[Frontiers in Bioscience 11, 1702-1715, May 1, 2006]

Matrix metalloproteinases: role in skeletal development and growth plate disorders

Charles J. Malemud

Department of Medicine/Division of Rheumatic Diseases and Department of Anatomy, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio 44106-5076

TABLE OF CONTENTS

1. ABSTRACT

Differentiation is the cellular process that regulates development of long bones and joint surface cartilage of synovial cavities. Growth plate cartilage development is commonly referred to as endochondral ossification which is the end stage of long bone development. Endochondral ossification proceeds as a continuum of chondrocyte proliferation cycles followed by non-proliferative phases coupled to extracellular matrix protein transformations that are regulated by proteins of the *hedgehog* family and by parathyroid-hormone-related peptide and its receptor, the parathyroid-hormone-related peptide receptor. A compelling body of evidence has now emerged implicating matrix metalloproteinases (MMPs) in the process of long bone lengthening and endochondral ossification. Among the MMPs, MMP-13 (collagenase-3), MMP-9 (92-kDa gelatinase; gelatinase B) and MMP-14 (MT1-MMP) are the most abundant proteinases that regulate cellular migration, alterations in the extracellular matrix and apoptosis in growth plate cartilage. Murine mutation or ablation models of growth plate development that target MMPs often result in skeletal abnormalities, indicating the critical role that MMPs play in these animal models and in skeletal maturation. Many of the MMPs that have been identified as regulating the spatial and temporal changes in rodent and rabbit endochondral ossification have also been identified by *in situ* hybridization and immunohistochemical analysis of human long bone development. Genetic manipulation to correct defective or dysfunctional MMP genes or MMP activity found in certain human chondrodysplasias may provide a novel strategy for treating medical disorders characterized by skeletal anomalies.

2. INTRODUCTION

2.1. Chondrocyte Differentiation Pathways in Skeletal Development

Differentiation is the cellular process whereby the genesis, development and stabilization of eukaryotic tissues and organs are established. Cartilaginous tissues emerge from embryonic mesenchyme (1). In the epiphyseal cartilage of the growth plate, a continuum of chondrocyte proliferative and non-proliferative cycles are coupled to extracellular matrix (ECM) transformations that are regulated by hormones and growth factors (2) resulting in a progressive lengthening of the skeleton followed by eventual cessation of skeletal growth over time, commonly referred to as growth plate closure. By contrast, in articular cartilage, (the cartilage located atop skeletal long bones), chondrocyte proliferation is an early embryologic event, but maturational events are dominated by gradual alterations in the composition of cartilage ECM as well as bony remodeling (3). Alterations in the programmed events related to skeletal development often result in skeletal abnormalities and dysplasias (4).

Chondrogenic stabilization is established when mesenchymal cells committed to the chondrogenic lineage synthesize proteins characteristic of cartilage ECM (5). Thus, the chondrogenic phenotype is characterized by the synthesis and integration of specific ECM macromolecules, namely, the proteoglycans aggrecan, decorin, biglycan, fibromodulin, and perlecan as well as the collagen isotypes, Type II, Type IX and Type XI (5, 6). Chondrocyte terminal differentiation is characterized, in part, by derepression of the Type X collagen gene (6).

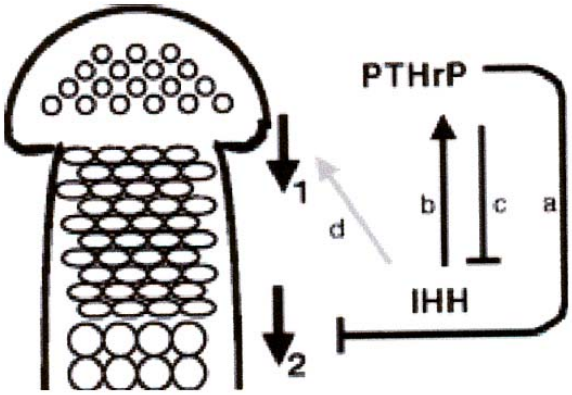

Figure 1. Growth plate cartilage development proceeds by the ordered transformation of "prehypertrophic" chondrocytes ($\downarrow_1$) to hypertrophic chondrocytes ($\downarrow_2$) and is regulated by the actions of IHH and PTHrP. Reproduced by permission from Company of Biologists, Ltd.

2.2. Ordered Molecular Signals Regulate Growth Plate Morphogenesis

The regulation of chondrocyte gene transcription for encoding ECM macromolecules and MMPs during skeletal development and articular cartilage morphogenesis involves specialized intracellular signaling pathways controlled via the activity of tyrosine kinases, mitogen-activated protein kinases (MAPKs), extracellular-signal regulated kinases (ERKs) and Janus kinases (JAKs) (7). In growth plate cartilage, signaling pathways resulting in chondrocyte differentiation are regulated by the *Hedgehog (HH)* protein gene family, namely, *Indian hedgehog* (*Ihh*) and *Sonic hedgehog* (*Shh*) as well as the parathyroid hormone-related peptide (PTHrP) and its receptor, PTHrP-receptor (PPR) (8, 9).

A sequence of morphologic transformations correlates with long bone lengthening and growth plate cartilage ossification (9). The morphologic transformations occurring during long bone development are governed by gene expressional events that eventually result in chondrocyte hypertrophy, synthesis of Type X collagen and cartilage ossification. In the first step in this process, proliferating "prehyppertrophic" chondrocytes cease to divide and express PPR (Figure 1, $\downarrow_1$). PRR signaling in columnar chondrocytes blocks terminal chondrocyte differentiation while also suppressing perichondrium differentiation to columnar cells (Figure 1, $\downarrow_2$). In the integrated model of PTHrP/Ihh feedback proposed by Kobayashi *et al*. (9), PTHrP-mediated signaling directly inhibits premature hypertrophic chondrocyte development (Figure 1, "a"). In turn, Ihh directly regulates PTHrP expression in cells of the perichondrium (Figure 1, "b"). In response to PTHrP, columnar chondrocytes develop from perichondrial cells between the periarticular zone and the zone of Ihh signaling (Figure 1, "c") where Ihh directly stimulates chondrocyte differentiation (Figure 1, "d"). In addition to the positive effects of Ihh on chondrocyte differentiation, perichondrial cells also express Patched (Ptc), a receptor for Shh, and the Gli oncogenes (i.e. Gli1, -2, 3) zinc-finger transcription factor GLI-Kruppel family members that mediate *HH* signaling in all vertebrates. Thus, PTHrP/PPR interactions negatively regulated terminal chondrocyte differentiation (9). The HH/Ptc/Gli pathway has also been implicated in modulating PTHrP/PPR interactions as well as in regulating Ihh synthesis itself (10).

Early in the studies of the Ihh/PTHrP/PPR signaling pathway, PTHrP appeared responsible for suppression of chondrocyte differentiation by delaying the synthesis of Ihh completing a negative feedback loop (8). This sequence of programmed events was thought to give rise to the development of hypertrophic chondrocytes wherein Ihh synthesis was suppressed. However, recent studies employing chondrocyte-specific PTHrP/PPR ablation models in mice with reduced PTHrP/PPR activity suggested that in these murine growth plates, Ihh positively influenced periarticular chondrocyte differentiation that was independent of PTHrP (9).

Additional experimental studies showed that the *Shh* gene was also induced during the formation of cartilage nodules *in vitro*. Thus, over-expression of the *Shh* gene induced by retroviral transfection of chicken limb bud micromass cultures resulted in alkaline phosphatase activity and Type X collagen synthesis (11).

Ihh and Shh appear to act in a coordinated fashion to regulate the transition from "prehypertrophic" to hypertrophic chondrocytes during growth plate maturation. In this regard, Stott and Chuong (11) proposed that Ihh was expressed principally in the late hypertrophic zone which promoted hypertrophy. In turn, Ihh inhibited chondrocyte maturation via PTHrP/PPR.

Smoothened (Smo), another key protein in signaling regulated by *HH* was also expressed during chondrocyte development (10) and Ihh, Ptc as well as Smo were synthesized in ATDC5 cells stimulated to undergo chondrocyte differentiation (12). Of note, a mouse recombinant N-terminal Ihh protein promoted Type X collagen gene expression in ATDC5 cells (10).

Specific skeletal abnormalities have been reported in growth plate cartilages deficient in PTH, PTHrP or PPR. Thus, Blomstrand chondrodysplasia is characterized by precocious endochondral ossification. In this disorder, Jobert *et al*. (13) found a loss of function point mutation in PTHrP which apparently accounted for premature ossification of human growth plate cartilage. Double knockout mice deficient in PTH or PTHrP/PPR also showed skeletal anomalies characterized by accelerated differentiation of growth plate chondrocytes as well as growth plate chondrocyte resistance to Ihh (14). Moreover, the PTH/PPR (-/-) bones showed a significant increase in osteoblast content as well as ECM accumulation without a delay in vascular invasion, suggesting that PTHrP decelerated vascular invasion, a process required for osteoid mineralization that was independent of PPR.

In addition to Ihh/PTHrP/PPR signaling, cyclic AMP-dependent kinase (cAMPk; kinase A) was also

Table 1. MMPs Involved In Growth Plate Chondrocyte Differentiation

Chondrocyte population	MMPs	Function(s)
Proliferating	MMP-8, MMP-13, MMP-14, MMP-1, -2, -3	Cell division/migration, Pro-MMP-2 activation, Apoptosis (?)
Prehypertrophic	MMP-1, -2, -3, MMP-8	ECM protein turnover, Activation of pro-MMP-13
Hypertrophic	MMP-7, MMP-9, MMP-10, MMP-12, MMP-13	Pro-MMP-1 activation, Type II collagen degradation, Type X collagen synthesis, "Chondropotosis",Cartilage vascularization

shown to play a salient role in long bone development. In that regard, Long *et al*. (15) showed that the cyclic-AMP response-element binding protein (CREB) was active in wild type mouse growth plate development where chondrocyte CREB activation was observed in the proliferative growth plate zone. Conversely, transgenic mice expressing a potent dominant-negative CREB inhibitor exhibited decreased chondrocyte proliferation and delayed hypertrophy.

The G-protein α-subunit ($G_s$alpha) couples receptors to adenylyl cyclase which is required for cAMP generation as well as cAMPk activation. Homozygous mice with complete $G_s$alpha deficiency died at birth and exhibited significant abnormalities in growth plate architecture with a shortened proliferative zone and advanced hypertrophic chondrocyte differentiation (16). The $G_s$alpha-deficient mice represented a phenotype similar to PTH-PTHrP knockout mice (17). The mouse phenotypic bore similarities to patients with Albright hereditary osteodystrophy, a heterozygous $G_s$alpha inactivation disorder of growth plate (18).

Recently, SOX9, a high mobility-group- domain–containing transcriptional factor critical to differentiation and cartilage development was shown to be phosphorylated by cAMPk *in vitro* (19). Immunohistochemical analysis showed that SOX9 was evenly distributed in growth plate cells in the resting and proliferative zones; SOX9 was absent in the hypertrophic zone. However, phospho-SOX9 was localized to the "prehypertrophic" chondrocytes. These studies indicated that SOX9 was a substrate for cAMPk. Of note, phospho-SOX9 showed enhanced transcriptional and DNA-binding activity to the 18-bp and 48-bp COL2A1 enhancer elements within the COL2A1 gene. Furthermore, Jia *et al*. (20) showed that cAMPk (in addition to casein kinase I) regulated Smo protein accumulation and activity in response to hedgehog proteins by the phosphorylation of Smo at several amino acid sites. Of note, phosphorylation-deficient forms of Smo failed to accumulate on the cell surface suppressing the transduction of hedgehog signals (20) while Shh was shown to up-regulate the expression of SOX9 (21) during mouse skeletal development.

3. MATRIX METALLOPROTEINASES (MMPs) IN SKELETAL DEVELOPMENT

3.1. MMPs in Skeletal Development: Overview

MMPs have been shown to play an integral role in the skeletal transformations that result in long bone maturation (Table 1). In this regard, MMPs were implicated in chondrocyte proliferation, ECM protein degradation and release of ECM protein degradation products, regulation of MMP and other proteinase activities as well as cell attachment, differentiation and programmed cell death (i.e. apoptosis) (22). Although in human fetal limb development, MMP-1, MMP-2, MMP-3 and MMP-9 as well as tissue inhibitor of metalloproteinases-1 (TIMP-1) were localized to different zones of the developing growth plate (23), MMP-14 (MT1-MMP), MMP-9 and MMP-13 appear to account for most of the proteinase-dependent transformations observed during endochondral ossification (22). By contrast, MMP-1,-2,-3 were seen chiefly in all proliferating chondrocytes and these MMPs were localized to regions distal to the joint line before synovial joint cavity formation. Mouse and rat limb development showed a similar pattern. Thus, MMP-1,-2, -3, -9 and -13 mRNA expressed during rat cartilage development in the tibial plateau was temporally and spatially dependent on the phase of mandible and/or limb development (24, 25).

The functional significance of MMPs dysfunction during skeletal development is related to the requirement for specific MMPs to cleave ECM proteins. Regulated ECM degradation promotes cell migration and tissue stabilization. In this regard, MMP-3, -7,-9, -13, -14 cleaved recombinant trimeric Type IIA collagen amino terminal pro-peptide *in vitro*, whereas MMP-1, -2 and -8 did not (26). Furthermore, MMP-7 was shown to cleave the amino-terminal propeptide from collagen fibrils in fetal cartilage ECM (26), indicating that some MMPs also played a critical role in procollagen processing to mature fibrils during skeletal development. Because the activity of MMPs is generally regulated by TIMPs, Joronen *et al*. (27) studied the extent to which TIMPs were expressed in the developing mouse skeleton and showed by immunohistochemical analyses a time-dependent change in TIMP-1, -2, -3 mRNA in articular and growth cartilages, synovial tissue and bone. However, the levels of TIMP-1, -2 mRNA in articular cartilage were lower than TIMP-1, -2 levels in growth plate suggesting that TIMPs played a more critical role in modulating MMP activity during growth plate development.

Studies by Dean *et al*. (28) and Maeda *et al*. (29) emphasized the modulatory effect of Vitamin D metabolites on growth plate development *in vivo* (28) and *in vitro* (29). These studies showed that 1alpha, 25(OH) 2D3 and 24R, 25(OH) 2D3 regulated neutral metalloproteinases (NMPs) and collagenase activity not only in growth plate cartilage development but also in cell maturation and mineralization as well. In cultures of resting zone chondrocytes, 24, 25(OH) 2D3 caused a dose-dependent increase in NMP activity, but had no effect on collagenase activity. By contrast, 1alpha, 25 (OH) 2D3 altered collagenase, but had no effect on NMP activity (28). The action of Vitamin D metabolites on NMP and collagenase activity during growth plate development appeared to be mediated by the activity of protein kinase C (29).

The MMPs to be discussed in detail below have been implicated in specific skeletal transformations during development. Several MMPs were shown to work independently; others were shown to act synergistically in the molecular events governing the activation of MMPs in skeletal development and maturation (Table 1).

3.2. MMP-13 (Collagenase-3)

The *MMP-13* gene is composed of 10 exons and 9 introns spanning over 12.5 kb in size. The *MMP-13* gene contains structural similarities to other MMP genes, such as those encoding fibroblast collagenase (*MMP-1)*, matrilysin (*MMP-7)* and macrophage metalloproteinase *(MMP-12)*, but *MMP-13* is somewhat distinct in its genomic organization from other MMP genes, namely, stromelysin-3 (*MMP-11),* gelatinase-A (*MMP-2)* and gelatinase-B (*MMP-9),* the latter genes flanking outside the chromosome 11q22 gene cluster where the *MMP-13* gene is located (30).

Hypertrophic chondrocyte maturation is characterized by an enrichment in Type II collagen, Type X collagen in the developing growth plate ECM as well as MMP-13 transcripts *in vivo* (31, 32) and *in vitro* (33) and during fracture callus healing (34). MMP-13 was also found as an enzyme marker for osteoclastic-derived tartrate-resistant alkaline phosphatase-containing cells (34). In addition, during skeletal long bone differentiation and maturation, hypertrophic chondrocytes degraded the NC4 globular domain of the alpha 1(IX) chain of Type IX collagen that protrudes from the collagen fibril (32). This event appeared to serve as a stimulus for *MMP-13* gene up-regulation temporally coinciding with Type X collagen gene expression. MMP-13 was also shown to degrade Type II collagen as well as the remaining COL2 domain of Type IX collagen alpha (IX) chains (32). Although aggrecan is a substrate for MMP-13 (6), the available evidence indicated that aggrecan was selectively spared degradation in this process and instead was retained intact in growth plate cartilage ECM (32). Moreover, MMP-13 inhibition by a non-toxic carboxylate inhibitor of MMP-13 resulted in suppression of Type X collagen gene expression and inhibition of Type II collagen degradation in addition to inhibition of ECM calcification (31).

Recent studies by D'Angelo *et al.* (33) showed that chondrocytes derived from the caudal zone of non-hypertrophying chick growth plate expressed neither Type X collagen nor MMP-13 transcripts, whereas chondrocytes from the hypertrophic zone expressed both markers of chondrocyte differentiation. Furthermore, the level of MMP-13 enzyme activity correlated with increasing levels of MMP-2 with time in culture, suggesting that pro-MMP-13 is activated by MMP-2 (30). In rat tibial growth plate development, expression of Types II and X collagen constituted constantly changing events in sequence resulting in terminal differentiation (34). By contrast, MMP-13 expression was shown to be biphasic; it was expressed at various times during the proliferative phase, but uniformly expressed at the hypertrophic stage (35).

The critical role for MMP-13 synthesis during growth plate cartilage development and maturation was emphasized by the profound deficiencies in morphological transformations preceding endochondral bone formation in an MMP-13-deficient (null) murine animal model (36). Additional evidence also supported the view that significant synergy existed between MMP-13 and MMP-9 in regulating growth plate development (37). Thus, mice deficient in both MMP-13 and MMP-9 had severely impaired endochondral bone formation, characterized by low ECM content, prolonged survival of "prehypertrophic" chondrocytes, delayed vascular formation and impaired trabecular bone formation.

The Wnt signaling protein family and its antagonist protein frizzbie-1 (Frzb-1) is expressed during skeletogenesis, especially during terminal differentiation and in joint formation (38). In that regard, Wnt/beta-catenin/lymphoid enhancer-binding factor (LEF)/T-cell transcription factor (TCF) activation was shown to promote chondrocyte hypertrophy and endochondral ossification (39). LEF belongs to the nuclear LEF/T-cell transcription factor (TCF) protein family and Wnt/beta-catenin/LEF binding in the nucleus promotes excessive Wnt/beta-catenin signaling. Recently, Tamamura *et al*. (40) studied transgenic mice expressing a mutant fusion protein of beta-catenin and LEF. These mice exhibited a totally disorganized growth plate structure characterized by a lack of maturing chondrocytes expressing neither Ihh nor Type X collagen. Furthermore, Wnt/beta-catenin activation in immature growth plate chondrocytes blocked chondrocyte maturation whereas in mature chondrocytes, Wnt/beta-catenin activation promoted the morphological transformations typical of chondrocyte hypertrophy and ECM mineralization as well as up-regulating MMP-13 and vascular endothelial growth factor (VEGF) gene expression (39). Taken together, these results showed that beta-catenin stabilization allowed a form of murine chondrodysplasia to occur (40). Furthermore, Wnt/beta-catenin signaling regulated both temporally and spatially the terminal chondrocyte differentiation pathway. Interactions between SOX9 and beta-catenin may also be critical for MMP-13 gene expression and integral to endochondral ossification (41).

The principal mediator of mammalian tissue hypoxic responses is the transcription factor, hypoxia-inducible factor-1 (HIF-1). Tissue-specific targeting to delete HIF-1 in mice resulted in HIF-1 null growth plates characterized by interruption of vascular flow (42). Of note, VEGF up-regulation was found to be HIF-1-independent in growth plate chondrocytes surrounding areas of cell death. Recently, Pufe *et al*. (43) showed that mechanical overload of bovine cartilage disks *in vitro* resulted in increased expression of HIF-1-alpha, known also to induce VEGF expression. Furthermore, mechanical overload increased MMP-1, MMP-3 and most notably, MMP-13 expression, but reduced TIMP-1, -2. MMP gene expression was also shown to be VEGF-dependent as kinase inhibition of VEGF-receptor-2 suppressed mechanically-induced MMP expression and restored TIMP expression to normal levels.

A large body of evidence has also implicated transforming growth factor-beta (TGF-beta) in chondrocyte

maturation and endochondral ossification (44). Members of the TGF-beta protein super family including the bone morphogenetic proteins (BMPs) transduce signals to the nucleus via cell membrane-specific Type I and Type II receptors and Smad proteins. Smad-1 and -2 mediate BMP signaling; Smad-3,-4, -5, and -6 regulate TGF-beta signaling (45). All 3 TGF-beta isoforms (i.e. TGF-beta-1, -2 and 3) were shown to regulate PTHrP expression in chick sternal chondrocytes via Smad-3 signaling (46). In growing human bone, TGF-beta-2 was detected in all cartilage zones of the growth plate, whereas TGF-beta-1 and -3 were restricted to the proliferative and hypertrophic zones (47). Smad-6, an inhibitor of TGF-beta/BMP signaling, when over-expressed in transgenic mice caused postnatal dwarfism characterized by normal growth plate chondrocyte proliferation, but significantly delayed chondrocyte hypertrophy and thin trabeculae (48). Smad ubiquitin regulatory factor-1 (Smurf-1) also blocks BMP signaling. Growth plate cartilage produced in Smad-6/Smurf-1 offspring had a greater delay in endochondral ossification than in Smad-6 mice (48).

TGF-beta was reported to induce MMP-2 and MMP-9 up-regulation via p38 kinase in human breast epithelial cells (49). Moreover, MMP-13 mRNA levels were up-regulated by TGF-beta and inhibited by BMP-2 in primary human fetal chondrocytes (50).

Other factors have been identified that control MMP-13 expressional events in addition to the reported temporally-regulated expression of MMP-13 during skeletal development, Thus, core binding protein alpha-1 (Cbfa1), a transcriptional factor of the runt gene family was shown to be critical for endochondral bone formation (51, 52). Cbfa1-deficient mice failed to express MMP-13 during fetal development (52). Experimental MMP-13 inhibition also suppressed matrix calcification which was shown to be dependent on Cbfa1 expression (32).

Defective endochondral ossification was also seen in mice with strongly reduced JunB expression, a member of the AP-1 complex required for MMP transcription during embryonic development (53). In this study (53), decreased JunB levels in growth plate chondrocytes and bone lining osteoblasts correlated with dysregulation of cyclins A and D1 that correlated with suppressed endochondral ossification.

Finally, the absence of another transcriptional factor, c-maf (i. e. c-maf-null mice) resulted in the prolongation of the chondrocyte hypertrophic state and marked suppression of MMP-13 activity, suggesting that c-maf was, in part, responsible for initiating terminal differentiation (54). By contrast, c-maf did not appear to be involved in chondrocyte proliferation and apoptosis during murine endochondral ossification.

3.3. MMP-8 (Neutrophil Collagenase)

Human MMP-8 (neutrophil collagenase) is a member of a family of MMPs capable of degrading all 3 alpha chains of Types I, II and III collagens (55). Cole *et al*. (56) showed that normal human articular chondrocytes expressed MMP-8 suggesting its role in ECM turnover. A 467 amino acid sequence was deduced from the nucleotide sequence of the human MMP-8 2.2 kb cDNA and was found to possess only 57% identity with fibroblast collagenase (MMP-1) and therefore represented a distinct MMP gene product (57). The role of MMP-8 in rat hind limb and mandible development was examined using *in situ* hybridization (58). The results showed that MMP-8 was expressed by osteoblastic precursors, differentiated osteoblasts, osteocytes and chondrocytes. The broad expression of MMP-8 compared to MMP-13 (58) suggested that MMP-8 may play a more diversified role in ECM turnover during many phases of endochondral ossification.

3.4. MMP-9 (92-kDa Type IV Collagenase; Human Neutrophil Gelatinase; Gelatinase B)

MMP-9 is a regulated MMP that plays an important role in connective tissue remodeling and basement membrane turnover (59). The cloned human *MMP-9* cDNA hybridized to a 2.8 kb mRNA from chronic granulocytic leukemia cells and was nearly identical to a 92-kDa gelatinase secreted by the HT1080 fibrosarcoma cell line (60). The predicted murine enzyme possessed 72% identity at the cDNA level and exon/intron structure with its human counterpart (61). The rat MMP-9 enzyme structure predicted from its cloned nucleotide sequence comprised 708 amino acids and showed 75% and 82% identity with the human and mouse MMP-9, respectively (61).

MMP-9 has been localized to the edge of endochondral cartilage isolated from 21 day old rats where MMP-9 was believed to participate in the degradation of Type II collagen (62), a step that precedes vascular formation and the replacement of cartilage by bone. MMP-9 was first observed during mouse development at an early stage (i.e. E13) of cartilage and tooth morphogenesis where the *MMP-9* gene was transiently expressed in surrounding mesenchymal cells (63). These studies concluded that MMP-9 was the gelatinase required for the removal of denatured collagen fragments generated by the action of interstitial collagenase (i.e. MMP-1). In this regard, Conot *et al*. (64) showed that MMP-9 mediated the vascular invasion of hypertrophic cartilage callus in developing murine long bones confirming the contention raised earlier by Vu *et al*. (65) who studied homozygous mice with a null mutation in the MMP-9 gene. These animals also exhibited abnormal patterns of growth plate vascular development. In addition, although MMP-9-null mice exhibited normal patterns of chondrocyte hypertrophy and apoptosis, ossification was delayed (65).

In situ hybridization and immunohistochemistry confirmed the predominant localization of MMP-9 mRNA and MMP-9 protein to osteoclasts and chondroclasts at the osteochondral junction in mouse fracture callus (34). In the same study (34), MMP-13 protein was found only in osteoblasts and individual hypertrophic chondrocytes near the cartilage-bone interface providing support for the view that MMP-9 and MMP-13 were mutually exclusive markers for cartilage resorption and endochondral

ossification, respectively (34, 66). *In situ* hybridization studies of avian growth plate development also showed that MMP-9 was expressed principally by cells surrounding blood vessels penetrating the growth plate and by chondrocytes located in front of these vascular invasion sites (67).

There is also additional data from studies in MMP-9-deficient animals which support a role for this enzyme in skeletal development. For example, chicken tibial chondrodysplasia was characterized, in part, by impairment of vascular penetration due to MMP-9 deficiency (68). However, Gustafsson *et al* (69) analyzed MMP-9 activity in the perlecan-null murine growth plate characterized by disorganized growth plate architecture and reduced collagen density. Immunohistochemical analysis revealed a weak, but definite expansion of MMP-9 deposition into the hypertrophic zone, but no alterations in MMP-9 activity, suggesting that MMP-9 did not contribute to the altered growth plates in these animals.

MMP-9 levels were also reduced in X-linked hypophosphatemic (Hyp) mice which is the murine homologue of the human disease caused by mutations in the *Phex* gene (70). This study (70) suggested that *Phex* may regulate mineralization and removal of hypertrophic chondrocytes by apoptosis as well as cartilage ECM degradation in growth plate by altering MMP-9 activity.

3.5. MMP-2 (72-kDa Gelatinase; Gelatinase A)

The mouse (71), chicken (72) and rabbit (73) *MMP-2* gene has been cloned. Rabbit MMP-2 cDNA was totally different from MMP-1, -3, -and -9 (73). The murine MMP-2 cDNA clone contained 662 amino acids and encoded a 72-kDa protein which was intensively expressed in mesenchymal cells (71). The mouse MMP-2 was also similar in many respects to the human MMP-2 cloned from human tumors (74). The importance of MMP-2 activity in skeletal development, however, remains contentious. MMP-2 activity was shown to be constitutive and associated with osteoblast phenotypic maturation (75). However, MMP-2 and MMP-9 levels were higher in growth plate than in articular cartilage *in vivo* (76).

3.6. MMP-14/MMP-15/MMP-16/MMP-17/MMP-18/MMP-25 (Membrane-type 1-6 matrix metalloproteinases; MT1-6 MMP)

The MT-MMPs are cell surface enzymes that appear to be critical for normal development as well as malignant transformation. The regulation of MT-MMP activity is complex involving autocatalytic processing, TIMP binding and enzyme internalization (77). In contrast to other MMPs which cluster on chromosome 11 (30), MT1-MMP (MMP-14) mapped to human or mouse chromosome 14, MT2-MMP (MMP-15) to human chromosome 16 and MT3-MMP (MMP-16) to human chromosome 8 (78, 79). Murine MT1-MMP consisted of 10 exons. The exon structure encoding the catalytic domain and pro-domain of MT1-MMP was distinct from all other MMPs (78). MT1-MMP and the *TIMP-2* gene were spatially and temporally co-expressed during mouse development and MT1-MMP/TIMP-2 was coordinated with the activation of MMP-2 (pro-gelatinase-A) (78). MMP-15 has also been shown to activate pro-MMP-2 and pro-MMP-13 (78). To date, MT4-MMP, MT5-MMP and MT6-MMP activity have not been specifically implicated in the regulation of growth plate cartilage development.

It is likely that the regulation of MT1-MMP catalytic activity involves vacuolar H(+)–ATPase-dependent degradation (80) and the MMP inhibitor membrane-anchored glycoprotein, reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) since mice defective in RECK die around stage E10.5 with defects in collagen fibrils, basal lamina and vascular development (81). In this regard, it was recently shown that MT1-MMP played a pivotal role in capillary-like tubular structures in a collagen-containing fibrin matrix *in vitro* (82).

MT1-MMP-deficient mice showed craniofacial abnormalities, arthritis, osteopenia and dwarfism in addition to tissue fibrosis as a result of suppressed collagenase activity (83). The shortened bones in MT1-MMP-deficient mice was not only traced to decreased chondrocyte proliferation in the growth plate, but also to delayed vascular development and enlargement of the hypertrophic zones with delayed formation of secondary centers of ossification (84). In addition, MT1-MMP deficiency also resulted in suppressed levels of MMP-2 suggesting that MT1-MMP was essential for pro-MMP-2 activation *in vivo* (84). However, to date, neither MT2-MMP nor MT3-MMP-deficiencies has been linked to either mouse or human growth plate disturbances.

3.7. MMP-12 (Macrophage Metalloelastase)

MMP-12 was cloned and purified from mouse (85) and human macrophages. The murine MMP-12 gene located on chromosome 9 predicted a molecular mass of the proenzyme of 53-kDa which shared only 33-48% amino acid homology with other MMPs (85). The rat MMP-12 catalytic domain protein expressed in *Escherichia coli* completely degraded Type V collagen, partially degraded Type I collagen, but was unable to digest Type IV collagen at neutral pH (86).

Although the precise role of MMP-12 in skeletal development remains to be fully established, Kerkela *et al* (87) employing *in situ* hybridization showed MMP-12 transcripts in human fetal hypertrophic chondrocytes in the vertebrae of the spinal column, in ribs, and in extremities undergoing ossification beginning at the gestational age of 8 weeks. However, peritoneal and alveolar macrophage over-expression of the human MMP-12 gene in transgenic rabbits was not associated with any particular skeletal anomalies (88) but did result in significantly enhanced arthritic lesions characterized by severe synovial thickening, pannus formation and marked articular cartilage destruction (89).

3.8. MMP-7 (Matrilysin)

Despite evidence that rat (90) and human (91) MMP-7 (matrilysin) cDNA were similar in many respects to the structure of other stromelysins in their substrate specificity, and to interstitial collagenases in the crystal

structure of its catalytic domain (92), MMP-7 was shown to be unique in that it lacked the carboxyterminal domains encoded by both MMP genes. However, along with several other MMP genes, MMP-7 mapped to chromosome region 11q22 (93).

Activated MMP-7 was shown to degrade cartilage ECM proteins such as aggrecan, fibronectin (92) and Type II collagen (25) *in vitro*. These results suggested that MMP-7 might play a role in ECM protein turnover under normal conditions and in accelerated ECM protein degradation in inflammation. Indeed, Ohta *et al* (94) found that MMP-7 was over-expressed in human osteoarthritic cartilage and the inflammatory cytokines, interleukin-1 and tumor necrosis factor-alpha enhanced MMP-7 gene expression in cultured human chondrocytes. More recently, experimental studies in a rodent femur model of fracture fixation with pure titanium implants showed evidence of MMP-7 immunoreactivity in addition to TIMP-3 and MMP-9 localized to hypertrophic chondrocytes and to the vascular component of the growth plate cartilage (95), suggesting a role for MMP-7 during intramedullary osteointegration.

3.9. MMP-1/MMP-3/MMP-10 (Interstitial collagenase-1; Stromelysin-1; Stromelysin-3)

Turnover of ECM proteins in connective tissues is largely governed by the activity of interstitial collagenase-1 (MMP-1) and the stromelysins, namely MMP-3 and MMP-10 (96). This view is supported by evidence that MMP-1 and MMP-3 (in addition to MMP-2) were localized by immunohistochemistry to chondrocytes in zones distant from the joint line during human fetal development (97). With respect to long bone development, Gack *et al* (98) found high levels of MMP-1 transcripts expressed in hypertrophic chondrocytes of the metaphyses and diaphyses of mouse long bones and osteoblast-like cells situated along the newly formed bony trabaculae, whereas MMP-3 and MMP-10 transcripts were not expressed at 7.5 days and 16.5 days after conception at these anatomic sites. Interestingly, the expression of TIMP-2 preceded MMP-1 expression in murine hypertrophic chondrocytes between mouse embryonic stages E6.5 and E17 of development (99) suggesting that regulation of MMP-1 activity during skeletal development was achieved not only at the transcriptional level, but at the post-translational level as well. Despite the fact that MMP-1 and MMP-10 share 82% sequence homology, activated MMP-10 measured by *in situ* zymography was found in human neonatal rib chondrocytes of the growth plate (100), whereas only an occasional MMP-3 signal was evident in that site. These results suggested that MMP-3 and MMP-10 were differentially synthesized and activated during human rib development.

4. PERSPECTIVE: FUTURE THERAPIES FOR TREATING DEVELOPMENTAL DISORDERS OF THE MUSCULOSKELETAL SYSTEM MAY REQUIRE CORRECTING MMP DYSFUNCTION

Skeletal development is dependent on mesenchymal cell recruitment, cellular commitment to the chondrogenic lineage and terminal chondrocyte differentiation within the growth plate; processes that are dependent on transcriptional factors, growth factors, cytokines, metabolites, hormones (101) protein kinases (6) and MMPs. Identification of fetal growth plate chondrocyte heterogeneity by Percoll gradient fractionation that showed varying gene expression of PTHrP, PPR, Ihh, Type X collagen and MMP-13 also determined that these molecules were restricted to either prehypertrophic or hypertrophic chondrocyte fractions (102). Restrictive expression of these factors, signature markers for chondrocyte terminal differentiation, support the view of cellular and molecular transformations prior to endochondral ossification first put forth by Vortkamp *et al*. (8) and later sustained by the studies of Kronenberg *et al*. (103).

A compelling body of evidence has implicated MMPs in the process of human skeletal development and maturation (104), including the processes resulting in endochondral ossification and synovial joint cavity formation. Among their other functions in connective tissue turnover, these studies (104-107) as well as others (108) also concluded that MMPs induced growth plate cartilage angiogenesis and apoptosis in conjunction with VEGF (104), a protein synthesized and secreted by vascular endothelial cells.

Although targeted murine mutation models resulting in skeletal defects have been useful in sorting out the cellular and molecular interactions required for skeletal development (106, 107), recent studies also emphasized the cooperative effects of fibroblast growth factor-2 (108) and bone morphogenetic proteins (109) in regulating chondrocyte proliferation as well as those interactions between Ihh, Ptc and PTHrP/PPR that precede MMP-13 gene expression (110). In this regard, because the precise timing of a temporal continuum in human long bone development is more difficult to study than in murine mutation models, immunohistochemical staining of discarded human growth plate cartilage have been employed. These studies concluded that Ptc, Ihh and PTHrP and several of the MMPs, including, MMP-9, MMP-13, MT1-MMP, were also critical components in human skeletal development (13, 97, 100, 110).

The recognition that MT1-MMP also plays a crucial role in uncoupling chondrocyte apoptosis from cartilage degradation (111) may provide an opportunity to explore the relevancy of targeted therapies designed to correct defects in MMPs that may contribute to skeletal anomalies such as premature growth plate closure. It was recently proposed that growth plate chondrocytes may be deleted by a process involving a special form of apoptosis, termed, “chondropotosis” (112, 113). The molecular signaling pathways regulating chondrocyte removal at the lower zone of the growth plate prior to ossification remains to be determined, but could involve the activity of MT1-MMP. In this regard, testican-1, a protein expressed in growth plate cartilage and its spliced variants that are members of a Ca^{2+}-binding proteoglycan family were identified as inhibitors of MT1-MMP and MT3-MMP-

mediated pro-MMP-2 activation (114, 115). Thus, testican-1 could regulate chondrocyte deletion in the growth plate.

Finally, normal skeletal development has been shown to be dependent on circulating systemic hormones including thyroid hormone (116). The mechanism underlying the mechanism by which thyroid hormone (117) enhances the activity of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like repeats) proteinase family that appear to be critical in regulating skeletal development may also factor into future medical strategies for treating long bone growth disorders. In this regard, Gao *et al*. (118) reported ADAMTS-4 (i.e. aggrecanase-1) activation was partially dependent on MT4-MMP suggesting that MT4-MMP played an important role in aggrecanase-1 activation in growth plate transformations. Two recent studies by Glasson *et al*. (119, 120) also have shed light on the potential role of the ADAMTS in skeletal development. One study reported that although ADAMTS-4 is the major aggrecanase in mouse growth plates, gene-targeted deletion of the ADAMTS-4 catalytic domain produced no abnormalities in long bone development, growth or remodeling nor was there any effect on the progression of surgically-induced OA pathology (119). Furthermore, Glasson *et al*. (120) also reported that ADAMTS-5 (i.e. aggrecanase-2) null mice also have normal growth plate morphology which was similar to wild-type mice. However, deletion of ADAMTS-5 activity prevented cartilage destruction in a murine model of OA. In addition, these studies also suggested that ADAMTS-4 activity (which is unaffected in ADAMTS-5 null mice) was not the enzyme mediating aggrecan degradation at the TEGE (373-374) ARGS aggrecanase site (119). Thus, the precise role of both ADAMTS-4 -5 in murine and human long bone development remains to be elucidated.

Kobayashi *et al*. (121) recently demonstrated that Ihh directly stimulated periarticular chondrocyte differentiation as well as regulating columnar cell mass independently of PTHrP.

5. ACKNOWLEDGEMENTS

The experimental studies reported in references 2, 3, 6 and 7 were supported, in part, by grants from the National Institutes of Health. The author thanks his many research and clinical colleagues in the Division of Rheumatic Diseases/Case Western Reserve University School of Medicine/University Hospitals of Cleveland for helpful discussions.

6. REFERENCES

1. P. Thorogood: Morphogenesis of cartilage. In: Cartilage. Development, differentiation and growth. Ed: Hall BK, Academic Press, NY 223-254 (1983)

2. C. J. Malemud: The role of growth factors in cartilage metabolism. *Rheum Dis Clin North Amer* 19(3), 569-580 (1993)

3. CJ Malemud & TM Hering: Regulation of chondrocytes in osteoarthrosis. In: Biological regulation of the chondrocytes. Ed: Adolphe M, CRC Press, FL 295-319 (1992)

4. P Goetinck: Mutations affecting limb cartilage. In: Cartilage. Biomedical aspects. Ed: Hall BK, Academic Press, NY 165-189 (1983)

5. EJ-MA Thonar, K Masuda, DH Manicourt & KE Kuettner. Structure and function of normal human adult articular cartilage. In: Osteoarthritis. Clinical and experimental aspects. Eds: Reginster J-Y, Pelletier J-P, Martel-Pelletier J, Henrotin Y, Springer, Germany 1-20 (1999)

6. C. J. Malemud, N. Islam & T. M. Haqqi: Pathophysiologic mechanisms in osteoarthritis lead to novel therapeutic strategies. *Cells Tissues Organs* 174(1-2), 34-48 (2003)

7. C. J. Malemud: Protein kinases in chondrocyte signaling and osteoarthritis. *Clin Orthop* 427S, S145-S151 (2004)

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

9. T. Kobayashi, U. I. Chung, E. Schipani, M. Starbuck, G. Karsenty, T. Katagiri, D. L. Goad, B. Lanske & H. M. Kronenberg: PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. *Development* 129(12), 2977-2986 (2002)

10. H. Akiyama, C. Shigeno, Y. Hiraki, C. Shukunami, H. Kohno, M. Akagi, J. Konishi & T. Nakamura: Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signaling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5. *Biochem Biophys Res Commun* 235(1), 142-147 (1997)

11. N. S. Stott & C. M. Chuong: Dual action of sonic hedgehog on chondrocyte hypertrophy: retrovirus mediated ectopic sonic hedgehog expression in limb bud micromass cultures induces novel cartilage nodules that are positive for alkaline phosphatase and type X collagen. *J Cell Sci* 110(Pt 21), 2691-2701 (1997)

12. H. Akiyama, C. Shigeno, K. Iyama, H. Ito, Y. Hiraki, J. Konishi & T. Nakamura: Indian hedgehog in late-phase differentiation in mouse chondrogenic EC cells, ATDC5: upregulation of type X collagen and osteoprotegerin ligand mRNAs. *Biochem Biophys Res Commun* 257(3), 814-820 (1999)

13. A. S. Jobert, P. Zhang, A Couivineau, J. Bonaventure, J Roume, M. Le Merrer & C. Silve: Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia. *J Clin Invest* 102(1), 34-40 (1998)

14. B. Lanske, M. Amling, L. Neff, J. Guiducci, R. Baron & H. M. Kronenberg: Ablation of the PTHrP gene and the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. *J Clin Invest* 104(4), 399-407 (1998)

15. F. Long, E. Schipani, H. Asahara, H. Kronenberg & M. Montminy: The CREB family of activators is required for endochondral bone development. *Development* 128(4), 541-550 (2001)

16. A. Sakamoto, M. Chen, T. Kobayashi, H. M. Kronenberg & L. S. Weinstein: Chondrocyte-specific knockout of G protein Gsalpha leads to epiphyseal and growth plate abnormalities and ectopic chondrocyte formation. *J Bone Miner Res* 20(4), 663-671 (2005)

17. E. Shipani, B. Lanske, J. Huzelman, A. Luz, C. S. Kovacs, K. Lee, A. Pirro, H. M. Kronenberg & H. Juppner: Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid-related peptide delays endochondral ossification and rescues mice that lack parathyroid hormone-related peptide. *Proc Natl Acad Sci USA* 94 (25), 13689-13694 (1997)

18. M. Bastepe, L. S. Weinstein, N. Ogata, H. Kawaguchi, H. Juppner, H. M. Kronenberg & U. I. Chung: Stimulatory G protein directly regulates hypertrophic differentiation of growth plate cartilage in vivo. *Proc Natl Acad Sci USA* 101(41), 14794-14799 (2004)

19. W. Huang, X. Zhou, V. Lefebvre & B. de Crombrugghe: Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transcribe a Col2a1 chondrocyte-specific enhancer. *Mol Cell Biol* 20(11), 4149-4158 (2000)

20. J. Jia, C. Tong, B. Wang, L. Luo & J. Jiang: Hedgehog signaling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. *Nature* 432(7020), 1045-1050 (2004)

21. S. Tavella, R. Biticchi, A. Schito, E. Minina, E. Di Martino, A. Pagano, A. Vortkamp, W. A. Horton, R. Cancedda & S. Garofalo: Targeted expression of SHH affects chondrocyte differentiation, growth plate organization and Sox9 expression. *J Bone Miner Res* 19(10), 1678-1688 (2004)

22. Z Werb & J. R. Chin: Extracellular matrix remodeling during morphogenesis. *Ann NY Acad Sci* 857, 110-118 (1998)

23. N. Ortega, D. Behonick, D. Stickens & Z. Werb: How proteases regulate bone morphogenesis. *Ann NY Acad Sci* 995, 109-116 (2003)

24. Y. Wang, F. Middleton, J. A. Horton, L. Reichel, C. E. Farnum & T. A. Damron: Microarray analysis of proliferative and hypertrophic growth plate zones identifies differentiation markers and signal pathways. *Bone* 35(6), 1273-1293 (2004)

25. A. Gepstein, S. Shapiro, G. Arbel, N. Lahat & E. Livne: Expression of matrix metalloproteinases in articular cartilage of temporomandibular and knee joints of mice during growth, maturation, and aging. *Arthritis Rheum* 46(12), 3240-3250 (2002)

26. N. Fukui, A McAlinden, Y. Zhu, E. Crouch, T. J. Broekelmann, R. P. Meacham & L. J. Sandell: Processing of type II procollagen propeptide by matrix metalloproteinases. *J Biol Chem* 277(3), 2193-2201 (2002)

27. K. Joronen, H. Salminen, V. Glumoff, M. Savontous & E. Vuorio: Temporospatial expression of tissue inhibitors of matrix metalloproteinases-1, -2, -3 during development, growth aging of the mouse skeleton. *Histochem Cell Biol* 114(2), 157-165 (2000)

28. D. D. Dean, B. D. Boyan, Z. Schwartz, O. E. muniz, M. R. Carreno, S. Maeda & D. S. Howell: Effect of 1alpha, 25-dihydroxyvitamin D3 and 24R, 25-dihydroxyvitamin D3 on metalloproteinase activity and cell maturation in growth plate cartilage in vivo. *Endocrine* 14(3), 311-323 (2001)

29. S. Maeda, D. D. Dean, V. L. Sylvia, B. D. Boyan & Z. Schwartz: Metalloproteinase activity in growth plate chondrocyte cultures is regulated by 1,25-(OH)(2)D(3) and 24,25-(OH)(2)D(3) and mediated by protein kinase C. *Matrix Biol* 20(2), 87-97 (2001)

30. A. M. Pendas, M. Balbin, E. Liano, M. G. Jimenez & C. Lopez-Ortin: Structural analysis and promoter characterization of the human collagenase-3-gene (MMP13). *Genomics* 40(2), 222-233 (1997)

31. C. W. Wu, E. V. Tchetina, F. Mwale, K. Hasty, I. Pidoux, A. Reiner, J. Chen, H. E. van Wart & A. R. Poole: Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. *J Bone Miner Res* 17(4), 639-651 (2002)

32. W. Wu, F. Mwale, E. Tchetina, T. Kojima, T. Yasuda & A. R. Poole: Cartilage matrix resorption in skeletogenesis. *Novartis Found Symp* 232, 158-166 (2001)

33. M. D'Angelo, Z. Yan, M. Nooreyazdan, M. Pacifici, D. S. Sarment, P. C. Billings & P. S. Leboy: MMP-13 is induced during chondrocyte hypertrophy. *J Cell Biochem* 77(4), 678-693 (2000)

34. H. Uusitalo, A. Hiltunen, M. Soderstrom, H. T. Aro & E. Vuorio: Expression of cathepsins B, H, K, L, and S and matrix metalloproteinases 9 and 13 during chondrocyte hypertrophy and endochondral ossification in mouse fracture callus. *Calcif Tissue Int* 67(5), 382-390 (2000)

35. J. Alvarez, M. Balbin, F. Santos, M. Fernandez, S. Ferrando & J. M. Lopez: Different bone growth rates are associated with the expression pattern of Types II and X collagens and collagenase-3 in proximal growth plates of the rat tibia. *J Bone Miner Res* 15(1), 82-94 (2000)

36. M. Inada, Y. Wang, M. H. Byrne, M. U. Rahman, C. Miyaura, C. Lopez-Ortin & S. M. Krane: Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci USA* 101(49), 17192-17197 (2004)

37. D. Stickens, D. J. Behonick, N. Ortega, B. Heyer, B. Hartenstein, Y. Yu, A. J. Fosang, M. Schorp-Kistner, P. Angel & Z. Werb: Altered endochondral bone formation in matrix metalloproteinase 13-deficient mice. *Development* 131(23), 5883-5895 (2004)

38. M. Enomoto-Iwamoto, J. Kitagiri, E. Koyama, V. Tamamura, C. Wu, N. Kanatani, T. Koike, H. Okada, T. Komori, T. Yoneda, V. Church, P.H. Francis-West, K. Kurisu, T. Nohno, M. Pacifici & M. Iwamoto: The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. *Dev Biol* 251(1), 142-156 (2002)

39. J. Kitagaki, M. Iwamoto, J. G. Liu, Y. Tamamura, M. Pacifici & M. Enomoto-Iwamoto: Activation of beta-catenin-LEF/TCF signal pathway in chondrocytes stimulates ectopic endochondral ossification. *Osteoarthritis Cartilage* 11(1), 36-43 (2003)

40. Y. Tamamura, T. Otani, N. Kanatani, E. Koyama, J. Kitagaki, T. Komori, Y. Yamada, F. Constantini, S. Wakisaka, M Pacifici, M. Iwamoto & M. Enomoto-Iwamoto: Developmental regulation of Wnt/beta-catenin signals is required for growth plate assembly, cartilage integrity and endochondral ossification. *J Biol Chem* 280(19), 19185-19195 (2005)

41. H. Akiyama, J. P. Lyons, Y. Mori-Akiyama, X. Zang, R. Zhang, Z. Zhang, J. M. Deng, M. M. Taket, T. Nakamura, R. R. Behringer, P. D. McCrea & B. de Crombrugghe: Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev* 18(9), 1072-1087 (2004)

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

43. T. Pufe, A. Lemke, B. Kurz, W. Petersen, B. Tillmann, A. J. Grodzinsky & R. Mentlein: Mechanical overload induces VEGF in cartilage discs via hypoxia-inducible factor. *Amer J Pathol* 164(1), 185-192 (2004)

44. M. D'Angelo, D. P. Sarment, P. C. Billings & M. Pacifici: Activation of transforming growth factor beta in chondrocytes undergoing endochondral ossification. *J Bone Miner Res* 16(12), 2339-2347 (2001)

45. T. Sakou, T. Onishi, T. Yamamoto, T. Nagamine, T. Sampath & P. Ten Dijke: Localization of Smads, the TGF-beta family intracellular signaling components during endochondral ossification. *J Bone Miner Res* 14(7), 1145-1152 (1999)

46. D. P. Pateder, C. M. Ferguson, A. M. Ionescu, E. M. Schwarz, R. N. Rosier, J. E. Puzas & R. J. O'Keefe: PTHrP expression in chick sternal chondrocytes is regulated by TGF-beta through Smad-mediated signaling. *J Cell Physiol* 188(3), 343-351 (2001)

47. A. Horner, P. Kemp, C. Summers, S. Bord, N. J. Bishop, A. W. Kelsall, N. Coleman & J. E. Compston: Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. *Bone* 23(2), 95-102 (1998)

48. M. Horiki, T. Imamura, M. Okamoto, M. Hayashi, J. Murai, A. Myoui, T. Ochi, K. Miyazono, H. Yoshikawa & N. Tsumaki: Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy and causes dwarfism with osteopenia. *J Cell Biol* 165(3), 433-445 (2004)

49. E. S. Kim, M. S. Kim & A. Moon: TGF-beta-induced upregulation of MMP-2 and MMP-9 depends on p38 MAPK, but not ERK signaling in MCF10A human breast epithelial cells. *Int J Oncol* 25(5), 1375-1382 (2004)

50. N. Johansson, U. Saarialho-Kere, K. Airola, R. Herva, L. Nissinen, J. Westermarck, E Vuori, J. Heino & V. M. Kahari: Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Dev Dyn* 208(3), 387-397 (1997)

51. M. J. Jimenez, M. Balbin, J. M. Lopez, J. Alarez, T. Komori & C. Lopez-Ortin: Collagenase-3 is a target of Cbfa1, a transcriptional factor of the runt gene family involved in bone formation. *Mol Cell Biol* 19(6), 4431-4442 (1999)

52. M. Inada, T. Yasui, S. Nomura, S. Mikake, K. Deguchi, M. Himeno, M. Sato, H. Yamagiwa, T. Kimura, N. Yasui, T. Ochi, N. Endo, Y. Kitamura, T. Kishimoto & T. Komori: Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev Dyn* 214(4), 279-290 (1999)

53. J. Hess, B. Hartenstein, S. Teurich, D. Schmidt, M. Schorp-Kistner & P. Angel: Defective endochondral ossification in mice with strongly compromised expression of JunB. *J Cell Sci* 116(Pt 22), 4587-4596 (2003)

54. H. E. MacLean, J. I. Kim, M. J. Glimcher, J. Wang, H. M. Kronenberg & L. H. Glimcher: Absence of transcription factor c-maf causes abnormal terminal differentiation of hypertrophic chondrocytes during endochondral bone development. *Dev Biol* 262(1), 51-63 (2003)

55. T. F. Ho, M. W. Qoronfleh, R. C. Wahl, T. A. Pulvino, K. J. Vavra, J. Falvo, J. Banks, P. G. Brake & R. B. Ciccarelli: Gene expression, purification and characterization of recombinant human neutrophil collagenase. *Gene* 146(2), 297-301 (1994)

56. A. A. Cole, S. Chubinskaya, B. Schumacher, K Huch, G Szabo, J. Yao, K. Mikecz, K. A. Hasty & K. E. Kuettner: Chondrocyte matrix metalloproteinase-8. Human articular chondrocytes express neutrophil collagenase. *J Biol Chem* 271(18), 11023-11026 (1996)

57. K. A. Hasty, T. F. Pouromotabbed, G. I. Goldberg, J. P. Thompson, D. G. Spinella, R. M. Stevens & C. L. Mainardi: Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. *J Biol Chem* 265(20), 11421-11424 (1990)

58. Y. Sasano, J. X. Zhu, M. Tsubota, I. Takahashi, K. Onodera, I Mizoguchi & M. Kagayama: Gene expression of MMP8 and MMP13 during embryonic development of bone and cartilage in the rat mandible and hind limb. *J Histochem Cytochem* 50(3), 325-332 (2002)

59. S. Masure, G. Nys, P. Fiten, J. Van Damme & G. Opdenakker: Mouse gelatinase B, cDNA cloning, regulation of expression and glycosylation in WEHI-3 macrophages and gene organisation. *Eur J Biochem* 218(1), 129-141 (1993)

60. P. Davarajan, J. J. Johnston, S. S. Ginsberg, H. E. van Wart & N. Berliner: Structure and expression of neutrophil gelatinase cDNA. Identity with type IV collagenase from HT1080 cells. *J Biol Chem* 267(35), 25228-25232 (1992)

61. A. Okada, M. Santavicca & P. Basset: The cDNA cloning and expression of the gene encoding rat gelatinase B. *Gene* 164(2), 317-321 (1995)

62. E. R. Lee, G. Murphy, M. el-Alfy, M.A. Davoli, L. Lamplugh L, A. J. Docherty & C. P. Leblond: Active gelatinase B is identified by histozymography in the cartilage resorption sites of developing long bones. *Dev Dyn* 215(3), 190-205 (1999)

63. P. Reponen, C. Sahlberg, C. Munaut, I Thesleff & K. Tryggvason: High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. *J Cell Biol* 124(6), 1091-1102 (1994)

64. C. Colnot, Z. Thompson, T. Miclau, Z. Werb & J. A. Helms: Altered fracture repair in the absence of MMP9. *Development* 130(17), 4123-4133 (2003)

65. T.H. Vu, J. M. Shipley, G. Bergers, J. E. Berger, J. A. Helms, D. Hanshan, S. D. Shapiro, R. M. Senior & Z. Werb: MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93(3), 411-422 (1998)

66. J.P. Tuckerman, K. Pittois, N.C. Partridge, J. Merregaert & P. Angel: Collagenase-3 (MMP-13) and integral membrane protein 2a (Itm2a) are marker genes of chondrogenic osteoblastic cells in bone formation: sequential temporal, and spatial expression of Itm2a, alkaline phosphatase, MMP-13, and osteocalcin in the mouse. *J Bone Miner Res* 15(7), 1257-1265 (2000)

67. A. Tong, A. Reich, O. Genin, M. Pines & E. Monsonego-Ornan: Expression of chick 75-kDa gelatinase-like enzyme in perivascular chondrocytes suggests its role in vascularization of the growth plate. *J Bone Miner Res* 18(8), 1443-1452 (2003)

68. C. A. Praul, B. C. Ford, C. V. Gay & M. Pines, R. M. Leach: Gene expression and tibial dyschondroplasia. *Poult Sci* 79(7) 1009-1013 (2000)

69. E. Gustaffsson, A. Aszodi, N. Ortega, E. B. Hunziker, H. W. Denker, Z. Werb & R. Fassler: Role of collagen type II and perlecan in skeletal development. *Ann NY Acad Sci* 995, 140-150 (2003)

70. D. Miao, X. Bai, D. K. Panda, A. C. Karaplis, D. Goltzman & M.D. McKee: Cartilage abnormalities are associated with abnormal Phex expression and with altered matrix protein and MMP-9 localization in Hyp mice. *Bone* 34(4), 638-647 (2004)

71. P. Reponen, S. Sahlberg, P. Huhtala, T. Hurskainen, I. Thesleff & K. Tryggvason: Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. *J Biol Chem* 267(11), 7856-7862 (1992)

72. R. T. Aimes, D. l. French & J. P. Quigley: Cloning of a 72 kDa matrix metalloproteinase (gelatinase) from chicken embryo fibroblasts using gene family PCR: expression of the gelatinase increases upon malignant transformation. *Biochem J* 300 (Pt 3): 729-736 (1994)

73. S. Matumoto, M. Katoh, T. Watanabe & Y. Masuho: Molecular cloning of rabbit matrix metalloproteinase-2 and its broad expression at several tissues. *Biochim Biophys Acta* 1307(2), 137-139 (1996)

74. P. Huhtala, L. T. Chow & K. Tryggvason: Structure of the human type IV collagenase gene. *J Biol Chem* 265(19), 11077-11082 (1990)

75. C. Filtani, G. R. Dickson, D. Di Martino, V. Ulivi, C. Sanguineti, P. Romano, C. Palermo & P. Manduca: The expression of metalloproteinase-2, -9 and -14 and tissue inhibitor 1 and -2 is developmentally modulated during osteogenesis in vitro, the mature osteoblastics phenotype expressing metalloproteinase-14. *J Bone Miner Res* 15(11), 2154-2168 (2000)

76. Y. Kawashima-Ohya, H. Satakeda, Y. Kuruta, T. Kawamoto, W. Yan, Y. Akagawa, T. Hayakawa, M. Noshiro, Y. Okada, S. Nakamura & Y. Kato: Effects of parathyroid hormone (PTH) and PTH-related peptide on expressions of matrix metalloproteinase-2, -3 and -9 in growth plate chondrocyte cultures. *Endocrinology* 139(4), 2120-2127 (1998)

77. M. G. Mattei, N. Roeckel, B. R. Olsen & S. S. Apte: Genes of the membrane-type matrix metalloproteinase (MT-MMP) gene family, MMP14, MMP15, and MMP16, localize to human chromosomes 14, 16 and 8, respectively. *Genomics* 40(1), 168-169 (1997)

78. S. S. Apte, N. Fukai, D. R. Beier & B. R. Olsen: The matrix metalloproteinase-14 (MMP-14) is structurally distinct from other MMP genes and is co-expressed with the TIMP-2 gene during mouse embryogenesis. *J Biol Chem* 272(41), 25511-25517 (1997)

79. Y. T. Konttinen, A. Ainola, H. Valleala, J. Ma, H. Ida, J. Mandelin, R. W. Kinne, S. Santavirta, T. Sorsa, C Lopez-Ortin & M. Takagi: Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann Rheum Dis* 58(11), 691-697 (1999)

80. E. Maquoi, K. Peyrollier, A. Noel, J. M. Foidart & F. Frankenne: Regulation of membrane-type 1 matrix metalloproteinase activity by vacuolar H+-ATPases. *Biochem J* 373(Pt 1), 19-24 (2003)

81. J. Oh, R. Takahashi, S. Kondo, A. Mizoguchi, E. Adachi, R. M. Sasahara, S. Nishimura, Y. Imamura, H. Kitayama, D. B. Alexander, C. Ide, T. P. Horan, T. Arakawa, H. Yoshida, S. Nishikawa, Y. Itoh, M. Seiki, S. Itohara, C. Takahashi & M. Noda: The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* 107(6), 789-800 (2001)

82. A. Collen, R. Hanemaajijer, F. Lupu, P. H. Quax, N. van Lent, J. Grimbergen, E. Peters, P. Koolwijk & V. W. van Hinsbergh: Membrane-type matrix metalloproteinase-mediated angiogenesis in a fibril-collagen matrix. *Blood* 101(5), 1810-1817 (2003)

83. K. Holmbeck, P. Bianco, J. Caterina, S. Yamada, M. Kromer, S. A. Kuznesov, M. Mankani, P. G. Robey, A. R. Poole, I. Pidoux, J. M. Ward & H. Birkedal-Hansen: MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis and connective tissue disease due to inadequate collagen turnover. *Cell* 99(1), 81-92 (1999)

84. Z. Zhou, S. S. Apte, R. Soininen, R. Cao, G. Y. Baaklini, R. W. Rauser, J. Wang, Y. Cao & K. Tryggvason: Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci USA* 97(8), 4052-4057 (2000)

85. S. D. Shapiro, G. L. Griffin, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, H. G. Welgus, R. M. Senior & T. J. Ley: Molecular cloning, chromosomal localization, and bacterial expression of a murine macrophage metalloelastase. *J Biol Chem* 267(7), 4664-4671 (1992)

86. J. Y. Fu, A. Lyga, H. Shi, M. L. Blue, B. Dixon & D. Chen: Cloning, expression, purification and characterization of rat MMP-2. *Protein Expr Purif* 21(2), 268-74 (2001)

87. E. Kerkela, T. Bohling, R. Herva, J. A. Uria & U. Saarialho-Kere: Human macrophage metalloelastase (MMP-12) expression is induced in chondrocytes during fetal development and malignant transformation. *Bone* 29(5), 487-493 (2001)

88. J. Fan, X. Wang, L. Wu, S. I. Matsumoto, J. Liang, T. Koike, T. Ichikawa, H. Sun, H. Shikama, Y. Sasaguri & T. Watanabe: Macrophage-specific overexpression of human matrix metalloproteinase-12 in transgenic rabbits. *Transgenic Res* 13(3), 261-269 (2004)

89. X. Wang, J. Liang, T. koike, H. Sun, T. Ichikawa, S. Kitajima, M. Morimoto, H. Shikama, T. Watanbe, Y. Sasaguri & J. Fan: Overexpression of human matrix metalloproteinase-12 enhances the development of inflammatory arthritis in transgenic rabbits. *Am J Pathol* 165(4), 1375-1383 (2004)

90. S. R. Abramson & J. F. Woessner, Jr: Characterization of rat matrilysin and its cDNA. *Ann NY Acad Sci* 732, 362-364 (1994)

91. M. Gaire, Z. Magbanua, S. McDonnell, L. McNeill, D. H. Lovett & L. M. Matrisian: Structure and expression of the human gene for the matrix metalloproteinase matrilysin. *J Biol Chem* 269(3), 2032-2040 (1994)

92. C. L. Wilson & L. M. Matrisian: Matrilysin: an epithelial matrix metalloproteinase with potentially novel functions. *Int J Biochem Cell Biol* 28(2), 123-136 (1996)

93. J. D. Knox, D. R. Boreham, J. A. Walker, D. P. Morrison, L. M. Matrisian, R. B. Nagle & G. T. Bowden: Mapping of the metalloproteinase gene matrilysin (MMP7) to human chromosome 11q21-->q22. *Cytogenet Cell Genet* 72(2-3), 179-182 (1996)

94. S. Ohta, K. Imai, K. Yamashita, T. Matsumoto, I. Azumano & Y. Okada: Expression of matrix metalloproteinase 7 (matrilysin) in human osteoarthritic cartilage. *Lab Invest* 78(1), 79-87 (1998)

95. V. I. Shubayev, B. Branemark, J. Steinauer & R. R. Myers: Titanium implants induce expression of matrix metalloproteinases in bone during osteointegration. *J Rehabil Res Dev* 41(6), 757-766 (2004)

96. R. L. Smith: Degradative enzymes in osteoarthritis. *Front Biosci* 4, d704-d712 (1999)

97. J. C. Edwards, L. S. Wilkinson, P. Soothill, R. M. Hembry, G. Murphy & J. J. Reynolds: Matrix metalloproteinases in the formation of human synovial joint cavities. *J Anat* 188(Pt 2), 355-360 (1996)

98. S. Gack, R. Vallon, J. Schmidt, A. Grigoriadis, J. Tuckerman, J. Schenkel, H. Weiher, E. F. Wagner & P. Angel: Expression of interstitial collagens during skeletal development of the mouse is restricted to osteoblast-like cells and hypertrophic chondrocytes. *Cell Growth Differ* 6(6), 759-767 (1995)

99. V. Mattot, M. B. Raes, P. Henriet, Y. Eeckhout, D. Stehelin, B. Vandenbunder & X. Desbiens: Expression of interstitial collagenase is restricted to skeletal tissue during mouse embryogenesis. *J Cell Sci* 108(Pt 2), 529-535 (1995)

100. S. Bord, A. Horner, R. M. Hembry & J. E. Compston: Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) expression in developing human bone: potential roles in skeletal development. *Bone* 23(1), 7-12 (1998)

101. R. S. Tuan: Biology of developmental and regenerative skeletogenesis. *Clin Orthop* 427 Suppl, S105-S117 (2004)

102. J. Weisser, S. Riemer, M. Schmidl, l. J. Suva, E. Poschi, R. Brauer & K. von der Mark: Four distinct chondrocyte populations in the fetal bovine growth plate: highest expression levels of PTH/PTHrP receptor, Indian hedgehog and MMP-13 in hypertrophic chondrocytes and their suppression by PTH (1-34) and PTHrP (1-40). *Exp Cell Res* 279(1), 1-13 (2002)

103. H. M. Kronenberg, L. Lee, B. Lanske & G. V. Segre: Parathyroid hormone-related protein and Indian hedgehog control the pace of cartilage differentiation. *J Endocrinol* 154 Suppl, S39-S45 (1997)

104. G. Haeusler, I. Walter, M. Helmreich & M. Egerbacher: Localization of matrix metalloproteinases (MMPs), their tissue inhibitors, and vascular endothelial growth factor (VEGF) in growth plates of children and adolescents indicates a role for MMPs in human postnatal development and skeletal maturation. *Calc Tissue Int* 76(5), 326-335 (2005)

105. M. Takahara, T. Naruse, M. Takagi, H. Orui & T. Ogino: Matrix metalloproteinase-9 expression, tartrate-resistant acid phosphatase activity, and DNA fragmentation in vascular and cellular invasion into cartilage preceding primary endochondral ossification in long bone. *J Orthop Res* 22(5), 1050-1057 (2004)

106. W. McLean & B. R. Olsen: Mouse models of abnormal skeletal development and homeostasis. *Trends Genet* 17(10), S38-S43 (2001)

107. D. Miao, H. Liu, P. Plut, M. Niu, R. Huo, D. Goltzman & J. E. Henderson: Impaired endochondral bone development and osteopenia in Gli2-deficient mice. *Exp Cell Res* 294(1), 210-222 (2004)

108. H. Nagai & M. Aoki: Inhibition of growth plate angiogenesis and endochondral ossification with diminished expression of MMP-13 in hypertrophic chondrocytes in FGF-2 -treated rats. *J Bone Miner Metab* 20(3), 142-147 (2002)

109. B. S. Yoon & K. M. Lyons: Multiple functions of BMPs in chondrogenesis. *J Cell Biochem* 93(1), 93-103 (2004)

110. T. Nakase, T. Miyaji, K. Kuriyama, N. Tamai, M. Horiki, T. Tomita, A Myoui, K. Shimada & H.Yoshikaw: Immunohistochemical detection of parathyroid hormone-related peptide, Indian hedgehog, and patched in the process of endochondral ossification in the human. *Histochem Cell Biol* 116(3), 277-284 (2001)

111. K. Holmbeck, P. Bianco, K. Chrysovergis, S. Yamada & H. Birkedal-Hansen: MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage, a critical process in skeletal growth. *J Cell Biol* 163(3), 661-671 (2003)

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

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

114. M. Nakada, A. Yamada, T. Takino, H. Miyamori, T. Takahashi, J. Yamashita & J. Sato: Suppression of membrane-type 1 matrix metalloproteinase (MMP)-mediated MMP-2 activation and tumor invasion by testican 3 and its spliced variant gene product, N-Tes. *Cancer Res* 61(24), 8896-8902 (2001)

115. H. J. Hausser, R. Deckling & R. E. Brenner: Testican-1, an inhibitor of pro-MMP-2 activation is expressed in cartilage. *Osteoarthritis Cartilage* 12(11), 870-877 (2001)

116. R. T. Ballock & R. J. O'Keefe: Physiology and pathophysiology of the growth plate. *Birth Defects Res C Embryo Today* 69(2), 123-143 (2003)

117. S. Makihira, W. Yan, H. Murikami, M. Furukawa, M. Kawai, N. Nikawa, E. Yoshida, T. Hamada, Y. Okada & Y. Kato: Thyroid hormone enhances aggrecanase-2/ADAM-TS5 expression and proteoglycan degradation in growth plate cartilage. *Endocrinology* 144(6), 2480-2488 (2003)

118. G. Gao, A. Plaas, V. P. Thompson, S. Jim, F. Zuo & J. D. Sandy: ADAMTS4 (aggrecanase-1) activation on the cell surface involves C-terminal cleavage by glycosylphosphatidyl inositol-anchored membrane type 4- matrix metalloproteinase and binding of the activated proteinase to chondroitin sulfate and heparan sulfate on syndecan. *J Biol Chem* 279(11), 10042-10051 (2004)

119. S. S. Glasson, R. Askew, B. Sheppard, B. A. Carito, T. Blanchet, H. L. Ma, C. R. Flannery, K. Kanki, E. Wang, D. Peluso, Z. Yang, M. K. Majumdar & E. A. Morris: Characterization of and osteoarthritic susceptibility in ADAMTS-4 knock out mice. *Arthritis Rheum* 50(8), 2547-2558 (2004)

120. S. S. Glasson, R. Askew, B Sheppard, B. Carito, T Blanchet, H-L Ma, C. R. Flannery, D. Peluso, K. Kanki, Z Yang, M. K. Majumdar & E. A. Morris: Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 434(7033), 644-648 (2005)

121. T. Kobayashi, D. W. Soegiarto, Y. Yang, B. Lanske, E. Schipani, A. P. McMahon & H. M. Kronenberg: Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP. *J Clin Invest* 115(7), 1734-1742 (2005)

Key Words: Growth plate cartilage; articular cartilage; matrix metalloproteinases; endochondral ossification; Chondrodysplasias

Send correspondence to: Charles J. Malemud, Ph.D. Department of Medicine/Division of Rheumatic Diseases Diseases, University Hospitals of Cleveland, 2061 Cornell Road, Cleveland, Ohio 44106-5076 Tel: 216844-7846; Fax.: 216-844-2288, E-mail: cjm4@cwru.edu

http://www.bioscience.org/current/vol11.htm