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Hypoxia, HIFs and bone development

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Abstract

Oxygen is not only an obviously important substrate, but it is also a regulatory signal that controls expression of a specific genetic program. Crucial mediator of the adaptive response of cells to hypoxia is the family of Hypoxia-Inducible Transcription Factors (HIFs). The fetal growth plate, which is an avascular structure of mesenchymal origin, has a unique out-in gradient of oxygenation. HIF-1 α is necessary for chondrogenesis *in vivo* by controlling a complex homeostatic response that allows chondrocytes to survive and differentiate in a hypoxic environment. Moreover, HIFs are also essential in osteogenesis and joint development. This brief Perspective summarizes the critical role of HIFs in endochondral bone development.

Introduction

In recent years, it has become increasingly clear that oxygen is not only an obviously important substrate, but it is also a regulatory signal that controls expression of a specific genetic program. Reduced availability of oxygen, or hypoxia, activates a transcriptional response that has an important role both in pathological conditions, such as ischemia or tumorigenesis, and in normal development [1,2].

Hypoxia, i.e. reduced availability of oxygen, is not an absolute but rather a relative concept. During development, all embryonic tissues experience a low oxygen tension before the circulatory system forms. This physiological hypoxia is essential for the vascularization of the placenta and of the embryo [3]. Once heart and blood vessels have formed and are functional, gradients of oxygenation are still present in the developing embryo [3]. The fetal growth plate is an outstanding example of how low availability of oxygen can activate a non-redundant genetic program with an essential homeostatic role during development. Hypoxia is also involved in the homeostasis of adult tissues that are physiologically hypoxic, like the articular joints. In this review, we will summarize the current knowledge of how the genetic program activated by hypoxia controls fetal growth plate development, osteoblast activity, and joint formation and homeostasis.

Hypoxia, HIFs and the others

Mammalian cells have developed sensors of low oxygen tension that trigger a complex, but elegant homeostatic response, which includes regulation of glucose utilization, iron transport, hematopoiesis, angiogenesis, cell survival and apoptosis [4]. The best characterized

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transcription factors involved in the cellular adaptation to hypoxia are the Hypoxia Inducible Transcription Factors (HIFs) [5].

HIF-1 is a heterodimer of two proteins, HIF-1 α and HIF-1 β ; HIF-1 β is constitutively expressed, whereas HIF-1 α is the hypoxic –responsive component of the complex. When oxygen drops below 5%, HIF-1 α translocates to the nucleus, heterodimerizes with the β subunit, and initiates its transcriptional program (Figure 1). Stability of HIF-1 α protein is hypoxia-sensitive, through oxygen-dependent hydroxylation of specific residues within its amino acid sequence. In particular, a family of HIF prolyl 4-hydroxylases is responsible for the hydroxylation of two proline residues (P402 and P564) in the oxygen dependent degradation domain (ODDD) of HIF-1 α [6]. The E3 ubiquitin ligase Von Hippel-Lindau (VHL) binds to the hydroxylated HIF-1 α and targets it to the proteasome for degradation [7,8]. In hypoxic conditions, hydroxylation of HIF-1 α does not occur, and the α subunit is free to migrate to the nucleus, bind its β counterpart, and thus act as a transcription factor along with other co-factors. An additional level of regulation mediated by cellular oxygen levels is given by the factor inhibiting HIF (FIH-1) [9], an asparagine hydroxylase that hydroxylates the N803 residue in the C-terminal transactivation domain of HIF-1 α [10,11]. In hypoxic conditions N803 is not hydroxylated by FIH-1; therefore, HIF-1 α can interact with its transcriptional coactivators, p300 and CBP (CREB binding protein), and hence further promote transcription of genes that have hypoxia responsive enhancer elements (HRE) in their promoter region [12,13]. Most of the genes regulated by hypoxia in a HIF-1 dependent fashion contain HRE sites in their promoter, and are activated through the mechanism described above. Interestingly, HIF-1 can interact with both the transcriptional coactivator histone acetyltransferase p300, and with histone deacetylases, like HDAC7 [14]. These findings might appear paradoxical at first glance, though it is not surprising that different target genes may require different cofactors.

Chromatin remodeling proteins are not only co-factors but also targets of HIF-1 action. Histone demethylases such as Jumonji-domain containing proteins (JMJ) are regulated by hypoxia in a HIF-1 dependent manner. JMJs are a class of enzymes that demethylate histone arginine and lysine residues through an oxidative reaction requiring the cofactors Fe(II) and α -ketoglutarate [15,16]. Recent findings have unveiled a role for JMJD1A in the upregulation of adrenomedullin and growth and differentiation factor 15 (GDF15) in hypoxia, effects which could ultimately positively modulate tumor growth [17].

Non-canonical modes of action of HIF-1 α , leading to down-regulation or repression of gene expression without direct interaction of HIF-1 α with HRE elements, have also been proposed. The “HIF-1 α -c-Myc” axis (reviewed in [18,19]) is a very interesting and intriguing example of HIF-1 α mediated, HRE-independent gene regulation. In particular, it has been reported that the PAS-B domain of HIF-1 α displaces c-Myc from specific promoter targets, and this results in significant changes of expression of c-Myc-dependent genes. An example of the dynamic interaction between HIF-1 α and c-Myc is the upregulation of the cell cycle checkpoint gene p21 in hypoxia [20]. p21 is encoded by a HRE deficient gene, and its expression is normally suppressed by c-Myc; however, in hypoxic conditions p21 mRNA and protein levels increase due to the HIF-1 α dependent displacement of c-Myc from its promoter region, which relieves the genetic repression [20].

Stimuli other than hypoxia also cause HIF-1 α to accumulate in normoxic cells, but the molecular mechanisms are not yet fully understood [21].

Notably, null mice lacking HIF-1 α die at very early stage of embryonic development, a finding which further proves the critical role of HIF-1 in development [22]. Other two HIF- α isoforms have been recently identified, HIF-2 α and HIF-3 α . The HIF-2 α isoform is regulated by oxygen with a modality very similar to HIF-1 α . However, the two α subunits differ in their

biological function. HIF-2 α and HIF-1 α have common targets as well as specific ones [23–26]. Moreover, they show different tissue localization: HIF-1 α is ubiquitous, whereas HIF-2 α can be found mainly in blood vessels, lung, kidney, interstitial cells, liver and neural crest [27]. Lastly, in some genetic backgrounds mice lacking HIF-2 α survive postnatally [28], where, as aforementioned, lack of HIF-1 α causes early embryonic lethality. Differently from HIF-1 α and HIF-2 α , the biological role of the HIF-3 α isoform is still largely unknown, though it has been proposed that this protein could have a dominant negative function, since it lacks the transactivation domains [29].

It is important to highlight that the hypoxic response is very complex, and it goes beyond stabilization and increased transcriptional activity of HIFs [30]. Along these lines, it is interesting to note that the genetic program activated by hypoxia also includes regulation of specific microRNAs. MicroRNAs are believed to play an important role in the down-regulation of genes in response to stress, and recently they have been described as active regulators of the HIF-mediated hypoxic response [31–33]. Even if microRNAs represent only 1% to 2% of the eukaryotic transcripts, they finely tune gene expression by regulating 30% of the coding mRNAs [34,35]. miRNAs are small non-coding RNAs of 19–24 nucleotides long, transcribed by RNA polymerase II and processed by Drosha and Dicer, a class of RNase III enzymes; they act as posttranscriptional repressors, by pairing to the 3' untranslated region of the target mRNA and degrading it or inhibiting its translation. In recent years their importance in many pathologies, like cancer, as well as in development has been unveiled [36,37]. Striking correlations have been found between microRNA expression and hypoxia [32], particularly in regard to miR-210 [38–40] and miR-21 [41]. Not surprisingly, microRNAs that specifically target HIF-1 α expression, like the miR-17-92 cluster and miR-199a, have also been identified [42,43].

HIFs and endochondral bone development

Bone can form through two different mechanisms, intramembranous and endochondral. While the flat bones of the skull develop from mesenchymal cells that directly differentiate into osteoblasts (intramembranous bone formation), the other skeletal bones are derived from the replacement of a chondrocyte anlage by bone, according to a very well-defined temporal and spatial pattern [44–47]. This latter process is called endochondral bone development [44–47], and an increasing body of evidence has highlighted the essential role of the genetic program turned on by hypoxia in regulating it [48]. Chondrocytes in the fetal growth plate synthesize collagen type II, are highly proliferative, and while they divide they also pile up to form a columnar layer (Figure 2). The most distal cells of the columnar layer stop proliferating, exit the cell cycle, and differentiate into hypertrophic chondrocytes, which produce collagen type X and mineralize their surrounding matrix (Figure 2). Programmed death of the hypertrophic chondrocytes allows blood vessels invasion, and replacement of cartilage by bone, the so-called *primary spongiosa* (Figure 2). The fetal growth plate is a unique mesenchymal tissue, since it is avascular for most of its length, though it does require the angiogenic switch in order to be replaced by bone. Consistent with its avascularity, the fetal growth plate contains hypoxic regions. As shown by immunohistochemistry for the EF5, a bio-reductive marker of hypoxia, the more hypoxic chondrocytes are found in the round proliferative layer, especially near the joint space, in the center of the columnar layer and the upper part of the hypertrophic zone, whereas the late hypertrophic chondrocytes at the border with the *primary spongiosa* are not hypoxic [49] (Figure 2). This specific spatial distribution of hypoxic areas is consistent with both the avascularity of cartilage and, conversely, the extensive vascularization of the surrounding soft tissue and of the *primary spongiosa*.

In the last few years, numerous studies have been conducted in the attempt to understand how the genetic program controlled by hypoxia modulates endochondral bone formation. The use

of genetically modified mice turned out to be a successful and informative strategy in elucidating the role of HIFs in growth plate and bone development. In vitro studies with primary chondrocytes or chondrogenic cell lines maintained in hypoxia, and/or in which the HIF pathway had been genetically or pharmacologically modified, further helped in dissecting out the complex interactome controlled by hypoxia in the developing bone. Taken together, an essential and non-redundant role of the HIFs in endochondral bone development has emerged. It has yet to be established, though, whether HIFs are not only necessary but also sufficient for cartilage formation, at least in specific settings in vivo. Notably, other genes, such as CCN2/CTGF and PTEN [50,51], have been shown to cooperate with HIF-1 in cartilage development, contributing to form a network of actions that is destined to expand in the future.

HIF-1 α and chondrocyte survival

Conditional deletion of HIF-1 α in limb bud mesenchyme or in chondrocytes, achieved by the use of the Cre-loxP strategy, has demonstrated that HIF-1 α is necessary for chondrogenesis *in vivo* by turning on a complex homeostatic response that allows chondrocytes to survive and differentiate in a hypoxic environment.

Lack of HIF-1 α causes massive cell death of the inner chondrocyte layer in the developing growth plate [49,52]. Numerous molecular mechanisms could be invoked as downstream mediators of HIF-1 α survival function. Consistent with its hypoxic status, the fetal growth plate lives on anaerobic glycolysis [49]. Notably, in HIF-1 α null growth plates, level of phosphoglycerate kinase (PGK-1), a key enzyme in anaerobic glycolysis and a classical downstream target of HIF-1 α , are extremely low, which suggests that anaerobic glycolysis could be affected in mutant chondrocytes [49].

In addition to metabolism, impaired up-regulation of vascular endothelial growth factor (VEGFA), another well-characterized downstream target of HIF-1 α , could be responsible, at least in part, for the massive cell death phenotype observed in HIF-1 α null growth plates. VEGF-A mRNA is expressed not only in late hypertrophic chondrocytes, where it is important for replacement of cartilage by bone [53–55], but also, although to a lesser extent, in the center of the proliferative layer and in the upper hypertrophic zone, i.e. in the hypoxic regions of the growth plate [56]. Notably, conditional knockout of VEGF-A in chondrocytes causes cell death in the center of mutant growth plates, indicating that VEGF-A is critical for chondrocyte survival [53]. This cell death is similar to the phenotype observed in chondrocytes lacking HIF-1 α , which suggests that HIF-1 α and VEGF-A could be part of a common pathway that supports chondrocyte survival in endochondral bone development. However, it is intriguing that VEGF-A mRNA is up-regulated in the viable chondrocytes surrounding the area of cell death in HIF-1 α null growth plates, raising questions about contribution of this growth factor to the overall HIF-1 α phenotype [49]. Moreover, both receptors for VEGF-A, VEGFR2 and VEGFR1, are not expressed in the fetal growth plate [53], whereas the co-receptors neuropilin-1&2 and VEGFR3, which does not bind VEGF-A [57], are [53]. Taken altogether, these findings imply that either VEGF-A produced by hypoxic chondrocytes diffuses out of the growth plate and controls angiogenesis in the surrounding soft tissue, or that this growth factor acts locally in chondrocytes through an autocrine/intracrine mechanism that does not require either VEGFR1 or VEGFR2. Additional studies are needed to reach a better understanding of these apparent paradoxes. More in general, it is intriguing that the proliferative layer of the fetal growth plate, despite expressing VEGF-A, is avascular, and that up-regulation of VEGF-A expression, as it occurs in mice lacking VHL in chondrocytes or overexpressing one of the VEGF isoforms (VEGF₁₆₄), does not cause ectopic vessel formation [56,58–60]. To date, we do not have a definitive explanation for this additional paradox, though slow remodeling of the extracellular matrix could be responsible for reduced availability of the active form of VEGF-A, and, therefore, for the intrinsic resistance of cartilaginous matrix

to be invaded by blood vessels [55,61,62]. Among the survival pathways controlled by HIF in chondrocytes, the role of autophagy is still ambiguous. It has been recently shown that autophagy is constitutively on in growth plate chondrocytes. Interestingly, hypoxia may trigger autophagy, with mechanisms that may or may not involve the transcription factor HIF-1 α [63–66]. Recently, in vitro evidence in support of a role for autophagy in chondrocyte survival has been provided [67]. Moreover, a relation between HIF-1 α and increased accumulation of autophagic proteins such as Beclin1 has been documented, at least in vitro [68]. Intriguingly, HIF-2 α , which is also expressed in chondrocytes, has been reported to be a suppressor of autophagy in vitro [69].

Taken together, it is possible that one of the mechanisms adopted by HIF-1 α to allow survival of chondrocytes in a hypoxic environment could indeed involve modulation of the autophagic process.

HIF-1 α and chondrocyte proliferation

HIF-1 α also modulates chondrocyte proliferation. In fetal growth plates deficient in HIF-1 α , the proliferation rate of viable chondrocytes is strikingly increased [49]. Conversely, chondrocyte proliferation rate is markedly reduced in mice that lack VHL in chondrocytes, which is concomitant with an increase of the cyclin-dependent kinase inhibitor p57 mRNA [56]. These latter findings are consistent with the notion that hypoxia leads to cell cycle arrest [70], at least in part through up-regulation of HIF-1 α transcriptional activity and inhibition of c-MYC activity, as reported above [20]. Overexpression of HIF-1 α in a model of fibrosarcoma derived by fibroblasts lacking VHL, also shows diminished proliferation rate due to an increase in the cyclin-dependent kinase inhibitors p21 and p27 [71]. Slow proliferation in VHL null chondrocytes and fibroblasts is paradoxical in light of the very well-known role of VHL as tumor suppressor, and more studies are necessary to reach a better understanding of these apparent incongruent findings.

HIF-1 α and chondrocyte differentiation

HIF-1 α is not required for the formation of precartilaginous condensation [52,72], but has a non-redundant and critical role in the differentiation of mesenchymal cells into chondrocytes. Lack of HIF-1 α in limb bud mesenchyme causes a remarkable delay in cartilage formation [52,72]. Further studies are required in order to understand whether hypoxia per se is needed for differentiation of mesenchymal cells into chondrocytes in vivo, or whether the critical role of HIF-1 α in early chondrogenesis is essentially homeostatic.

In any event, the involvement of HIF-1 α in cell differentiation is tissue-specific, because HIF-1 α maintains stem cells in an undifferentiated state [23,73–77], inhibits differentiation of mesenchymal cells into osteoblasts, adipocytes and myocytes [23,78–80], yet stimulates differentiation of trophoblastic cells and dopaminergic neurons and chondrocytes [23,81–83]. HIF-1 α may positively modulate chondrogenesis by up-regulating expression of Sox9 [52, 84], a master regulator of chondrogenesis [47,85–87]. In mouse bone marrow stromal (ST2) cells, in particular, hypoxia increases nuclear accumulation of HIF-1 α and Sox9 transcription [84]. Similar findings have been reported in limb bud micromass cultures [52], but have not been confirmed in primary chondrocytes or in ex-vivo metatarsal explants [72].

HIF-1 α and the cartilaginous matrix

Recent experimental evidence has indicated that regulation of post-translational modification of collagens, with hydroxylation of collagen prolines in particular, could be one of the modalities by which HIF-1 α regulates chondrocyte survival and differentiation. Prolyl-4-hydroxylases I and II (P4HaI and P4HaII) are the enzymes responsible for generating 4-hydroxyprolines in the collagens; these are essential for the formation of triple-helical collagens

[88]. P4Has have much lower K_m for O_2 than the PHDs, which trigger HIF-1 α degradation (20 vs. 250, respectively)[88]. This indicates that P4Has require a minimal amount of O_2 for proper function, i.e. they still function enzymatically at low O_2 levels. The alpha subunits of P4HaI and P4HaII are targets of hypoxia in chondrocytes and other cell types in a HIF-1 α -dependent fashion. Proper accumulation of extracellular matrix is not only essential for organ development, but also promotes cell differentiation and survival through specific cell-matrix interactions [89]. HIF-1 α may thus operate as a survival and differentiation factor in chondrocytes, improving the efficiency of post-translational modifications of collagen type II and, in so doing, promoting the formation of a proper extracellular matrix.

The positive effect of HIF-1 α on matrix accumulation in chondrocytes is consistent with the role of hypoxia in promoting fibrosis in pathological conditions. Moreover, it has been reported that lysyl oxidase, which is responsible for the formation of cross-links between collagen molecules, is induced by hypoxia and is essential for metastasis of highly malignant and hypoxic tumors [90,91].

HIF-1 α , chondrocytes and microRNAs

Not surprisingly, microRNAs have been recently involved in chondrocyte differentiation [92–94]. In particular, mouse genetics has demonstrated that Dicer is necessary for chondrocyte proliferation and for overall skeletal development [95]. Notably, mir-199a, has been independently implicated in both chondrogenesis and hypoxic response. Mir-199a down regulates HIF-1 α and, inhibits early chondrogenesis by targeting Smad1 [43,92,93]. To this end, it is unknown whether mir-199a regulates chondrogenesis also by modifying HIF-1 α activity.

HIFs and osteogenesis

As hypertrophic chondrocytes in the developmental growth plate undergo apoptosis, blood vessels invade the hypertrophic cartilage and the primary ossification center begins to form. A tight coupling between osteogenesis and angiogenesis is therefore essential for bone formation, and the genetic program regulated by hypoxia considerably impacts this mechanism. Notably, whereas cartilage is an avascular and hypoxic mesenchymal tissue [48,49,58,72,96], bone is highly vascularized, though the bone marrow is relatively hypoxic when compared to other adult organs (see below) [23].

Osteoblasts express HIF proteins and, not surprisingly, hypoxia stimulates VEGF-A mRNA production in this cell type [97]. If the HIF pathway is manipulated in osteoblasts, bone is deeply affected, both in volume and in vascular architecture [98,99]. The conditional upregulation of HIF proteins in osteoblasts, obtained by the deletion of VHL in osteocalcin expressing cells (Δ VHL), leads to an increase in VEGF-A protein. Strikingly, bone mass in the long bones of Δ VHL mice is augmented and this is mainly due to an increase in osteoblast number and in bone formation rate, and not to an impairment of osteoclast number or activity [98]. Conversely, a considerable decrease of both bone volume and VEGF-A expression is observed if HIF-1 α or HIF-2 α are disrupted in osteoblasts (Δ HIF-1 α and Δ HIF-2 α) [98,99]. Consistent with the VEGF-A data, a striking correlation between degree of ossification and vascularization, both in the Δ VHL and Δ HIFs has been identified, which imply that changes in bone volume and architecture observed in these mutant mice could be indeed secondary, at least in part, to changes in vasculature. The pericytic mesenchymal stem cells- like cells (MSCs) could indeed represent the physiological link between osteogenesis and angiogenesis. These cells reside in the bone marrow vascular niche, have osteogenic potential and may account for the increased osteoblast number in highly vascularized ossification centers [100,101].

In bone repair, angiogenesis and osteogenesis coupling also plays a pivotal role. At fracture sites, inflammatory signals and mechanical stimuli, along with hypoxia, are the key factors that stimulate the physiological processes involved in bone repair. Angiogenesis in this setting is essential and, if it is delayed, chondrocytes and not osteoblasts will constitute the new tissue [102]. An interesting model to study bone repair is distraction osteogenesis (DO). In DO a close temporal and spatial relationship between angiogenesis and osteogenesis is established, with an external fixation device that gradually applies a mechanical separation across the fracture [103]. Interestingly, in DO all the expression of the three VEGF isoforms and both VEGFR-1 and VEGFR-2 mRNAs are enhanced, and if VEGFR-1 and VEGFR-2 are depleted, there is a considerable decrease in blood vessel number and, remarkably, in bone repair [104]. Notably, lack of VHL considerably accelerates bone repair in a DO model, and this is parallel to a significant increase in blood vessels [105].

Despite all these experimental evidence that invoke a critical role for angiogenesis in the increased bone volume observed when the HIF-1/VEGF pathway is up regulated, it is likely that cell autonomous mechanisms are as important. In support of this alternative mechanism, osteoblast lacking HIF-1 α show impaired proliferation *in vitro*, whereas lack of HIF-2 α does not affect osteoblast function *in vitro* [99]. Moreover, osteoblasts isolated from a mutant mouse expressing only the VEGF₁₂₀ isoform do not properly differentiate *in vitro* [106]. Lastly, overexpression of the isoform VEGF₁₆₅ in osteoblasts results in an increase of bone mass, and this effect appears to be secondary to activation of beta catenin in osteoblasts [58]. Curiously, intermittent hypoxia inhibits Runx2, the master transcription factor of osteoblastogenesis [107], in osteoblasts *in vitro* [78], a paradox requiring further investigation. And talking about paradoxes, whereas endochondral bone is clearly affected by lack of VHL or HIFs, intramembranous bone is definitively less so [98].

HIFs and joints

The synovial joint is an avascular tissue both in development and in adult life [108]. It experiences a high degree of hypoxia and the HIF proteins and VEGFA are highly expressed at this level [72]. Moreover, hypoxia has been involved in the pathogenesis of osteoarthritis (OA) [109].

In agreement with these findings, mice lacking HIF-1 α in limb bud mesenchyme delays joint development with yet unknown mechanisms [72]. Notably, hypoxia upregulates matrix accumulation in Sox9-dependent fashion in articular surface chondrocytes *in vitro* [110,111]. The relevance of this finding for articular cartilage surface homeostasis *in vivo* needs to be established.

Conclusions

In this brief Perspective, we have highlighted the critical role of hypoxia and HIFs in chondrocyte survival and differentiation, in bone development and in joint formation and homeostasis. It will be now important to identify the molecular mechanisms that mediate the complex and multifaceted action of this family of transcription factors in each of these processes. The identification of such mechanisms may significantly expand our understanding of both cellular adaptation to hypoxia and cartilage formation, bone modeling and remodeling and articular surface homeostasis.

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Figure 1. Diagram of the HIF-1 α pathway

In normoxia prolyl-hydroxylases (PHDs) hydroxylates prolines 402 and 564 in the O₂ – dependent degradation domain (ODDD) of HIF-1 α , leading to its proteosomal degradation mediated by the von Hippel-Lindau (VHL) ubiquitin ligase. Also the factor inhibiting HIF (FIH) can hydroxylate HIF-1 α on asparagine 803 in its carboxy-terminal transactivation domain (C-TAD), leading to transcriptional inactivation. When HIF is stabilized in normoxia, a plethora of genes that contain hypoxia responsive elements (HRE, 5'-RCGTG-3') are expressed. Among the hypoxia-induced genes there are microRNAs, genes involved in metabolism, angiogenesis, erythropoiesis, chondrocyte differentiation and collagen matrix.

Figure 2. Schematic representation of the fetal growth plate

Mesenchymal cells differentiate into round proliferative chondrocytes and when they divide are piled up in a layer of columnar proliferative chondrocytes. Both layers produce collagen type II. The latest chondrocytes are the hypertrophic ones that express collagen type X and mineralize their matrix. When hypertrophic chondrocytes undergo apoptosis, the space is invaded by blood vessels and colonized by osteoblasts, which produce the primordial trabecular bone, also called *primary spongiosa*. The most hypoxic areas in the growth plate are shown in red.