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PROSPECT

Expression of Bone-Specific Genes by Hypertrophic Chondrocytes: Implications of the Complex Functions of the Hypertrophic Chondrocyte During Endochondral Bone Development

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Abstract Endochondral bone formation is one of the most extensively examined developmental sequences within vertebrates. This process involves the coordinated temporal/spatial differentiation of three separate tissues (cartilage, bone, and the vasculature) into a variety of complex structures. The differentiation of chondrocytes during this process is characterized by a progressive morphological change associated with the eventual hypertrophy of these cells. These cellular morphological changes are coordinated with proliferation, a columnar orientation of the cells, and the expression of unique phenotypic properties including type X collagen, high levels of bone, liver, and kidney alkaline phosphatase, and mineralization of the cartilage matrix. Several studies indicate that hypertrophic chondrocytes also express osteocalcin, osteopontin, and bone sialoprotein, three proteins which until very recently were widely believed to be restricted in their expression to osteoblasts. Recent studies suggest that the hypertrophic chondrocytes are regulated by the calcitropic hormones, morphogenic steroids, and local tissue factors. These considerations are based on the regulation by $1,25(OH)_2D_3$ and retinoids of the cartilage specific genes as well as osteopontin and osteocalcin expression in hypertrophic chondrocytes. They are also based on the effects on growth plate development caused by 1) transgenic ablation of autocrine/paracrine regulators such as PTHrP and of the transcriptional regulator c-fos and 2) naturally occurring genetic mutations of the FGF receptor. These studies further suggest that specific transcriptional factors mediate exogenous regulatory signals in a coordinated manner with the development of bone. While it has been widely demonstrated that the majority of hypertrophic chondrocytes undergo apoptosis during terminal stages of the developmental sequence, their response to specific exogenous regulatory signals and their expression of bone-specific proteins give rise to questions about whether all growth chondrocytes have the same developmental fates and have identical functions. Furthermore, specific questions arise as to whether there are similar mechanisms of regulation for commonly expressed genes found in both cartilage and bone or whether these genes have unique regulatory mechanisms in these different tissues. These recent findings suggest that hypertrophic chondrocytes are functionally coupled during endochondral bone formation to the recruitment of osteoblasts, vascular cells, and osteoclasts.

Key words: hypertrophic chondrocytes, endochondral development, bone gene expression, cartilage, osteoblasts

Much of skeletal formation during embryogenesis and postnatal growth occurs by a process referred to as the endochondral sequence. This is a developmentally regulated process which occurs in a highly coordinated temporal and spatial manner, in which there is a sequential recruitment and differentiation of cells which form cartilage, vascular, and bone tissues. During the initial phases of this process, mesenchymal stem cells are recruited and then condense and differentiate into chondrocytes forming an initially unmineralized and avascular cartilage model of a developing bone. Subsequently, the chondrocytes within this cartilage model further differentiate, and the matrix in specific areas mineralizes. As mineralization proceeds, vascularization of the tissue is initiated with blood vessels accompanied by undifferentiated mesenchymal cells entering the hypertrophic

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chondrocyte lacunae. The chondrocytes within the lacunae die; bone is synthesized by the perivascular cells on the calcified cartilage cores, and metaphyseal remodeling occurs as the mineralized extracellular matrix of both bone and cartilage is resorbed and replaced with bone alone [Bidder 1906; Ham and Cormack, 1979; Holtrop 1972; Hunziker et al., 1984; Shapiro et al., 1977].

Numerous research studies have focused on the morphological and biochemical phenotypic changes of cartilage tissue during the process of endochondral bone formation. In particular, the morphological characteristics of the endochondral process in long bone epiphyseal growth plates of vertebrates have been thoroughly studied [Buckwalter et al., 1986; Hunziker et al., 1984; Farnum and Wilsman, 1987], and results from such studies have been extrapolated to aspects of the endochondral sequence of bone formation in general. The growth plates have been characterized as a series of morphologically discrete zones progressing from the epiphyseal to the metaphyseal and diaphyseal regions. As the cells progress from the resting or germinal zone at the epiphyseal surface to the proliferating and pallisading zone and then towards the hypertrophic zone, the chondrocytes become progressively larger, display more mitoses, and are more active metabolically [Breur et al., 1991; Farnum and Wilsman, 1993]. Each of the triad of increased proliferation, increased extracellular matrix (ECM) biosynthesis, and increased cellular size contributes to the overall growth in the length and shape of a given bone [Breur et al., 1991; Hunziker and Schenk, 1989]. Cross-species comparisons have demonstrated that in mammals the relative contribution of increased cell volume is the major factor related to long bone growth, while in avian long bones both increases in cell number and cell size share an almost equal role in bone elongation [Baretto and Wilsman, 1994]. During postembryonic growth, which occurs in the epiphyseal regions of bones, the contributory roles of these developmental changes involving shaping and growth in skeletal tissue have been well characterized. However, whether the various contributory roles that proliferation, morphological change, and extracellular matrix deposition play during embryologic, fetal, and postnatal skeletal formation, in non-long bone formation, or in postnatal fracture repair mediated by the endochondral sequence are the same has as yet not been as clearly defined.

Examinations of the biochemical and biosynthetic changes during endochondral development have focused primarily on the gene expression and synthesis of the major extracellular matrix proteins in cartilage. Collagen gene expression and synthesis have been intensively studied because of the large quantities of these proteins in the cartilage extracellular matrix. At least three collagen types (II, IX, and X) are found predominantly in cartilage; these have been examined both in vivo and in vitro during the endochondral sequence [Gerstenfeld et al., 1989; Leboy et al., 1989; Nishimura et al., 1989; Poole et al., 1989; Schmid and Linsenmayer, 1985; Tacchetti et al., 1987]. Collagen type X has been localized immunologically and biochemically only within those areas of cartilage where the chondrocytes are hypertrophic and there are dense foci of mineralization. Other studies have shown that the expression of type X is under tight developmental regulation [Gerstenfeld and Landis, 1991; Leboy et al., 1989; Schmid and Linsenmayer, 1985; Tacchetti et al., 1987]. Such characteristics suggest that collagen type X may be important in either mineralization, matrix resorption, or vascular invasion of the hypertrophic tissue [Gerstenfeld and Landis, 1991]. Collagen types II and IX, on the other hand, are expressed throughout the endochondral developmental sequence, including areas of chondrocyte proliferation in the growth plate as well as articular cartilage which persists in the mature animal [Nishimura et al., 1989; Gerstenfeld et al., 1989; Leboy et al., 1988]. Other phenotypic characteristics associated with hypertrophic chondrocytes are the expression of high levels of alkaline phosphatase and the accumulation of type II C-pro-peptide (chondrocalcin) in the extracellular matrix [Poole et al., 1989].

Numerous studies examining the expression and turnover of the large proteoglycan aggrecans have also been carried out. Decreased proteoglycan content and reduced proteoglycan synthesis in zones of cartilage hypertrophy and calcification have been reported [Buckwalter 1983; Campo and Romano, 1986; Ehrlich et al., 1982; Poole et al., 1982]. While such data suggest that matrix remodeling in the hypertrophic zone is mediated by proteolytic turnover of the proteoglycan [Poole et al., 1982], results from studies in vitro indicate that matrix composition is also regulated by quantitative changes in the

ratio of matrix components that are synthesized by the chondrocytes [Gerstenfeld and Landis, 1991]. Other data may imply this same possibility since morphometric analysis of growth cartilage cell and matrix volumes demonstrated an increased matrix volume in the hypertrophic zones [Buckwalter et al., 1986]. Moreover, biochemical studies have shown quantitative losses of proteoglycans in hypertrophic zones that are not associated with qualitative changes in either their size, aggregation with hyaluronic acid, or immunoreactivity [Poole et al., 1982]. Thus, factors that primarily affect one of the three components controlling growth cartilage development (cell proliferation, morphological maturation, or extracellular matrix formation) probably have reciprocal effects within the cartilage on each of the other components in both normal and pathologic states.

Recent studies have demonstrated that cells within areas of mineralized cartilage share many molecular characteristics with osteoblasts and express a subset of common gene products including alkaline phosphatase [Leboy et al., 1989; Gerstenfeld and Landis, 1991], osteopontin [Ilwamoto et al., 1993; Lian et al., 1993; McKee et al., 1992; Strauss et al., 1990; Takahashi et al., 1992], bone sialoprotein [Bianco et al., 1991], and osteocalcin [Kesterson et al., 1993; Lian et al., 1993; Neugebauer et al., 1995; Strauss et al., 1990]. An explanation of the presence of these proteins in these very different tissues is probably related to their similar functions at the molecular level within the ECM in both the processes of mineralization and resorption. However, the expression of these proteins by cells within growth cartilage tissue has raised a number of questions about the nature of the cells which are producing them. The first set of questions revolves around the developmental origins of these cells. Three potential hypotheses can be put forward: 1) the "transdifferentiation" of cells in the growth cartilage sequence to an osteoblastic phenotype [Kahn and Simmons, 1977; Roach, 1992; Thesingh et al., 1991] (such a process implies the dedifferentiation of a chondrocyte to an early lineage stage followed by redifferentiation to an osteoblast; 2) the presence of a unique branch of the skeletal lineage that has both cartilage and bone phenotypes [Silberman et al., 1983]; and 3) a continued progression of the growth chondrocyte lineage to an end stage that normally synthesizes these genes [Cancedda et al., 1992; Manduca et al., 1992; Galotto et al., 1994; Lian et al., 1993] (Fig. 1). Morphological data clearly indicate that the majority of terminal growth chondrocytes undergo cell death, although whether this is by apoptosis is not definitively known [Farnum and Wilsman, 1989; Yoshioka and Yagi, 1988]. Therefore, the former two hypotheses would be supported only if a subpopulation of the total population of cells in a growth cartilage expressed the bone-specific gene products. In contrast, the third hypothesis could be consistent with both the whole population or a fraction of the whole population of growth chondrocytes expressing these phenotypic properties. Data that would support the latter two hypotheses but not transdifferentiation would involve definitive determination of the overlapping expression of the hypertrophic cartilage phenotype such as type X collagen with specific properties such as the expression of osteopontin, bone sialoprotein, or osteocalcin. At this point, however, such experiments have not been performed.

The other major question related to expression of osteopontin, bone sialoprotein, and osteocalcin by cells within the cartilage growth plate is whether genes for these proteins are regulated in response to the systemic calcitropic hormones or local autocrine/paracrine factors in the same fashion in both cartilage and bone. Owing to the very different functions of these two tissues, it seems reasonable to speculate that these tissues do indeed regulate their common genes in very different fashions. Growth cartilage is a transient tissue that serves a developmental growth and scaffold function within embryonic and immature animals, while bone has definitive structural and metabolic functions related to its role in mineral homeostasis once it is synthesized. Data supporting this contention are from studies demonstrating that hypertrophic chondrocytes regulate these bone-specific genes in response to $1,25(OH)_2D_3$ [Schwartz et al., 1989; Gerstenfeld et al., 1990] in a fashion more consistent with that seen for immature osteoblasts or even in a different fashion; indeed, the expression of type I collagen is stimulated by $1,25(OH)_2D_3$ in chondrocytes in comparison to the inhibitory effects that this hormone has on this gene in osteoblasts [Gerstenfeld et al., 1990; Broess et al., 1995]. Other studies also show that the addition of retinoic acid promotes the further maturation of hypertrophic chondrocytes with the expression of bone-related genes including osteopontin and

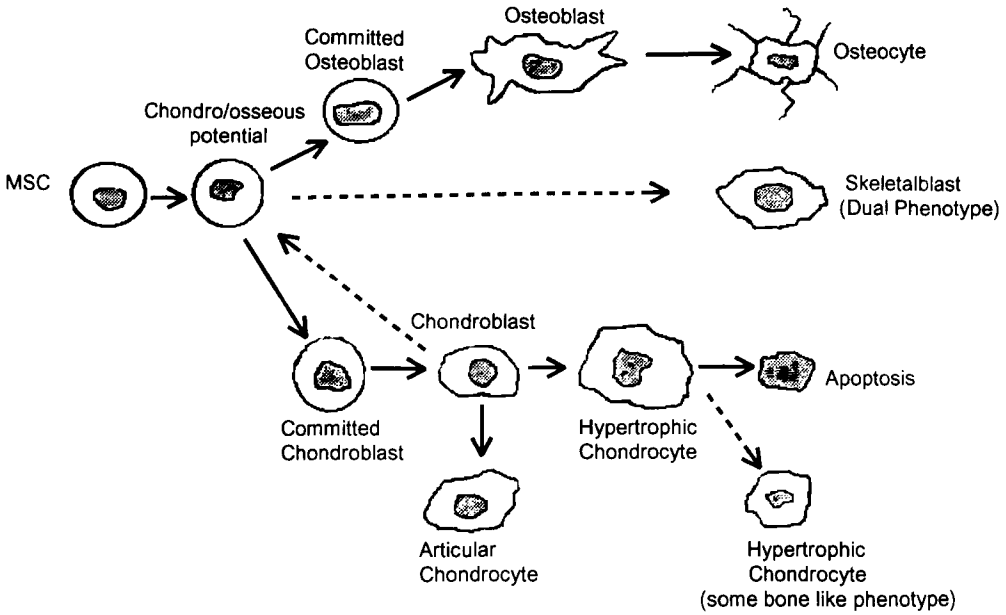

Fig. 1. Schematic diagram summarizing known and possible lineage progressions of the undifferentiated mesenchymal stem cells (MSC) including the hypertrophic chondrocytes which express an osteogenic phenotype. The top progression denotes the well-established differentiation to committed osteoblast, osteoblast, and osteocyte. The next includes a separate progression from a skeletal lineage stem cell to a unique cell type designated a skeletalblast [Silbermann et al., 1983] which has the dual potential of expressing both endochondral cartilage and osteogenic phenotypes. The final lineages include cell progression of a committed chondroblast to chondroblast, chondrocyte, and then to the hypertrophic cell which has an end stage with osteoblast-like functions. This end stage cell could either have continued viability within the growth plate or become a terminal apoptotic cell normally seen within the hypertrophic and mineralizing zones of the growth plate. A possible progression is that of transdifferentiation which implies a chondrocyte differentiation, dedifferentiation and redifferentiation to an osteoblast. A subset of chondrocytes (bottom progression) differentiates to articular chondrocytes. The possible origins of osteogenic cells in endochondral tissues are denoted by the dotted lines.

osteonectin with downregulation of type II collagen and upregulation of type X collagen [Ilwamoto et al., 1993]. These results suggest that either systemic or local effectors of mineral homeostasis and tissue morphogenesis are also specifically targeted to the developmental aspects of endochondral differentiation. Consistent with this suggestion are the studies [Jacenko and Tuan, 1986] of calcium deficiency in avian embryos lacking shells that resulted in the development of cartilage tissue within the calvaria. Such data clearly indicate that systemic calcium deficiency has an effect on the skeletal lineage progression during embryogenesis.

Considerable data have accumulated, from naturally occurring pathological conditions such as rickets and from genetically engineered defects that affect growth cartilage development, that provide strong evidence that growth cartilage differentiation is functionally coupled to the progression of bone development. While the vitamin D–deficient-induced rickets results in osteomalcia in adult animals, in growing animals its major pathological manifestation is a failure of mineralization and replacement of the cartilage growth plate associated with a lack of progressive osteogenic differentiation [Cole et al., 1989]. Extensive new data have emerged with the use of genetic engineering that provide further clues to the functional coupling between cartilage and bone development during embryogenesis. In studies of the effects of the ablation of the PTHrP (parathyroid hormone related peptide) gene, an osteochondrodsyplasia, primarily manifested as an acceleration in chondrocyte hypertrophy [Karaplis et al., 1993], was observed that provides further evidence that local autocrine/paracrine regulators of mineral homeostasis may play a crucial role in embryonic development. In transgenic animals in which c-fos expression was ablated, an osteochondrodysplasia was observed that is qualitatively different than that seen for the ablation of PTHrP. Unlike the PTHrP ablated animals, which had a premature replacement of the growth cartilage, c-fos ablation led to an overproduction of hyper-

trophic cartilage tissues and a failure of bone replacement producing an embryonic osteopetrotic-like condition [Wang et al., 1992]. In the naturally occurring genetic defect that causes human achondroplasia there is a mutation in FGF (fibroblast growth factor) receptor 3 (FGFR3) which results in the absence of the hypertrophic zones in the growth plate, although in this defect normal bone formation and remodeling occur [Shiang et al., 1994]. Finally, numerous reports demonstrate that bone morphogenetic factors or the implantation of skeletogenic precursor cells invariably lead to an endochondral sequence of bone development, while a direct recruitment of osteogenic precursors with the direct formation of membranous bone is almost never observed by itself [Wozney et al., 1988; Reddi and Cunningham, 1993; Baylink et al., 1993; Nathanson 1994].

Important questions related to the mechanisms by which osteogenesis is functionally coupled to growth cartilage maturation will be decided by a correlation of molecular studies with structural features of the endochondral sequence, particularly in relation to events at the lower epiphyseal-metaphyseal continuum (Figs. 2, 3). The first of these questions is related to the exact temporal/spatial sequence of morphogenesis as observed by light and electron

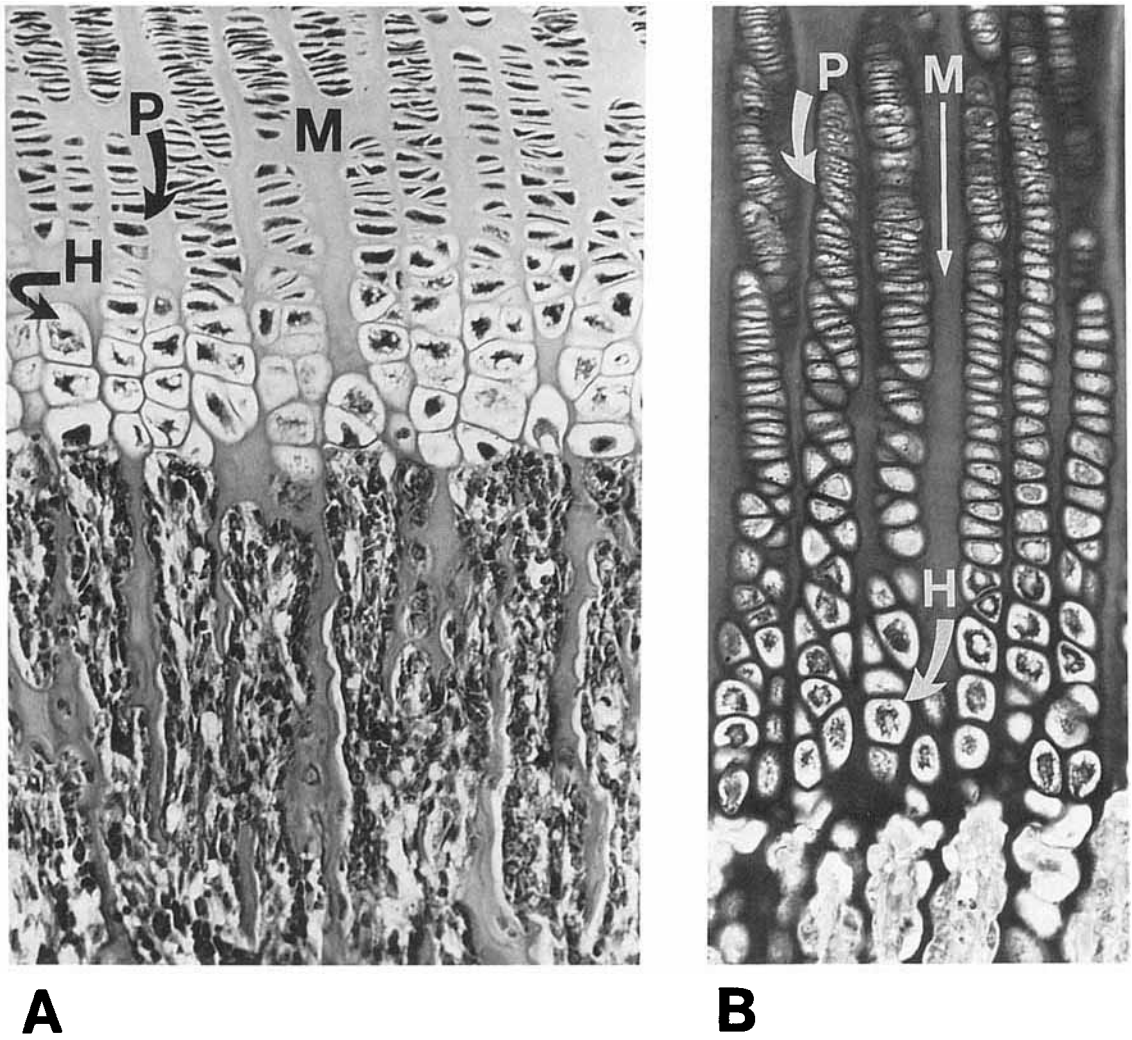

Fig. 2. Photomicrographs illustrating growth plate structure. **A:** Photomicrograph from a metatarsal growth plate of a 1-month-old rabbit shows the linear arrays of proliferating (P) or columnar cells at top, the hypertrophic (H) cells below, the cartilage matrix (M), and the adjacent metaphyseal region at bottom (Paraffin embedded, H and E stained section). **B:** Photomicrograph from the proximal tibial growth plate of a 1-month-old rabbit shows the linear arrays of proliferating (P) or columnar cells at top, the hypertrophic (H) cells below, the cartilage matrix (M), and the adjacent metaphyseal region. (JB4 plastic embedded, 1% toluidine blue stained section).

microscopic structural studies at the zone of mineralized cartilage. To what degree is the progression of mineralization, vascularization, bone synthesis, and bone and cartilage resorption concurrent, or is there an as yet to be defined temporal/spatial sequence to the process? Answers to these structural questions may hasten resolution of the second question concerning which cell types are coupled. Do growth chondrocytes elaborate signals that recruit or promote the differentiation of vascular, osteoclastic, or osteogenic cells? Do vascular, osteogenic, or resorptive cells elaborate signals that effect chondrocyte development and programmed cell death? Do the molecular or mineral components of the extracellular matrix, by themselves, provide signals that mediate these processes? The last series of questions concerns the nature of the systemic and local factors that mediate the coupling. What are these factors, and how are they regulated? If the signals reside in the matrix, what structural aspects of the extracellular matrix or specific proteins provide the necessary cues for vascularization, osteoinduction, and resorption? These latter questions are beginning to be answered as various autocrine/paracrine or growth factors have been identified in the growth plate such as PTHrP [Karaplis et al., 1994; Lee et al., 1995], FGFs [Shiang et al., 1994], specific BMPs [Vukicevic et al., 1990; Jones et al., 1991], and transcriptional factors [Closs et al., 1990; Lee et al., 1994; Ruther et al., 1987; Wang et al., 1992].

CONCLUSION

Extensive morphological, biochemical, and molecular data exist which have defined the developmental progression of growth cartilage. Recent data demonstrating considerable phenotypic overlap between osteogenic cells and cells within the hypertrophic and mineralizing zones of the growth cartilage suggest that there are specific mechanisms of functional coupling between the growth cartilage and the osteogenic, vasculariza-

Fig. 3. Morphological characteristics at the growth plate cartilage–metaphyseal bone interface. Diagramatic illustration and surrounding high power photomicrographs are shown. A diagramatic outline of the major morphological and cellular events at the hypertrophic chondrocyte–metaphyseal interface is presented (**top middle**). RBC, red blood cell; OB, osteoblast; OC, osteocyte; OCL, osteoclast; V, vessel. The major morphological landmarks are denoted as I–V which are depicted in the adjacent micrographs. **Area I (upper right):** Photomicrograph illustrates a portion of an undecalcified growth plate–metaphyseal junction from a 2-week-old rabbit metatarsal. The hypertrophic cells are seen above. Note the dark staining mineral in the longitudinal cartilage septae with very little or no mineral in the transverse septae. Vascular invasion of the hypertrophic cell lacunae has occurred (*solid arrow*) on one side of a mineralized cartilage trabeculum, while on the opposite side there are newly differentiating mesenchymal cells designed to become osteoblasts and synthesize osteoid on the calcified cartilage core. The dots represent tritiated proline in this autoradiograph with sacrifice performed 20 min after injection. **Area II (lower right):** High power photomicrograph shows the lower regions of the hypertrophic zone at top and the vascular and mesenchymal cell invasion from below. This histologic section and those depicting areas III, IV, and V are all demineralized preparations. The hypertrophic cells from the lowest layers of the hypertrophic zone (*white arrow*) are seen. Red blood cells from the metaphysis are seen adjacent to the last persisting hypertrophic cell lacunae. A multinucleated osteoclast (*curved arrow*) is seen, as are undifferentiated mesenchymal cells which will shortly begin to synthesize an osteoid matrix on the persisting cores of calcified matrix (*open arrow*). (Proximal tibial growth plate–metaphyseal junction in 1-month-old rabbit; plastic embedded JB4 section stained with 1% toluidine blue). **Area III (upper left):** Photomicrograph of metaphyseal tissue immediately adjacent to the hypertrophic zone of the growth plate from a 1-month-old rabbit proximal tibial metaphysis. The persisting cartilage cores (C) are black. Newly synthesized bone (B) can be seen adjacent to them. Osteoblasts (*curved arrow*) line the surface of the newly synthesized bone. Osteocytes are within. We refer to these as mixed trabeculae encompassing both bone and cartilage tissue. A vessel (V) is also seen. A multinucleated osteoclast is seen centrally where it is resorbing both cartilage and bone (*black arrow*). (JB4 plastic section stained with 1% toluidine blue). **Area IV (lower left):** Photomicrograph of tissue deeper within the metaphyseal region. There are areas of persisting cartilage (black) but much more newly synthesized light staining bone. Surface osteoblasts are seen (*black arrows*), as are osteocytes and osteoclasts (*open arrow*). **Area V (middle lower level):** A high power view of metaphyseal bone and cartilage trabeculae is seen. Osteoclasts (arrows) can be seen resorbing both bone and cartilage.

The major cellular events of this process include the recruitment and induction of both osseous and vascular tissues. Presumptive osteoblasts are recruited and line the trabeculae of mineralized cartilage to synthesize osteoid. The term *mixed trabeculum* refers to the presence of mineralized cartilage cores surrounded by newly synthesized bone. Vascular elements invade and line the empty lacunae of the lowermost hypertrophic chondrocytes which have undergone cell death. Pertinent questions relative to the osseous and vascular induction within these zones include the nature of the functional coupling among the three tissues: cartilage, bone, and vascular. Signals elaborated by the endochondral cells are targeted to the subsequent development of the chondrocytic components of the growth plate (autocrine regulation) and towards the osteogenic and vascular elements (paracrine regulators). Extracellular matrix components of the mineralized growth plate may elaborate signals or be a permissive substrate for osseous and vascular induction. The surrounding and resorption of the mixed trabeculae by osteoclastic cells is the terminal event in the remodeling process of the endochondral tissue. Signals elaborated from the mineralized cartilage/bone trabeculae, as well as the cells lining this trabeculae, are involved in this recruitment process.

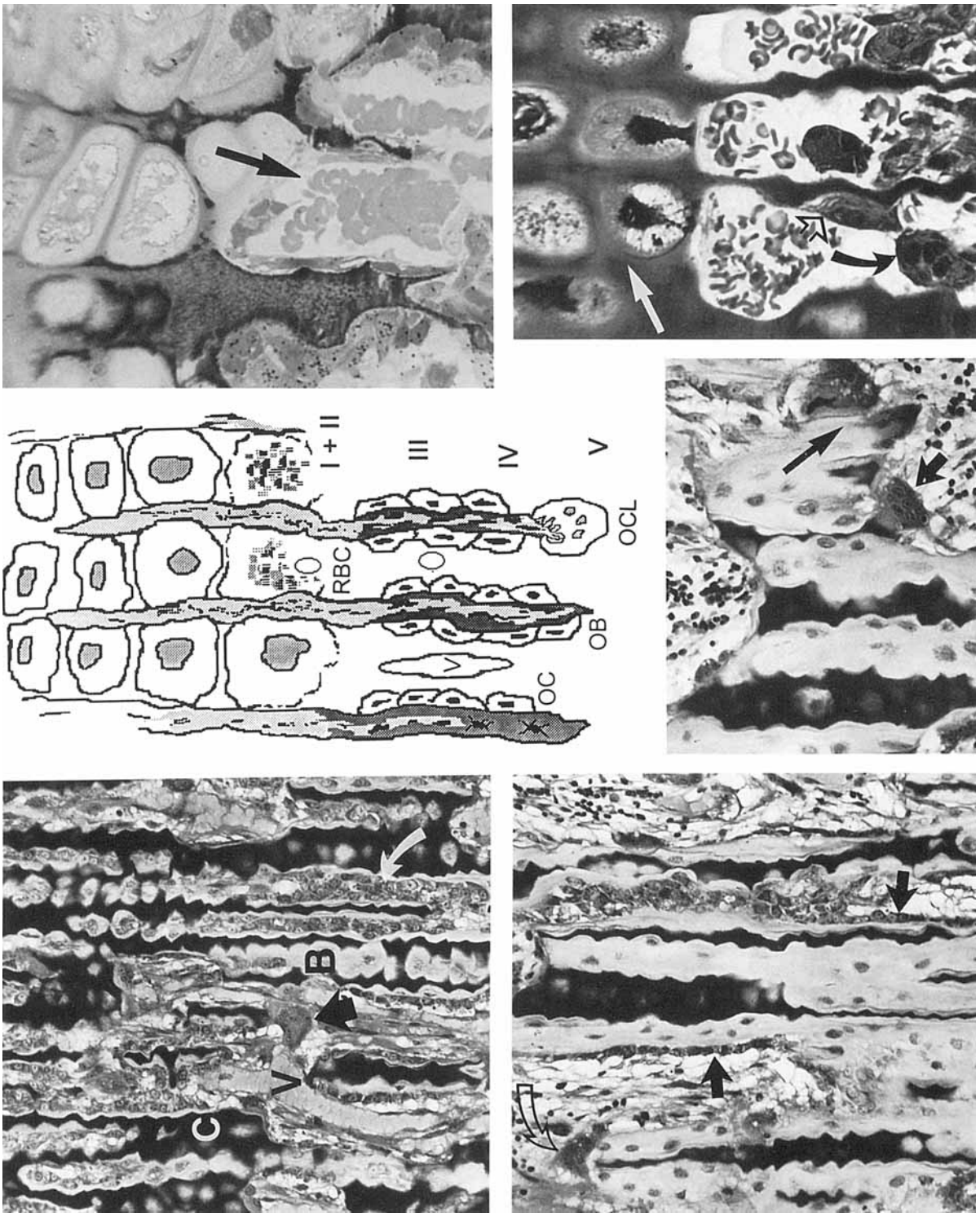

Figure 3.

tion, and resorption processes. Areas of future research include 1) a determination of the developmental origin of the cells within growth cartilage that express the osteogenic phenotypic properties, 2) whether the unique structural features of the mineralized ECM of growth cartilage or systemic and local factors regulate these cells, 3) the target cell populations within the metaphysis which respond to these signals, and 4) the molecular mechanisms of the coupling process.

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