Although there is evidence that both MMP9 and MMP13 cleave aggrecan in growth cartilage *in vivo* (Stickens et al., 2004), in mice in which aggrecan is rendered resistant to MMP cleavage there are no morphological defects in growth cartilage, and aggrecan does not accumulate (Little, Meeker et al., 2005).

Another group of enzymes, the aggrecanases, are capable of cleaving aggrecan; these enzymes are members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of enzymes (Cawston & Wilson, 2006). Mice lacking ADAMTS-1, ADAMTS-4 or ADAMTS-5 grow normally, and show no defects in growth cartilage (Little, Mittaz et al., 2005; Stanton et al., 2005). These are thought to be the most important aggrecanases in cartilage, and thus it has been concluded that none plays a significant non-redundant role in aggrecan removal in growth cartilage. It is possible that a number of enzymes (of the MMP and ADAMTS families) are able to substitute for each other, thus preventing growth defects in animals with manipulation of a single ADAMTS gene. An alternative conclusion that brings together the results of studies on *Mmp9*-null and *Mmp13*-null mice with those on mice with MMP-resistant aggrecan and the various aggrecanase-null strains of mice is that whereas removal of fibrillar collagen is critical for normal invasion of the ossification front, removal of aggrecan may not be important.

These studies also raise the question: how is pericellular matrix degraded to allow for chondrocyte hypertrophy? Genetic manipulation of none of the MMPs or ADAMTSs appears to perturb this process, suggesting that other proteases may be responsible. Other proteases that could possibly assist with removal of cartilage matrix to make room for expanding chondrocytes include some of the cathepsins and calpain; these are candidates for such a role because of their expression by chondrocytes and matrix-degrading activity (Cawston & Wilson, 2006). Cathepsin L-null mice have defective metaphyseal ossification, but the cellular defect appears to be a lack of osteoclasts (Potts et al., 2004). The growth plate was only examined in cathepsin L-null mice approaching skeletal maturity; perhaps a defect in cartilage matrix degradation exists in younger mice. Similarly, the plasminogen activators could potentially also play a role in degradation of the matrix surrounding chondrocytes undergoing hypertrophy. Mice deficient in both urokinase-type and tissue plasminogen activators have longer bones than wildtype mice suggesting an alteration in growth kinetics, but their growth plates show normal morphology, indicating that these serine proteinases are not required for chondrocyte hypertrophy (Daci et al., 2003).

5. Invasion of growth cartilage by the ossification front

As mentioned above, expression of MMP13 by chondrocytes is a prerequisite for invasion of growth cartilage by blood vessels, osteoclasts and osteogenic cells, thus it appears that these cells cannot enter the empty lacunae recently made vacant by the death of hypertrophic chondrocytes until their transverse septa are degraded by MMP13. It has been known for many years that blood vessels invade the growth cartilage in parallel with

osteoclasts (Fig. 2A and E), and some observers have noted that blood vessels appear to precede osteoclasts (Schenk, Spiro, & Wiener, 1967). In mice treated with a bisphosphonate to inhibit osteoclast activity, as well as in mice lacking osteoclasts due to mutations in critical osteoclastogenic factors, blood vessels are able to invade a cartilage model, indicating that osteoclasts are not required for vascular invasion to occur (Deckers et al., 2002). Osteoclasts are, however, required for establishment of the primary centre of ossification, since in their absence most cartilage matrix is not removed, and there is no invasion by bone marrow cells or deposition of bone matrix on cartilage remnants. Vascular invasion of growth cartilage is facilitated by vascular endothelial growth factor (VEGF), which is expressed by chondrocytes and up-regulated with hypertrophy under the control of Runx2 (Zelzer et al., 2001). In mice that fail to express VEGF in chondrocytes, both vascular invasion and ossification in primary ossification centres is substantially delayed (Zelzer et al., 2004). It may be incorrect to conclude on the basis of these observations that vascular invasion is required for osteoclastic invasion, since VEGF acts not only on vascular endothelial cells to promote angiogenesis, but also on osteoclasts to stimulate resorption and survival (Nakagawa et al., 2000). FGF18 induces *Vegf* expression in chondrocytes, and in *Fgf18*-null mice, in which vascular invasion is delayed, *Vegf* expression in hypertrophic chondrocytes is reduced (Liu, Lavine, Hung, & Ornitz, 2007). This aspect of the phenotype of FGF18-null mice is different from that of FGFR3-null mice, suggesting that a different FGFR mediates induction of VEGF.

6. Conclusion

The process by which endochondral bones develop is unusual, both because it involves the gradual replacement of a temporary structure with a permanent one better suited to the mechanical and other needs of the adult, and because this process of organogenesis is maintained until growth ceases. Endochondral ossification is a complex process, involving a carefully regulated sequence of changes in chondrocyte behaviour coordinated with the actions of blood vessels, osteoclasts and the other cells that they bring with them. Much has been learnt in recent years about the molecular and cellular mechanisms guiding these events. Intriguing questions that remain to be answered, and that have been raised in this review, include the following: What are the functional differences between 'light' and 'dark' hypertrophic chondrocytes? How do hypertrophic chondrocytes die? Is it necessary for hypertrophic chondrocytes to die for the orderly invasion of the ossification front to occur, and conversely, if the ossification front does not invade due to failure of cartilage matrix degradation, do hypertrophic chondrocytes still die? What is the role of the cytoskeleton in chondrocyte hypertrophy and death? What are the structural effects of extracellular matrix molecules such as collagen type X, as opposed to their effects on chondrocyte behaviour? How is aggrecan removed from cartilage matrix at the ossification front, and is this important for normal endochondral ossification?

In recent years, the availability of improved experimental methods such as microarray and transgenic animal technologies, together with information about the human and mouse genomes, have led to an increase in the rate of identification of molecules that are critical for normal endochondral ossification. As discussed above, these methods have identified essential roles for extracellular factors, receptors, intracellular signalling molecules, transcription factors and cytoskeletal components in regulating the behaviour of chondrocytes in such a way as to prepare the cartilage matrix for invasion by the cells of the ossification front. In many cases, however, although specific molecules have been identified as being required, their precise functional roles have not yet been elucidated. For some of these factors, more detailed experiments will be required, such as gene silencing or over-expression in chondrocytes at specific stages of differentiation. In many cases, the up-stream and/or down-stream components of signalling pathways have not yet been identified. It is likely that gene discovery approaches such as whole-of-genome arrays will bring to light a large number of interesting molecules that are not yet known to be expressed in the skeleton. Moreover, the identification of the molecular basis for yet to be elucidated human skeletal dysplasias will make further contributions to knowledge in this area. The knowledge base and methods are in place for a rapid expansion of understanding of the process of endochondral ossification in the coming years.

References

Adams, C. S., & Shapiro, I. M. (2002). The fate of the terminally differentiated chondrocyte: Evidence for microenvironmental regulation of chondrocyte apoptosis. *Crit. Rev. Oral Biol. Med.*, *13*(6), 465–473.

Ahmed, Y. A., Tatarczuch, L., Pagel, C. N., Davies, H. M., Mirams, M., & Mackie, E. J. (2007). Physiological death of hypertrophic chondrocytes. *Osteoarthritis Cartilage*, *15*(5), 575–586.

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., & de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation path-

way and is required for expression of Sox5 and Sox6. *Genes Dev.*, *16*(21), 2813–2828.

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., et al. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.*, *18*(9), 1072–1087.

Alvarez, J., Sohn, P., Zeng, X., Doetschman, T., Robbins, D. J., & Serra, R. (2002). TGFbeta2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression. *Development*, *129*(8), 1913–1924.

Anderson, H. C. (1969). Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J. Cell Biol.*, *41*(1), 59–72.

Anderson, H. C., Sipe, J. B., Hessle, L., Dhanyamraju, R., Atti, E., Camacho, N. P., et al. (2004). Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am. J. Pathol.*, *164*(3), 841–847.

Andrade, A. C., Nilsson, O., Barnes, K. M., & Baron, J. (2007). Wnt gene expression in the post-natal growth plate: Regulation with chondrocyte differentiation. *Bone*, *40*(5), 1361–1369.

Arnold, M. A., Kim, Y., Czubryt, M. P., Phan, D., McAnally, J., Qi, X., et al. (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev. Cell*, *12*(3), 377–389.

Baker, J., Liu, J. P., Robertson, E. J., & Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, *75*(1), 73–82.

Ballock, R. T., Heydemann, A., Wakefield, L. M., Flanders, K. C., Roberts, A. B., & Sporn, M. B. (1993). TGF-beta 1 prevents hypertrophy of epiphyseal chondrocytes: Regulation of gene expression for cartilage matrix proteins and metalloproteases. *Dev. Biol.*, *158*(2), 414–429.

Ballock, R. T., & Reddi, A. H. (1994). Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. *J. Cell Biol.*, *126*(5), 1311–1318.

Barnard, J. C., Williams, A. J., Rabier, B., Chassande, O., Samarut, J., Cheng, S. Y., et al. (2005). Thyroid hormones regulate fibroblast growth factor receptor signaling during chondrogenesis. *Endocrinology*, *146*(12), 5568–5580.

Bastepe, M., Weinstein, L. S., Ogata, N., Kawaguchi, H., Juppner, H., Kronenberg, H. M., et al. (2004). Stimulatory G protein directly regulates hypertrophic differentiation of growth plate cartilage in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, *101*(41), 14794–14799.

Benjamin, M., Archer, C. W., & Ralphs, J. R. (1994). Cytoskeleton of cartilage cells. *Microsc. Res. Technol.*, *28*(5), 372–377.

Bitgood, M. J., & McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Dev. Biol.*, *172*(1), 126–138.

Bohme, K., Conscience-Egli, M., Tschan, T., Winterhalter, K. H., & Bruckner, P. (1992). Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. *J. Cell Biol.*, *116*(4), 1035–1042.

Burch, W. M., & Lebovitz, H. E. (1982). Triiodothyronine stimulates maturation of porcine growth-plate cartilage in vitro. *J. Clin. Invest.*, *70*(3), 496–504.

Byers, P. D., & Brown, R. A. (2006). Cell columns in articular cartilage physes questioned: A review. *Osteoarthritis Cartilage*, *14*(1), 3–12.

Cawston, T. E., & Wilson, A. J. (2006). Understanding the role of tissue degrading enzymes and their inhibitors in development and disease. *Best Pract. Res. Clin. Rheumatol.*, *20*(5), 983–1002.

Chan, D., & Jacenko, O. (1998). Phenotypic and biochemical consequences of collagen X mutations in mice and humans. *Matrix Biol.*, *17*(3), 169–184.

Colnot, C., Sidhu, S. S., Balmain, N., & Poirier, F. (2001). Uncoupling of chondrocyte death and vascular invasion in mouse galectin 3 null mutant bones. *Dev. Biol.*, *229*(1), 203–214.

Daci, E., Everts, V., Torrekens, S., Van Herck, E., Tigchelaar-Gutterr, W., Bouillon, R., et al. (2003). Increased bone formation in mice lacking plasminogen activators. *J. Bone Miner. Res.*, *18*(7), 1167–1176.

DeChiara, T. M., Robertson, E. J., & Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*, *64*(4), 849–859.

Deckers, M. M., Van Beek, E. R., Van Der Pluijm, G., Wetterwald, A., Van Der Wee-Pals, L., Cecchini, M. G., et al. (2002). Dissociation of angiogenesis and osteoclastogenesis during endochondral bone formation in neonatal mice. *J. Bone Miner. Res.*, *17*(6), 998–1007.

Ehlen, H. W., Buelens, L. A., & Vortkamp, A. (2006). Hedgehog signaling in skeletal development. *Birth Defects Res. C: Embryo Today*, *78*(3), 267–279.

Enomoto-Iwamoto, M., Kitagaki, J., Koyama, E., Tamamura, Y., Wu, C., Kanatani, N., et al. (2002). The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. *Dev. Biol.*, *251*(1), 142–156.

Fedde, K. N., Blair, L., Silverstein, J., Coburn, S. P., Ryan, L. M., Weinstein, R. S., et al. (1999). Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J. Bone Miner. Res.*, *14*(12), 2015–2026.

Fell, H. B., & Mellanby, E. (1955). The biological action of thyroxine on embryonic bones grown in tissue culture. *J. Physiol.*, *127*(2), 427–447.

Gao, B., Guo, J., She, C., Shu, A., Yang, M., Tan, Z., et al. (2001). Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1. *Nat. Genet.*, *28*(4), 386–388.

Gibson, G. (1998). Active role of chondrocyte apoptosis in endochondral ossification. *Microsc. Res. Technol.*, *43*(2), 191–204.

Grimsrud, C. D., Romano, P. R., D'Souza, M., Puzas, J. E., Reynolds, P. R., Rosier, R. N., et al. (1999). BMP-6 is an autocrine stimulator of chondrocyte differentiation. *J. Bone Miner. Res.*, *14*(4), 475–482.

Grimsrud, C. D., Romano, P. R., D'Souza, M., Puzas, J. E., Schwarz, E. M., Reynolds, P. R., et al. (2001). BMP signaling stimulates chondrocyte maturation and the expression of Indian hedgehog. *J. Orthop. Res.*, *19*(1), 18–25.

Guevara-Aguirre, J., Rosenbloom, A. L., Vasconez, O., Martinez, V., Gargosky, S. E., Allen, L., et al. (1997). Two-year treatment of growth hormone (GH) receptor deficiency with recombinant insulin-like growth factor I in 22 children: Comparison of two dosage levels and to GH-treated GH deficiency. *J. Clin. Endocrinol. Metab.*, *82*(2), 629–633.

Guo, J., Chung, U. I., Yang, D., Karsenty, G., Bringhurst, F. R., & Kronenberg, H. M. (2006). PTH/PTHrP receptor delays chondrocyte hypertrophy via both Runx2-dependent and -independent pathways. *Dev. Biol.*, *292*(1), 116–128.

Hirao, M., Tamai, N., Tsumaki, N., Yoshikawa, H., & Myoui, A. (2006). Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. *J. Biol. Chem.*, *281*(41), 31079–31092.

Huang, W., Chung, U. I., Kronenberg, H. M., & de Crombrugghe, B. (2001). The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones. *Proc. Natl. Acad. Sci. U.S.A.*, *98*(1), 160–165.

Huang, W., Zhou, X., Lefebvre, V., & de Crombrugghe, B. (2000). Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase

A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Mol. Cell. Biol.*, *20*(11), 4149–4158.

Hunziker, E. B., Wagner, J., & Zapf, J. (1994). Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes in vivo. *J. Clin. Invest.*, *93*(3), 1078–1086.

Hwang, W. S. (1978). Ultrastructure of human foetal and neonatal hyaline cartilage. *J. Pathol.*, *126*(4), 209–214.

Inada, M., Wang, Y., Byrne, M. H., Rahman, M. U., Miyaura, C., Lopez-Otin, C., et al. (2004). Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc. Natl. Acad. Sci. U.S.A.*, *101*(49), 17192–17197.

Jensen, C. G., Poole, C. A., McGlashan, S. R., Marko, M., Issa, Z. I., Vujcich, K. V., et al. (2004). Ultrastructural, tomographic and confocal imaging of the chondrocyte primary cilium in situ. *Cell Biol. Int.*, *28*(2), 101–110.

Kaneshige, M., Suzuki, H., Kaneshige, K., Cheng, J., Wimbrow, H., Barlow, C., et al. (2001). A targeted dominant negative mutation of the thyroid hormone alpha 1 receptor causes increased mortality, infertility, and dwarfism in mice. *Proc. Natl. Acad. Sci. U.S.A.*, *98*(26), 15095–15100.

Karaplis, A. C., He, B., Nguyen, M. T., Young, I. D., Semeraro, D., Ozawa, H., et al. (1998). Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Blomstrand chondrodysplasia. *Endocrinology*, *139*(12), 5255–5258.

Kennedy, A. M., Inada, M., Krane, S. M., Christie, P. T., Harding, B., Lopez-Otin, C., et al. (2005). MMP13 mutation causes spondyloepimetaphyseal dysplasia, Missouri type (SEMD(MO)). *J. Clin. Invest.*, *115*(10), 2832–2842.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, *26*(4), 239–257.

Kirsch, T. (2006). Determinants of pathological mineralization. *Curr. Opin. Rheumatol.*, *18*(2), 174–180.

Kirsch, T., Nah, H. D., Shapiro, I. M., & Pacifici, M. (1997). Regulated production of mineralization-competent matrix vesicles in hypertrophic chondrocytes. *J. Cell Biol.*, *137*(5), 1149–1160.

Kluppel, M., Wight, T. N., Chan, C., Hinek, A., & Wrana, J. L. (2005). Maintenance of chondroitin sulfation balance by chondroitin-4-sulfotransferase 1 is required for chondrocyte development and growth factor signaling during cartilage morphogenesis. *Development*, *132*(17), 3989–4003.

Koyama, E., Leatherman, J. L., Noji, S., & Pacifici, M. (1996). Early chick limb cartilaginous elements possess polarizing activity and express hedgehog-related morphogenetic factors. *Dev. Dyn.*, *207*(3), 344–354.

Koziel, L., Wuelling, M., Schneider, S., & Vortkamp, A. (2005). Gli3 acts as a repressor downstream of Ihh in regulating two distinct steps of chondrocyte differentiation. *Development*, *132*(23), 5249–5260.

Kronenberg, H. M. (2006). PTHrP and skeletal development. *Ann. N.Y. Acad. Sci.*, *1068*, 1–13.

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., et al. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology*, *137*(11), 5109–5118.

Lefebvre, V., & Smits, P. (2005). Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Res. C: Embryo Today*, *75*(3), 200–212.

Leitinger, B., & Hohenester, E. (2007). Mammalian collagen receptors. *Matrix Biol.*, *26*(3), 146–155.

Leitinger, B., & Kwan, A. P. (2006). The discoidin domain receptor DDR2 is a receptor for type X collagen. *Matrix Biol.*, *25*(6), 355–364.

Little, C. B., Meeker, C. T., Hembry, R. M., Sims, N. A., Lawlor, K. E., Golub, S. B., et al. (2005). Matrix metalloproteinases are not essential for aggrecan turnover during normal skeletal growth and development. *Mol. Cell. Biol.*, *25*(8), 3388–3399.

Little, C. B., Mittaz, L., Belluoccio, D., Rogerson, F. M., Campbell, I. K., Meeker, C. T., et al. (2005). ADAMTS-1-knockout mice do not exhibit abnormalities in aggrecan turnover in vitro or in vivo. *Arthritis Rheum.*, *52*(5), 1461–1472.

Liu, Z., Lavine, K. J., Hung, I. H., & Ornitz, D. M. (2007). FGF18 is required for early chondrocyte proliferation, hypertrophy and vascular invasion of the growth plate. *Dev. Biol.*, *302*(1), 80–91.

Long, F., Joeng, K. S., Xuan, S., Efstratiadis, A., & McMahon, A. P. (2006). Independent regulation of skeletal growth by Ihh and IGF signaling. *Dev. Biol.*, *298*(1), 327–333.

Luckman, S. P., Rees, E., & Kwan, A. P. (2003). Partial characterization of cell-type X collagen interactions. *Biochem. J.*, *372*(Pt 2), 485–493.

Mak, K. K., Chen, M. H., Day, T. F., Chuang, P. T., & Yang, Y. (2006). Wnt/beta-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. *Development*, *133*(18), 3695–3707.

McGlashan, S. R., Haycraft, C. J., Jensen, C. G., Yoder, B. K., & Poole, C. A. (2007). Articular cartilage and growth plate defects are associated with chondrocyte cytoskeletal abnormalities in Tg737 (orpk) mice lacking the primary cilia protein polaris. *Matrix Biol.*, *26*(4), 234–246.

Minina, E., Kreschel, C., Naski, M. C., Ornitz, D. M., & Vortkamp, A. (2002). Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. *Dev. Cell*, *3*(3), 439–449.

Minina, E., Wenzel, H. M., Kreschel, C., Karp, S., Gaffield, W., McMahon, A. P., et al. (2001). BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. *Development*, *128*(22), 4523–4534.

Morishima, A., Grumbach, M. M., Simpson, E. R., Fisher, C., & Qin, K. (1995). Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J. Clin. Endocrinol. Metab.*, *80*(12), 3689–3698.

Nakagawa, M., Kaneda, T., Arakawa, T., Morita, S., Sato, T., Yomada, T., et al. (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. *FEBS Lett.*, *473*(2), 161–164.

Nakamura, T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Pacifici, M., et al. (1997). Induction of osteogenic differentiation by hedgehog proteins. *Biochem. Biophys. Res. Commun.*, *237*(2), 465–469.

Nilsson, O., Marino, R., De Luca, F., Phillip, M., & Baron, J. (2005). Endocrine regulation of the growth plate. *Horm. Res.*, *64*(4), 157–165.

Nurminsky, D., Magee, C., Faverman, L., & Nurminskaya, M. (2007). Regulation of chondrocyte differentiation by actin-severing protein adseverin. *Dev. Biol.*, *302*(2), 427–437.

Ornitz, D. M. (2005). FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.*, *16*(2), 205–213.

O'Shea, P. J., Bassett, J. H., Sriskantharajah, S., Ying, H., Cheng, S. Y., & Williams, G. R. (2005). Contrasting skeletal phenotypes in mice with an identical mutation targeted to thyroid hormone receptor alpha1 or beta. *Mol. Endocrinol.*, *19*(12), 3045–3059.

Poole, C. A., Flint, M. H., & Beaumont, B. W. (1985). Analysis of the morphology and function of primary cilia in connective tissues: A cellular cybernetic probe? *Cell Motil.*, *5*(3), 175–193.

Poole, C. A., Zhang, Z. J., & Ross, J. M. (2001). The differential distribution of acetylated and detyrosinated alpha-tubulin in the microtubular cytoskeleton and primary cilia of hyaline cartilage chondrocytes. *J. Anat.*, *199*(Pt 4), 393–405.

Potts, W., Bowyer, J., Jones, H., Tucker, D., Freemont, A. J., Millest, A., et al. (2004). Cathepsin L-deficient mice exhibit abnormal skin and bone development and show increased resistance to osteoporosis following ovariectomy. *Int. J. Exp. Pathol.*, *85*(2), 85–96.

Provot, S., Kempf, H., Murtaugh, L. C., Chung, U. I., Kim, D. W., Chyung, J., et al. (2006). Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development*, *133*(4), 651–662.

Roach, H. I., Aigner, T., & Kouri, J. B. (2004). Chondroptosis: A variant of apoptotic cell death in chondrocytes? *Apoptosis*, *9*(3), 265–277.

Roach, H. I., & Clarke, N. M. (1999). "Cell paralysis" as an intermediate stage in the programmed cell death of epiphyseal chondrocytes during development. *J. Bone Miner. Res.*, *14*(8), 1367–1378.

Roach, H. I., & Clarke, N. M. (2000). Physiological cell death of chondrocytes in vivo is not confined to apoptosis. New observations on the mammalian growth plate. *J. Bone Joint Surg. Br.*, *82*(4), 601–613.

Schenk, R. K., Spiro, D., & Wiener, J. (1967). Cartilage resorption in the tibial epiphyseal plate of growing rats. *J. Cell Biol.*, *34*(1), 275–291.

Schipani, E., Kruse, K., & Juppner, H. (1995). A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science*, *268*(5207), 98–100.

Schipani, E., Ryan, H. E., Didrickson, S., Kobayashi, T., Knight, M., & Johnson, R. S. (2001). Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes Dev.*, *15*(21), 2865–2876.

Serra, R., Karaplis, A., & Sohn, P. (1999). Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor beta (TGF-beta) on endochondral bone formation. *J. Cell Biol.*, *145*(4), 783–794.

Shao, Y. Y., Wang, L., & Ballock, R. T. (2006). Thyroid hormone and the growth plate. *Rev. Endocr. Metab. Disord.*, *7*(4), 265–271.

Shapiro, I. M., Adams, C. S., Freeman, T., & Srinivas, V. (2006). Fate of the hypertrophic chondrocyte: Microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate. *Birth Defects Res. C: Embryo Today*, *75*(4), 330–339.

Sims, N. A., Clement-Lacroix, P., Da Ponte, F., Bouali, Y., Binart, N., Moriggl, R., et al. (2000). Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. *J. Clin. Invest.*, *106*(9), 1095–1103.

Smith, E. P., Boyd, J., Frank, G. R., Takahashi, H., Cohen, R. M., Specker, B., et al. (1994). Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N. Engl. J. Med.*, *331*(16), 1056–1061.

Smits, P., Dy, P., Mitra, S., & Lefebvre, V. (2004). Sox5 and Sox6 are needed to develop and maintain source, columnar, and hypertrophic chondrocytes in the cartilage growth plate. *J. Cell Biol.*, *164*(5), 747–758.

Song, B., Haycraft, C. J., Seo, H. S., Yoder, B. K., & Serra, R. (2007). Development of the post-natal growth plate requires intraflagellar transport proteins. *Dev. Biol.*, *305*(1), 202–216.

Stanton, H., Rogerson, F. M., East, C. J., Golub, S. B., Lawlor, K. E., Meeker, C. T., et al. (2005). ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature*, *434*(7033), 648–652.

St-Jacques, B., Hammerschmidt, M., & McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.*, *13*(16), 2072–2086.

Stevens, D. A., Hasserjian, R. P., Robson, H., Siebler, T., Shalet, S. M., & Williams, G. R. (2000). Thyroid hormones regulate hypertrophic chondrocyte differentiation and expression of parathyroid hormone-related peptide and its receptor during endochondral bone formation. *J. Bone Miner. Res.*, *15*(12), 2431–2442.

Stickens, D., Behonick, D. J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., et al. (2004). Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development*, *131*(23), 5883–5895.

Sutter, N. B., Bustamante, C. D., Chase, K., Gray, M. M., Zhao, K., Zhu, L., et al. (2007). A single IGF1 allele is a major determinant of small size in dogs. *Science*, *316*(5821), 112–115.

Tamamura, Y., Otani, T., Kanatani, N., Koyama, E., Kitagaki, J., Komori, T., et al. (2005). Developmental regulation of Wnt/beta-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. *J. Biol. Chem.*, *280*(19), 19185–19195.

Tchetina, E. V., Kobayashi, M., Yasuda, T., Meijers, T., Pidoux, I., & Poole, A. R. (2007). Chondrocyte hypertrophy can be induced by a cryptic sequence of type II collagen and is accompanied by the induction of MMP-13 and collagenase activity: Implications for development and arthritis. *Matrix Biol.*, *26*(4), 247–258.

Terkeltaub, R. A. (2001). Inorganic pyrophosphate generation and disposition in pathophysiology. *Am. J. Physiol. Cell Physiol.*, *281*(1), C1–C11.

Thorp, B. H., & Dixon, J. M. (1991). Cartilaginous bone extremities of growing monotremes appear unique. *Anat. Rec.*, *229*(4), 447–452.

van der Eerden, B. C., Karperien, M., & Wit, J. M. (2003). Systemic and local regulation of the growth plate. *Endocr. Rev.*, *24*(6), 782–801.

Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., et al. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell*, *119*(4), 555–566.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., & Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science*, *273*(5275), 613–622.

Vu, T. H., Shipley, J. M., Bergers, G., Berger, J. E., Helms, J. A., Hanahan, D., et al. (1998). MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell*, *93*(3), 411–422.

Weise, M., De-Levi, S., Barnes, K. M., Gafni, R. I., Abad, V., & Baron, J. (2001). Effects of estrogen on growth plate senescence and epiphyseal fusion. *Proc. Natl. Acad. Sci. U.S.A.*, *98*(12), 6871–6876.

Whyte, M. P. (2006). Chondrodystrophies and Mucopolysaccharidoses. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (6th ed., pp. 421–425). Washington, D.C.: American Society for Bone and Mineral Research.

Wilsman, N. J., Farnum, C. E., Hilley, H. D., & Carlson, C. S. (1981). Ultrastructural evidence of a functional heterogeneity among physeal chondrocytes in growing swine. *Am. J. Vet. Res.*, *42*(9), 1547–1553.

Wilsman, N. J., Farnum, C. E., & Reed-Aksamit, D. K. (1980). Incidence and morphology of equine and murine chondrocytic cilia. *Anat. Rec.*, *197*(3), 355–361.

Wilsman, N. J., & Van Sickle, D. C. (1972). Cartilage canals, their morphology and distribution. *Anat. Rec.*, *173*(1), 79–93.

Yakar, S., Rosen, C. J., Beamer, W. G., Ackert-Bicknell, C. L., Wu, Y., Liu, J. L., et al. (2002). Circulating levels of IGF-1 directly regulate bone growth and density. *J. Clin. Invest.*, *110*(6), 771–781.

Yamada, T., Kawano, H., Koshizuka, Y., Fukuda, T., Yoshimura, K., Kamekura, S., et al. (2006). Carminerin contributes to chondrocyte calcification during endochondral ossification. *Nat. Med.*, *12*(6), 665–670.

Yang, C., Li, S. W., Helminen, H. J., Khillan, J. S., Bao, Y., & Prockop, D. J. (1997). Apoptosis of chondrocytes in transgenic mice lacking collagen II. *Exp. Cell Res.*, *235*(2), 370–373.

Yang, Y., Topol, L., Lee, H., & Wu, J. (2003). Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development*, *130*(5), 1003–1015.

Yates, K. E., Shortkroff, S., & Reish, R. G. (2005). Wnt influence on chondrocyte differentiation and cartilage function. *DNA Cell Biol.*, *24*(7), 446–457.

Yoon, B. S., Pogue, R., Ovchinnikov, D. A., Yoshii, I., Mishina, Y., Behringer, R. R., et al. (2006). BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways. *Development*, *133*(23), 4667–4678.

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., et al. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.*, *18*(8), 952–963.

Zelzer, E., Glotzer, D. J., Hartmann, C., Thomas, D., Fukai, N., Soker, S., et al. (2001). Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mech. Dev.*, *106*(1/2), 97–106.

Zelzer, E., Mamluk, R., Ferrara, N., Johnson, R. S., Schipani, E., & Olsen, B. R. (2004). VEGFA is necessary for chondrocyte survival during bone development. *Development*, *131*(9), 2161–2171.

Zhang, P., Jobert, A. S., Couvineau, A., & Silve, C. (1998). A homozygous inactivating mutation in the parathyroid hormone/parathyroid hormone-related peptide receptor causing Blomstrand chondrodysplasia. *J. Clin. Endocrinol. Metab.*, *83*(9), 3365–3368.

Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., et al. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, *103*(50), 19004–19009.