THEMATIC REVIEW

The skeleton: a multi-functional complex organ. The growth plate chondrocyte and endochondral ossification

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Abstract

Endochondral ossification is the process that results in both the replacement of the embryonic cartilaginous skeleton during organogenesis and the growth of long bones until adult height is achieved. Chondrocytes play a central role in this process, contributing to longitudinal growth through a combination of proliferation, extracellular matrix (ECM) secretion and hypertrophy. Terminally differentiated hypertrophic chondrocytes then die, allowing the invasion of a mixture of cells that collectively replace the cartilage tissue with bone tissue. The behaviour of growth plate chondrocytes is tightly regulated at all stages of endochondral ossification by a complex network of interactions between

circulating hormones (including GH and thyroid hormone), locally produced growth factors (including Indian hedgehog, WNTs, bone morphogenetic proteins and fibroblast growth factors) and the components of the ECM secreted by the chondrocytes (including collagens, proteoglycans, thrombospondins and matrilins). In turn, chondrocytes secrete factors that regulate the behaviour of the invading bone cells, including vascular endothelial growth factor and receptor activator of NFκB ligand. This review discusses how the growth plate chondrocyte contributes to endochondral ossification, with some emphasis on recent advances.

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Introduction

Bones in different parts of the skeleton develop through two distinct processes, intramembranous ossification and endochondral ossification. Intramembranous ossification, which occurs in the flat bones of the skull, involves direct differentiation of embryonic mesenchymal cells into the bone-forming osteoblasts. In contrast, endochondral ossification, which occurs in the remainder of the skeleton, involves the replacement of a cartilage model by bone tissue.

The cartilage model of a prospective bone is formed as embryonic mesenchymal cells condense and differentiate into chondrocytes, which secrete the various components of cartilage extracellular matrix (ECM), including collagen type II and the proteoglycan aggrecan. The model expands through chondrocyte proliferation. Ossification of the cartilage model is preceded by hypertrophy of the chondrocytes in the prospective mid-shaft of the bone, and

deposition of a periosteal bone collar by recently differentiated osteoblasts surrounding the mid-shaft (Fig. 1). Blood vessels, osteoclasts (cartilage- and bone-resorbing cells), as well as bone marrow and osteoblast precursors then invade the model from the bone collar and proceed to form the primary centre of ossification. The primary centre expands towards the ends of the cartilage model, as the osteoclasts remove cartilage ECM and osteoblasts deposit bone on cartilage remnants. In long bones, a secondary ossification centre subsequently forms at each end of the cartilage model, leaving a cartilaginous growth plate between the primary and secondary ossification centres, as well as the prospective permanent articular cartilages at each end of the bone. The growth plate is responsible for longitudinal growth of bones. Skeletal maturity occurs when the expanding primary centre of ossification meets the secondary centre of ossification, thus obliterating the growth plate (Fig. 1).

Endochondral ossification is initiated during foetal life, and continues until growth ceases in early adulthood. Although endochondral ossification is dependent on the concerted actions of a number of cell types, it is the chondrocyte that drives the process. This review will focus on the role of the chondrocyte in endochondral ossification, with some emphasis on areas in which recent advances have been made.

This paper is one of three papers that form part of a thematic review section on the skeleton: a multi-functional complex organ. The Guest Editor for this section was Colin Farquharson, Roslin Institute, University of Edinburgh, UK.

Figure 1 Overview of development of a bone by endochondral ossification. (A) The cartilage model of the future bone. (B) A periosteal bone collar has formed and formation of the primary centre of ossification has been initiated. (C) The primary centre of ossification is starting to expand towards the ends of the cartilage model. (D) The secondary centres of ossification have formed at each end of the bone, leaving a cartilaginous growth plate between primary and secondary centres of ossification. (E) Skeletal maturity has been achieved, with complete replacement of the growth plate cartilage by bone. The only cartilage remaining is the articular cartilage at the ends of the bone.

The growth plate chondrocyte models its own environment

At all stages of endochondral ossification, from the initiation of formation of the primary centre to the final stages of adolescent growth, the chondrocytes contributing to this process participate in an orderly sequence of events that are reflected in their morphology; the different stages of the chondrocyte lifespan are visible in distinct zones in sections through the growth plate (Fig. 2). First, chondrocytes undergo proliferation, which is observed as the presence of pairs of chondrocytes in a single lacuna within the cartilage ECM, before their separation from each other by secretion of ECM (Figs 2 and 3). Following proliferation, the chondrocytes undergo a period of high secretory activity, as they deposit the typical cartilage ECM components around themselves, while remaining in multicellular clusters, often arranged in columns parallel to the long axis of the bone. These cells gradually undergo hypertrophy, modelling their surrounding ECM as they expand, and then mineralising it. Following hypertrophy, chondrocytes undergo physiological death, and the transverse septa of the cartilage ECM surrounding them are removed, allowing entry of the mixture of cells responsible for the expansion of the ossification centre. Thus, the growth plate chondrocyte plays multiple important roles during its lifespan. It constructs the transient growth plate tissue, which has the necessary capacity to move in space through continued self-renewal and localised degradation, but simultaneously maintains the mechanical stability of the growing bone. Accumulating evidence indicates that the

growth plate chondrocyte orchestrates the invasion of its own domain by the ossification front not only through preparation of the cartilage tissue, but also by secreting soluble molecules that regulate the behaviour of the invading cells.

Contribution to bone elongation

The growth plate chondrocyte contributes to bone elongation through a combination of proliferation, ECM secretion and hypertrophy. The relative contributions of these parameters vary with growth rate, which varies with anatomical location, age and species; the higher the growth rate the greater the contribution from cellular hypertrophy and the smaller the contribution from matrix synthesis (Wilsman *et al.* 1996). These functions of the chondrocyte are tightly controlled by circulating molecules such as hormones, as well as by substances produced locally by the chondrocytes themselves (summarised in Fig. 4). Mutations in the genes encoding these regulatory molecules result in abnormal chondrocyte behaviour and skeletal dysplasias, most of which are associated with dwarfism, i.e. inadequate growth of the bones that form by endochondral ossification (Krakow & Rimoim 2010).

Proliferation

An important stimulator of chondrocyte proliferation in the growth plate is GH, which is produced in the pituitary gland. GH exerts its effects on the growth plate predominantly

Figure 2 Growth plate morphology. Section through the proximal growth plate of the tibia from a 3-week-old mouse; the section is oriented longitudinally with respect to the bone; bar = 50 µm.

Figure 3 The changing morphology of growth plate chondrocytes. Chondrocytes in a section through the proximal growth plate of a 4-week-old rat tibia, examined by transmission electron microscopy. (A) Zone of proliferative chondrocytes, where pairs of cells that have recently undergone proliferation have not yet separated from each other. (B) Zone of early hypertrophic chondrocytes, where ECM now separates the cells. (C) Zone of late hypertrophic chondrocytes, including the last lacunae before the ossification front, which can be identified by the presence of erythrocytes (arrow). The majority of cells are light (li) chondrocytes, but occasional dark (da) chondrocytes are visible in all zones. All figure parts are the same magnification; bar = 10 µm.

through stimulation of secretion of insulin-like growth factor 1 (IGF1), both by liver cells and by growth plate chondrocytes (Nilsson *et al.* 2005, Pass *et al.* 2009). The importance of the GH/IGF1 axis is underlined by the excessive growth observed in children with elevated GH levels due to pituitary tumours, and the inadequate growth observed in children with GH deficiency or mutations in the genes encoding components of the GH response pathway. It is likely that suppression of activity of the GH/IGF1 axis by inflammatory cytokines is partly responsible for the growth defects observed in children with chronic inflammatory conditions, who generally show reduced levels of IGF1 (Pass *et al.* 2009). A second IGF, IGF2, is expressed in the growth plate independently of GH, and is required for normal embryonic growth (DeChiara *et al.* 1991). All of IGF1's effects and most of IGF2's effects are mediated by the type I IGF receptor, and mice lacking this receptor demonstrate a more severe growth defect than mice lacking either of the IGFs (Baker *et al.* 1993).

Indian hedgehog (IHH) is secreted by chondrocytes undergoing the transition from proliferation to hypertrophy, known as 'prehypertrophic' chondrocytes; IHH is required for normal chondrocyte proliferation during both embryonic and postnatal growth (Koyama *et al.* 1996, St-Jacques *et al.* 1999, Maeda *et al.* 2007). Substantial differences in *Ihh* expression levels are associated with inter-breed differences in growth rate in dogs (Tryfonidou *et al.* 2010). IHH exerts its effects by binding to its cell surface receptor patched 1 (Ptch1), which normally suppresses signalling through the membrane protein smoothened (Smo), but binding of Hh to Ptch1 reverses inhibition of Smo and allows IHH pathway signalling to occur. Responses of chondrocytes to IHH are mediated by members of the Gli family of transcriptional regulators (Wuelling & Vortkamp 2010). In the presence of

IHH signalling, these proteins promote transcription of IHH target genes, but when IHH is absent, Gli2 and Gli3 are subject to proteolytic conversion into transcriptional repressors. IHH stimulates chondrocyte proliferation through inactivation of the repressor form of Gli3, in particular, as demonstrated by the phenotype of double IHH-null/Gli3-null mice (Ehlen *et al.* 2006). IHH binds to aggrecan through its chondroitin sulphate side chains, and in the mouse growth plate normal sulphation of chondroitin sulphate is required for normal IHH protein distribution and signalling, and for chondrocyte proliferation (Cortes *et al.* 2009). IHH signalling, and thus proliferation of growth plate chondrocytes, is also dependent on the presence of an intact primary cilium, a structure consisting of a basal body and a ciliary axoneme that extends several micrometres from the surface of the chondrocyte and most other cells (Wilsman *et al.* 1980, Poole *et al.* 1985, Koyama *et al.* 2007).

WNTs are a family of secreted proteins that play multiple roles in skeletal development and maintenance (Yates *et al.* 2005). Binding of WNTs to their receptor Frizzled (Frz) in combination with the co-receptor Lrp5 or Lrp6 leads to activation of the canonical WNT signalling pathway which involves accumulation of β -catenin, whereas binding to Frz alone leads to activation of the non-canonical (calcium-dependent kinase C-mediated) pathway which can result in degradation of β -catenin. A number of WNTs are expressed by growth plate chondrocytes and are capable of stimulating their proliferation, but there is disagreement in the literature as to whether β -catenin mediates this effect (Yang *et al.* 2003, Akiyama *et al.* 2004, Mak *et al.* 2006, Andrade *et al.* 2007, Chen *et al.* 2008).

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF β) superfamily.

Figure 4 Overview of the effects of factors secreted by chondrocytes on growth plate cell function and invasion. Under the control of circulating hormones, chondrocytes secrete growth factors that act on chondrocytes to regulate their proliferation and hypertrophy (upper two panels), and on cells of the ossification front (lower panel), to regulate their invasion of the growth plate cartilage. Arrows indicate stimulatory pathways, and crossed lines indicate inhibitory pathways. Not all effects mentioned in the text are included in this figure.

Activation of BMP signalling in the growth plate results in chondrocyte proliferation (Minina *et al.* 2001, Yoon *et al.* 2006). BMP receptors are complexes of type I and type II serine/threonine kinase receptors, which when activated phosphorylate receptor-Smads, causing their translocation to the nucleus. The receptor-Smads responsible for BMP signalling in chondrocytes are Smads 1 and 5, which appear to have overlapping functions (Retting *et al.* 2009). Phosphorylation of Smads 1 and 5 in chondrocytes is dependent on the presence of neogenin, which is a receptor for the neuronal axon guidance cues netrins and repulsive guidance molecules (Zhou *et al.* 2010). Neogenin's role in this process appears to be to mediate the association of the BMP receptor with membrane microdomains (lipid rafts). Schnurri proteins are large zinc finger adapter proteins that can bind to and cooperate with Smads in induction of BMP-responsive genes (Jin *et al.* 2006). A recent study has demonstrated that mice that lack both *Shn2* and *Shn3* (but not each gene individually) show defects in endochondral ossification including inadequate chondrocyte proliferation, an effect that is likely to be due to failure of BMP signalling (Jones *et al.* 2010). BMPs induce IHH expression and IHH induces expression of various BMPs in growth cartilage, but neither of these factors is absolutely dependent on expression of the other for its effects on chondrocyte proliferation (Grimsrud *et al.* 2001, Minina *et al.* 2001).

Fibroblast growth factors (FGFs) acting through FGF receptor 3 (FGFR3) are important regulators of chondrocyte

proliferation, but activation of this receptor represses proliferation rather than promoting it (Ornitz 2005). Achondroplasia, the most common form of short-limbed dwarfism in humans, as well as some other human skeletal dysplasias, result from activating mutations in *FGFR3* (Ornitz 2005, Krakow & Rimoim 2010). A number of FGFs are capable of activating FGFR3, and on the basis of expression and gene manipulation studies in mice, it has been concluded that the most important activator in the growth plate is likely to be FGF18 (Ornitz 2005). In the human growth plate, however, the predominantly expressed FGFs are FGFs 1, 2, 15 and 19 (Krejci *et al.* 2007). FGFR3 is a tyrosine kinase receptor, and its suppression of chondrocyte proliferation in the growth plate appears to be mediated by STAT1 (Ornitz 2005). The activity of FGFs, like that of IHH, is modulated by glycosaminoglycans. Mice that lack sulphatase-modifying factor 1, which activates sulphatases (and thus proteoglycan desulphation) show dwarfism, which can be rescued by crossing with FGF18-null mice (Settembre *et al.* 2008). Thus, it appears that FGF-induced repression of chondrocyte proliferation is limited by the desulphation of glycosaminoglycans. The relevant glycosaminoglycan is most likely to be heparan sulphate, found in proteoglycans such as perlecan in the cartilage ECM (Bishop *et al.* 2007, Rodgers *et al.* 2008, Chuang *et al.* 2010). BMP signalling antagonises the inhibition of chondrocyte proliferation caused by activation of FGFR3 (Minina *et al.* 2002).

Chondrocyte proliferation in response to growth factors including IHH is mediated by cyclins which form complexes with cyclin-dependent kinases, ultimately leading to activation of E2F transcription factors and cell cycle progression (Wuelling & Vortkamp 2010). Cyclin D1, in particular, is required for normal proliferation of growth plate chondrocytes, as demonstrated by studies in cyclin D1-null mice (Beier *et al.* 2001). The ability of chondrocytes to express normal levels of cyclin D1, and thus undergo normal proliferation, is dependent on the presence of the transcriptional repressor TRPS1. This protein derives its name from tricho-rhino-phalangeal syndrome, a condition in humans involving skeletal malformations, which results from mutations in the *TRPS1* gene. TRPS1-null mice demonstrate abnormally low levels of proliferation in growth plate chondrocytes, which can be attributed to a role for TRPS1 in repression of STAT3 expression, which in turn allows for elevated expression of cyclin D1 (Suemoto *et al.* 2007). It is interesting to note that TRPS1 binds the transcriptional activator form of Gli3, but the implications of this binding for chondrocyte proliferation in the growth plate are as yet unclear (Wuelling *et al.* 2009, Wuelling & Vortkamp 2010).

Conversely, inhibition of chondrocyte proliferation in response to activation of FGFR3 is accompanied by induction of inhibitors of cyclin-dependent kinase including p21, presumably thereby causing cell cycle arrest (Dailey *et al.* 2003). Further information about the mechanism by which FGFR3 causes early cell cycle exit is provided by the results of expression array studies comparing chondrocytes isolated from human foetuses affected by thanatophoric dysplasia caused by activating FGFR3 mutations with chondrocytes isolated from normal foetuses; among other observations concerning cell cycle-related genes, the FGFR3 mutations are associated with lower levels of cell cycle-related E2F target genes (Schibler *et al.* 2009).

Extracellular matrix

Secretion of ECM by growth plate chondrocytes makes an important contribution to growth. Cartilage ECM consists primarily of large aggregates of aggrecan and the glycosaminoglycan hyaluronan, packed in amongst fibrils of collagen type II (Gentili & Cancedda 2009, Heinegard 2009). These three components of cartilage ECM confer on the growth plate the mechanical stability required by this integral component of the growing skeleton. The collagen fibrils provide the framework for the tissue and the strongly hydrophilic hyaluronan-aggrecan aggregates allow the tissue to withstand compression. Both collagen type II and aggrecan are almost exclusively expressed in cartilage.

Cartilage ECM also contains a number of less abundant collagens, proteoglycans and other non-collagenous proteins, which, together with the three major cartilage constituents, form a complex network of interacting molecules (Gentili & Cancedda 2009, Heinegard 2009). The relative abundance of many of the cartilage ECM constituents varies between zones

of the growth plate. Minor cartilage collagens include collagen types VI, IX, X, XI, XII and XIV. Cartilage proteoglycans, in addition to aggrecan, include the small leucine-rich proteoglycans decorin, biglycan and fibromodulin, as well as the large proteoglycan perlecan. Other non-collagenous proteins found in cartilage ECM include the matrilins and thrombospondin family members, such as thrombospondin-5, also known as cartilage oligomeric matrix protein (COMP). Many of the less abundant molecules make important contributions to cartilage ECM assembly and, together with the major cartilage constituents, influence the behaviour of chondrocytes (Heinegard 2009, Klatt *et al.* 2011).

The importance of the complex interactions between cartilage ECM components for growth is illustrated by the effects of mutations in humans or mice in the genes encoding a number of the proteins. For some of these molecules, the complete absence of the protein has no detrimental effect, but mutations causing a failure of secretion from the endoplasmic reticulum (ER) result in retention of the mutant protein and its binding partners in the ER as well as growth defects (Zaucke & Grässel 2009, Klatt *et al.* 2011). Pseudoachondroplasia in humans is caused by mutations in *COMP*, and the milder multiple epiphyseal dysplasia can be caused by mutations in the genes encoding *COMP*, collagen type IX or matrilin-3 (Briggs & Chapman 2002). Many of the *COMP* mutations result in misfolding of *COMP*, and the chondrocytes from these patients retain not only *COMP* but also collagen type IX and matrilin-3 in their ER, and the ECM is depleted of all these proteins (Hecht *et al.* 2005). Secretion of aggrecan and collagen type II is not affected by the mutations, but the collagen does not form organised fibril bundles, indicating that the *COMP*/collagen IX/matrilin-3 complexes are required for normal cartilage matrix organisation and growth plate structure. Mice lacking *COMP* or matrilin-3 show no abnormality in skeletal development or growth, and mice lacking collagen type IX exhibit a mild growth defect, suggesting that these proteins are at least partly able to substitute for each other (Fässler *et al.* 1994, Hagg *et al.* 1997, Svensson *et al.* 2002, Ko *et al.* 2004). Generation of mice lacking both *COMP* and collagen type IX did not show any greater disturbance of the growth plate than did mice lacking collagen type IX alone, but the ECM of these growth plates still contains some matrilin-3, so perhaps the additional deletion of matrilin-3 and/or possibly other proteins is required for the demonstration of functional redundancy (Blumbach *et al.* 2008, Posey *et al.* 2008). It is interesting to note that in these mice the additional deletion of the *COMP*-related protein thrombospondin-3, which alone causes no obvious growth plate defect, caused a significantly greater disruption of growth plate organisation and limb length reduction (Posey *et al.* 2008).

In addition to the protein components of cartilage ECM, hyaluronan plays an important role in the contribution of ECM secretion to growth. Mice in which the gene for hyaluronan synthase 2 (*Has2*) is inactivated in tissues derived from limb bud

mesoderm possess abnormally short limbs (Matsumoto *et al.* 2009). The growth plates from these mice contain abnormally low levels of hyaluronan; they show a decrease in the deposition of aggrecan and a decrease in the amount of matrix separating the chondrocytes, manifest as an increase in cell density in the absence of any effect on proliferation.

Expression and secretion of components of cartilage ECM, including collagen type II and aggrecan are stimulated by a variety of soluble factors present in the growth plate, including IGF1, BMPs and other members of the TGF β superfamily, and are absolutely dependent on the transcription factor SOX9 (Bi *et al.* 1999, Lefebvre & Smits 2005, Tew *et al.* 2008). SOX9-activated transcription appears to be modulated by epigenetic mechanisms, since it occurs predominantly in hyperacetylated chromatin; the histone acetyltransferase p300 associates with SOX9 and enhances SOX9-dependent transcription. Moreover, inhibition of histone deacetylases (HDACs) stimulates expression of SOX9-activated cartilage ECM genes and induces histone acetylation in the region of the *Col2a1* enhancer in primary chondrocyte cultures (Furumatsu *et al.* 2005). Overexpression of HDAC1 or 2 in chondrocytes results in down-regulation of expression of *Aggrecan* and *Col2a1*, providing further evidence for epigenetic control of this aspect of chondrocyte function (Hong *et al.* 2009).

Hypertrophy

As post-proliferative chondrocytes undergo hypertrophy, they experience changes in gene expression that allow them to modify the structure and composition of the surrounding ECM. The synthesis of collagen type II is down regulated and the synthesis of the non-fibrillar collagen type X, expression of which is specific to hypertrophic chondrocytes, is initiated (van der Eerden *et al.* 2003). Hypertrophic chondrocytes also selectively express matrix metalloproteinase 13 (MMP13), a collagenase capable of degrading fibrils of collagen type II (Johansson *et al.* 1997, Cawston & Young 2010). Post-proliferative growth plate chondrocytes exist as two populations, which are described as light and dark cells on the basis of their appearance when viewed by transmission electron microscopy (Fig. 3; Anderson 1964, Hwang 1978, Wilsman *et al.* 1981, Roach & Clarke 2000, Ahmed *et al.* 2007). The fact that these two populations could only be identified by electron microscopy has made it difficult to study their functional differences, but the recent identification of periostin as a dark chondrocyte-associated protein will assist in research in this area (Chen *et al.* 2010).

It may seem logical to assume that chondrocytes would need to degrade the ECM immediately surrounding themselves to undergo the enormous increase in volume described as hypertrophy (Fig. 3), however, none of the enzymes known to both degrade the components of cartilage ECM and be expressed by hypertrophic chondrocytes are required for the process of hypertrophy. The chondrocytes of MMP13-null mice undergo apparently normal hypertrophy, indicating that collagen degradation is not necessary (Inada

et al. 2004, Stickens *et al.* 2004). Similarly, aggrecan degradation does not appear to be required. There are no morphological defects in the growth cartilage of mice in which aggrecan is rendered resistant to MMP cleavage (Little *et al.* 2005a). A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family of enzymes includes ADAMTS-1, -4 and -5, which are capable of cleaving aggrecan; mice lacking each of these individually (Little *et al.* 2005b, Stanton *et al.* 2005) or ADAMTS-4 and -5 in combination (Rogerson *et al.* 2008) show normal growth plate morphology. The results of the latter study indicate that an additional, as yet unidentified, aggrecan-degrading activity is present in mouse cartilage, thus a role for aggrecan cleavage in chondrocyte hypertrophy cannot yet be ruled out. It seems likely, however, that ECM degradation is not in fact required for chondrocytes to expand in volume. Observations on the role of hyaluronan in the growth plate may provide some insight into this problem of how the chondrocyte is able to expand so dramatically. Results of studies in which growth plate organ cultures were treated with hyaluronidase have provided evidence for a role for hyaluronan in the enlargement of the lacunae of hypertrophic chondrocytes (Pavasant *et al.* 1996). Moreover, in the *Has2*-null mice mentioned-above, as well as having reduced matrix volume, the growth plates show an almost complete failure of chondrocyte hypertrophy, based on both morphology and expression of hypertrophy-associated genes (Matsumoto *et al.* 2009). As noted by these authors, the role of hyaluronan may be to initiate hypertrophy-inducing intracellular signalling in chondrocytes emerging from the proliferative phase. Given the importance of hyaluronan-aggrecan complexes for swelling of the growth plate ECM, however, it is likely that this swelling *per se* is required for the concomitant expansion of the chondrocyte. Recently proliferated chondrocytes are flattened in the longitudinal axis of the growing bone, and the subsequent increase in cell volume is manifest as a disproportionate increase in height, relative to width of the cell, that is, in the direction of bone growth (Fig. 3; Breur *et al.* 1994). Perhaps the role of hyaluronan is to provide sufficient space between collagen fibrils to ensure that the chondrocyte can displace the surrounding matrix as it expands.

Just as proliferation of the growth plate chondrocyte is regulated, both positively and negatively, by systemic and locally produced extracellular molecules, so is the transition from the proliferative to the hypertrophic state. Thyroid hormones are important systemic regulators of chondrocyte hypertrophy (Shao *et al.* 2006). *In vitro*, the thyroid hormone triiodothyronine (T₃) stimulates morphological hypertrophy as well as molecular markers of hypertrophy, without stimulating proliferation (Burch & Lebovitz 1982, Bohme *et al.* 1992, Ballock & Reddi 1994, Wang *et al.* 2007). Hypothyroidism in humans results in slowing of longitudinal bone growth, with abnormally thin growth plates and impaired chondrocyte hypertrophy. Studies in genetically manipulated mice have demonstrated that the receptor

responsible for these effects is thyroid hormone receptor α (and not thyroid hormone receptor β ; Kaneshige *et al.* 2001).

The mediators of thyroid hormone-induced chondrocyte hypertrophy are starting to be identified, but the mechanism of this response has not yet been fully elucidated. One such pathway appears to be WNT/ β -catenin signalling. WNTs not only stimulate chondrocyte proliferation as noted above, but also promote chondrocyte hypertrophy (Enomoto-Iwamoto *et al.* 2002, Dong *et al.* 2006). A number of studies in genetically manipulated mice with either constitutive activation or depletion of components of the β -catenin signalling pathway have demonstrated that activation of this pathway promotes chondrocyte hypertrophy; thus, it appears most likely that the effect of WNTs on chondrocyte hypertrophy is mediated by the canonical WNT signalling pathway (Tamamura *et al.* 2005, Chen *et al.* 2008, Kawasaki *et al.* 2008). In chondrocytes *in vitro*, T_3 up-regulates *Wnt4* mRNA and protein expression as well as cellular accumulation of β -catenin, and inhibition of WNT signalling by secreted WNT antagonists inhibits T_3 -induced hypertrophy, providing strong evidence that WNT signalling contributes to thyroid hormone-induced hypertrophy (Wang *et al.* 2007). In addition, these effects of T_3 are partially inhibited by inhibitors of IGF1 signalling, indicating that IGF1 also makes some contribution to thyroid hormone-induced hypertrophy (Wang *et al.* 2010). T_3 also induces expression of FGFR3 in chondrocyte-like cells and the growth plates of thyroid hormone receptor α -null mice express substantially lower levels of FGFR3 than do those of wild-type animals (Barnard *et al.* 2005). Since activation of FGFR3 results in acceleration of hypertrophy, this receptor appears to be another mediator of thyroid hormone-induced chondrocyte hypertrophy (Minina *et al.* 2002, Ornitz 2005).

The IHH secreted by prehypertrophic chondrocytes is another factor that not only influences proliferation, but also hypertrophy, which it is generally considered to inhibit (Vortkamp *et al.* 1996, Maeda *et al.* 2007). This effect of IHH is mediated by Gli2, and Gli2-dependent stimulation of secretion of parathyroid hormone-related peptide (PTHrP; Vortkamp *et al.* 1996, Kronenberg 2006, Joeng & Long 2009). PTHrP suppresses hypertrophy, thus keeping the cells in a proliferative state (Lee *et al.* 1996). PTHrP exerts this effect at least in part through inhibition of expression of the transcription factor RUNX2; RUNX2 induces chondrocytic transcription of hypertrophy-associated genes including *col10a1* and promotes hypertrophy (Lefebvre & Smits 2005, Guo *et al.* 2006). The suppression of hypertrophy by PTHrP is probably also mediated by SOX9, which is activated by PTHrP through protein kinase A-dependent phosphorylation and indirectly inhibits RUNX2 expression (Huang *et al.* 2000, 2001, Akiyama *et al.* 2002, Yamashita *et al.* 2009). PTHrP stimulates cyclin D1 expression in chondrocytes, and is unable to down-regulate RUNX2 expression in chondrocytes from cyclin D1-null mice, apparently because cyclin D1 contributes to proteasomal degradation of RUNX2 (Beier *et al.* 2001, Zhang *et al.* 2009). The transcriptional co-regulator Zfp521,

which is induced by PTHrP, has also recently been identified as an effector of PTHrP's actions in the growth plate; the growth cartilage of mice with chondrocyte-specific deletion of Zfp521 resembles that of PTHrP-null mice, and PTHrP is unable to stimulate cyclin D1 expression or inhibit RUNX2 expression in the absence of Zfp521 (Correa *et al.* 2010).

Expression of PTHrP by growth plate chondrocytes is inhibited by TRPS1, as demonstrated by studies in TRPS1-null mice and accompanying *in vitro* studies (Nishioka *et al.* 2008). Mice with a heterozygous in-frame deletion of the DNA-binding domain of TRPS1 show elevated expression of RUNX2 in the growth plate; moreover, TRPS1 directly interacts with RUNX2 to inhibit its function (Napierala *et al.* 2008). These observations suggest that TRPS1 acts to fine-tune cell cycle exit and progression to the hypertrophic state, by limiting both the antihypertrophic activity of PTH and the pro-hypertrophic activity of RUNX2, while at the same time supporting proliferation in a cyclin D1-dependent manner (mentioned above in the section on proliferation; Suemoto *et al.* 2007).

In vivo studies in rats indicate that thyroid hormones suppress expression of both PTHrP and its receptor, thus providing another potential mechanism by which thyroid hormones induce chondrocyte hypertrophy (Stevens *et al.* 2000). It has recently been demonstrated that in the absence of PTHrP, IHH promotes chondrocyte hypertrophy, an effect that is likely to be mediated by WNT and BMP signalling pathways (Mak *et al.* 2008). Furthermore, complex interactions between the locally secreted factors that regulate chondrocyte behaviour in the growth plate include the fact that FGFs antagonise BMP-induced IHH expression, an effect that is relevant to the FGFs' effect on hypertrophy, but not proliferation (Minina *et al.* 2002, Retting *et al.* 2009).

Degradation and invasion of growth plate cartilage

Mineralisation

Hydroxyapatite crystals (composed primarily of calcium and phosphate) are deposited in the ECM surrounding late hypertrophic chondrocytes. The matrix vesicles released by these cells contain a combination of proteins including phosphate transporters, phosphatases and annexins and provide the nucleation site for mineralisation (Anderson 1969, Kirsch *et al.* 1997, Kirsch 2006). *In vitro* studies identified annexins 5 and 6 as calcium channels with a potential role in calcium deposition in matrix vesicles, however, the results of a recent study in mice lacking both of these proteins have ruled out an essential role for them in mineralisation of growth plate cartilage (Kirsch *et al.* 2000, Genge *et al.* 2007a,b, Belluoccio *et al.* 2010). It may be that other members of the annexin family expressed by hypertrophic chondrocytes compensate for the loss of these two proteins. The phosphatases PHOSPHO1 and tissue

non-specific alkaline phosphatase, both contribute to mineralisation of cartilage ECM. Recent studies involving individual and combined ablation of the genes encoding these phosphatases in mice suggest that PHOSPHO1 plays a role in the initiation of matrix vesicle mineralisation, while alkaline phosphatase activity is required for normal progression of mineralisation beyond the confines of the matrix vesicle (Fedde *et al.* 1999, Anderson *et al.* 2004, Yadav *et al.* 2011). The role of alkaline phosphatase activity in mineralisation in the growth plate is thought to be to remove extracellular pyrophosphate, which is a putative inhibitor of mineralisation (Kirsch 2006). Carminerin, a transcriptional inhibitor of nucleotide pyrophosphatase/phosphodiesterase 1, is also required for normal mineralisation of growth plate ECM; carminerin-null mice show no abnormality in growth plate morphology other than reduced mineralisation (Yamada *et al.* 2006). This observation suggests that growth plate mineralisation is not required for normal function of the growth plate itself. The importance of growth plate mineralisation may lie in provision of an appropriate composition for the remnants of cartilage matrix on which bone is deposited in the primary centre of ossification, since bone volume in the newly formed bone of the metaphysis is reduced in the absence of carminerin (Yamada *et al.* 2006).

Chondrocyte death

Most hypertrophic chondrocytes appear to undergo rapid death in the last row of lacunae before the ossification front. A number of publications have described these cells as dying by apoptosis, but the evidence for this conclusion is based on the detection of molecular features known to be associated with apoptosis, such as DNA strand breaks and caspase activation, rather than on the more definitive morphological changes observed on ultrastructural examination (Gibson 1998, Adams & Shapiro 2002, Correa *et al.* 2010). Cells undergoing apoptosis show intense condensation of chromatin into geometric shapes, and fragmentation of the nucleus and cytoplasm into membrane-bound apoptotic bodies (Kerr *et al.* 1972). A number of careful ultrastructural studies have failed to identify chondrocytes undergoing apoptosis in growth plates of several species (Roach & Clarke 1999, Colnot *et al.* 2001, Roach *et al.* 2004, Emons *et al.* 2009). In fact, light and dark hypertrophic chondrocytes appear to die by cell type-specific (non-apoptotic) mechanisms (Ahmed *et al.* 2007). Each of these cell types undergoes a distinctive series of morphological changes following hypertrophy: light chondrocytes appear to disintegrate within their cell membrane and dark chondrocytes progressively extrude their cytoplasm into the extracellular space. Nuclear condensation occurs very late and is irregular.

Matrix degradation

Degradation of the cartilage matrix surrounding growth plate chondrocytes does not appear to be required for hypertrophy,

as noted in the section on chondrocyte hypertrophy above, but it is required for invasion of the growth plate by the cells of the centre of ossification. In the lacunae closest to the ossification front, the transverse septa separating the chondrocytes from the invading cells are broken down, leaving many of the vertical septa intact to provide a scaffold for deposition of bone matrix (Fig. 2). Both the collagenase MMP13, expressed by hypertrophic chondrocytes, and MMP9, which cleaves denatured collagens and aggrecan and is expressed by cells within the ossification front, are required for normal growth plate cartilage removal (Vu *et al.* 1998, Inada *et al.* 2004, Stickens *et al.* 2004). It appears that the removal of collagen rather than of aggrecan is the limiting factor in this process, given the lack of an abnormal growth plate phenotype in mice with MMP-resistant aggrecan and mice lacking the aggrecan-degrading ADAMTSs (Little *et al.* 2005a,b, Stanton *et al.* 2005, Rogerson *et al.* 2008).

Osteoclasts at the ossification front are also required for normal growth plate cartilage matrix degradation, as demonstrated in mice treated with pharmacological inhibitors of osteoclast activity (bisphosphonates) and in osteopetrotic mouse mutants, which lack osteoclasts (Deckers *et al.* 2002). In the absence of osteoclastic activity, the growth plate is elongated because, with the exception of blood vessels, the cells of the ossification centre are unable to invade it (Deckers *et al.* 2002). Osteoclastic resorption of bone is primarily dependent on the lysosomal cysteine proteinase cathepsin K, but growth plate cartilage degradation is not dramatically altered by failure to express cathepsin K (Saftig *et al.* 1998). Since osteoclasts express MMP9, and there is abnormal retention of growth plate cartilage in MMP9-null mice, it appears that osteoclasts depend more on this enzyme than on cathepsin K for their degradation of cartilage matrix (Reponen *et al.* 1994, Vu *et al.* 1998).

Osteoclasts are not, however, the first cells to invade the growth plate. It has been noted that blood vessels appear to precede osteoclasts as the first cells that enter the lacunae recently vacated by dying hypertrophic chondrocytes, thus it is not particularly surprising that they are able to invade the growth plate in the absence of osteoclast activity (Schenk *et al.* 1967, Deckers *et al.* 2002). Another cell type that has recently been described as accompanying the blood vessels at the ossification front is the 'septoclast', a perivascular cell that expresses cathepsin B; it has been proposed that septoclasts assist in the degradation of the transverse cartilage septa, thus allowing entry of capillaries (Lee *et al.* 1995, Gartland *et al.* 2009).

Regulation of behaviour of invading cells

In addition to osteoclasts, septoclasts and capillaries and their contents, the invading cells of the primary ossification centre include osteoblasts and bone marrow cells. The osteoblasts deposit bone matrix on the remaining vertical septa of cartilage, resulting in the formation of the trabecular (spongy) bone of the metaphysis. A number of recent studies have

provided evidence that growth plate chondrocytes produce factors that regulate the behaviour of these invading cells (summarised in Fig. 4), thus presumably ensuring that the rate of replacement of cartilage by bone is matched to the preparation of the growth plate for invasion.

As chondrocytes undergo hypertrophy, under the control of RUNX2 they increase their expression of vascular endothelial growth factor, which promotes the vascular invasion of the growth plate (Zelzer *et al.* 2001, 2004). High-mobility group box 1 protein (HMGB1) is secreted by hypertrophic chondrocytes, and acts as a chemoattractant for endothelial cells, osteoclasts and osteoblasts; HMGB1-null mice display delayed invasion of the growth plate by cells of the ossification centre (Taniguchi *et al.* 2007). Another factor expressed by hypertrophic chondrocytes that promotes invasion by osteoclasts is receptor activator of NFκB ligand (RANKL), which is essential for osteoclast differentiation (Kishimoto *et al.* 2006). Hypertrophic chondrocytes stimulate osteoclast differentiation *in vitro* in a RANKL-dependent manner, and RANKL expression by cultured chondrocytes is stimulated by BMP2 and mediated by RUNX2 (Usui *et al.* 2008). A possible role for WNTs released by chondrocytes in increasing osteoblast numbers has been suggested by the finding that levels of β-catenin protein and markers of osteoblast differentiation are abnormally low in the primary spongiosa of mice with a postnatally induced chondrocyte-specific knockout of *IHH* (Maeda *et al.* 2007). Furthermore, evidence for regulation of osteoblast behaviour by growth plate chondrocytes has been provided by studies in mice expressing an activating *Fgfr3* mutant under the control of the *Col2a1* (chondrocyte-specific) or *Col1a1* (osteoblast-specific) promoter (Matsushita *et al.* 2009). Activation of FGFR3 signalling in chondrocytes, but not osteoblasts, caused an increase in osteoblast numbers at the chondro-osseous junction, an effect that is likely to be mediated by the stimulation of BMP ligand expression and inhibition of BMP antagonist expression observed in these mice (Matsushita *et al.* 2009).

Collectively, these observations indicate that chondrocytes of the growth plate release soluble factors that allow them to delicately control the behaviour of the invading vascular endothelial cells, osteoclasts and osteoblasts, which all have important roles in transforming growth plate cartilage into bone tissue.

Growth plate closure

As skeletal maturity approaches, the rate of advancement of the ossification front is greater than the rate at which growth plate chondrocytes replace themselves. The growth plate cartilage disappears as the primary centre of ossification meets the secondary centre and bony fusion occurs (Fig. 1). In human males as well as females, oestrogen regulates this process, as demonstrated by the failure of normal growth plate closure in patients with oestrogen deficiency or oestrogen resistance (Smith *et al.* 1994, Morishima *et al.* 1995). It is thought that growth plate closure occurs when the chondrocytes exhaust

their proliferative potential, and that the role of oestrogen is to accelerate this process of senescence (Weise *et al.* 2001). Oestrogen receptors expressed by growth plate chondrocytes are likely to mediate these effects (Nilsson *et al.* 2005).

Conclusion

As demonstrated in this review, the understanding of the role of the growth plate chondrocyte in endochondral ossification has increased enormously in recent years, largely as a result of the identification of gene mutations responsible for human chondrodysplasias, and discoveries making use of recent advances in gene manipulation technologies for *in vitro* and *in vivo* studies, supported by careful morphological characterisation. There is still much to learn about this process. The molecular bases of some chondrodysplasias remain undiscovered, and for many of those for which the responsible genes have been identified, the links between mutation and disease are incompletely elucidated. Although chondrocytes, the major structural components of the matrix they produce and components of the major signalling pathways that regulate their behaviour have been extensively studied, there are still many gaps in our knowledge. The process by which a single cell type, the chondrocyte, provides the impetus for the growth of rigid bones from embryonic life to adulthood is a fascinating area of study, which will no doubt keep us occupied for many years to come.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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