Growth Plate and Cellular Growth

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The growth plate is a highly organized cartilage structure with a complex cellular biology. Studies have been limited by the relative lack of relevant experimental models. Thanks to new technologies, e.g. targeted deletions of genes, knowledge is rapidly expanding. The first paper selected representing the **mechanism of the year** describes how estradiol can rescue skeletal growth during GH resistance through a novel mechanism of targeted GH receptor-independent stimulation of IGF-I synthesis in the liver. Under **new paradigms**, we chose a paper showing that the skeletal effects of estrogen are mediated by opposing actions of classical and non-classical estrogen receptor pathways. In addition, two papers were selected to demonstrate contrasting skeletal phenotypes in mice with mutations in thyroid hormone receptor- α or β . The possibility of modulating proteins and genes involved in angiogenesis gives **new hope** for therapeutic intervention of different growth disorders. **Concepts revised or re-centered** are represented by three reports focusing on the regulation of growth plate chondrocyte differentiation. **Important observation for clinical practice** is represented by a report demonstrating that hypophosphatemia may lead to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes. Three different papers were selected describing **new mechanisms** involved in the normal development of the growth plate. The section **new genes** includes a paper confirming the genetic linkage of human height to two regions, 9q22 and Xq24. The **review** of the year entitled 'Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate' presents a new concept: hypertrophic cells die through the induction of autophagy. Finally, we selected two papers supplying some **food for thought**.

Mechanism of the year

Growth without growth hormone receptor: estradiol is a major growth hormone-independent regulator of hepatic IGF-I synthesis

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J Bone Miner Res 2006;20:2138–2149

Background: Growth hormone (GH) and estrogen play a pivotal role in pubertal growth and bone mineral acquisition. Estrogens can affect GH secretion and thereby provide a GH-dependent mechanism for their effects on skeletal growth. It is presently unclear if or to what extent estrogens are able to regulate pubertal growth and bone mineral accrual independent of GH and its receptor.

Methods: Estradiol (E_2 ; 0.03 μ g/day by subcutaneous silastic implants) was administered to orchidectomized (ORX) male mice with disrupted GHR (GHRKO) and corresponding WT mice during late puberty (6–10 weeks). Longitudinal and radial bone growth, serum IGF-I and IGF-I expression in liver, muscle, bone, and liver were studied by histomorphometry, RIA, RT-PCR, microarrays, and Western blotting.

Results: E_2 stimulated longitudinal (femur length and growth plate thickness) and radial growth (cortical thickness and periosteal perimeter) in ORX GHRKO, whereas no significant changes occurred in WT. E_2 upregulated serum IGF-I and liver IGF-I synthesis in ORX GHRKO, whereas IGF-I synthesis in the femur or muscle was unaffected. Study of the underlying mechanism of the stimulation of hepatic IGF-I expression showed that E_2 restored downregulated receptor signaling systems. E_2 thereby recovered the Janus kinase (JAK)/signal transducers and activators of the transcription (STAT) pathway as evidenced by a significantly increased activation of the transcription factor STAT5 in ORX GHRKO.

Conclusion: The data show a stimulation of skeletal growth through upregulation of hepatic IGF-I by a hormone other than GH. E_2 rescues skeletal growth during GH resistance through a novel mechanism of GHR-independent stimulation of IGF-I synthesis in the liver.

The authors demonstrate that in the absence of a functional GHR and in the context of very low serum IGF-I levels, E_2 stimulates liver IGF-I production. The anabolic effects of the subsequent E_2 -induced rise in serum IGF-I on longitudinal and radial bone growth provide evidence for the importance of endocrine IGF-I in mediating growth. The data presented demonstrate that E_2 has the capacity to rescue growth during GH resistance through a novel mechanism of GHR-independent stimulation of IGF-I production in the liver. Liver-derived IGF-I is not only important for bone metabolism but also for carbohydrate and lipid metabolism as well as blood pressure regulation. Future research should address to what extent treatment with estrogen or estrogen-like compounds (e.g., selective estrogen receptor modulators) might be clinically useful for the restoration of hepatic IGF-I synthesis in patients with disease-related or age-dependent GH/IGF-I deficiency and/or GH-resistant conditions. In the last year or two we have learned that the crucial role of the STATs is the signaling of the GHR, and that STAT mutations [1], or GHR mutations that affect STAT docking [2], result in GHIS (or its new name given by the industry IGF-deficiency; IGFD). It turns out that estrogen can bypass the receptor and activate STAT directly. Whereas estrogens have many other effects, the pharmaceutical modulation of STAT seems to be closer than ever.

New paradigms

Skeletal effects of estrogen are mediated by opposing actions of classical and nonclassical estrogen receptor pathways

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J Bone Miner Res 2005;20:1992–2001

Introduction: Estrogen receptor- α (ER α) can activate gene transcription either through classical estrogen response elements (EREs) or through binding with other transcription factors (e.g., AP-1, SP-1, NF κ B) and modulating their transcriptional activity (non-classical pathways). To investigate the involvement of classical versus non-classical estrogen receptor pathways in bone biology, mice heterozygous for a knock-in mutation abolishing ERE binding (non-classical ER α knock-in [NERKI]) were crossed with heterozygote ER α knockout mice and the resulting female ER $\alpha^{+/+}$, ER $\alpha^{+/NERKI}$, and ER $\alpha^{-/NERKI}$ mice were investigated. Thus, obtained ER $\alpha^{-/NERKI}$ mice harbor an ER α , which is unable to activate ERE-dependent promoters but can stimulate non-classical estrogenic pathways. The ER $\alpha^{+/NERKI}$ mice have altered balance between classical and non-classical signaling pathways activated by estrogens, although to a lesser extent than ER $\alpha^{-/NERKI}$ mice.

Materials and Methods: The mice were either sham-operated or ovariectomized (OVX). OVX mice were treated with 17 β -estradiol (0.25 and 1.0 μ g/day; n = 10–12/group) and bone density and structure were studied by DXA, pQCT and μ CT.

Results: Cortical, but not trabecular BMD was reduced in 3-month-old female ER $\alpha^{+/NERKI}$ and ER $\alpha^{-/NERKI}$ mice. Estrogen replacement did not reverse OVX-induced trabecular bone loss in ER $\alpha^{-/NERKI}$ mice but completely reversed it in ER $\alpha^{+/+}$ mice. Surprisingly, OVX increased and estrogen treatment decreased cortical BMD in ER $\alpha^{-/NERKI}$ mice, an effect which is completely opposite to the one observed in ER $\alpha^{+/+}$ mice. Estrogen also had opposite effects on bone formation and resorption parameters on endocortical surfaces in ER $\alpha^{-/NERKI}$ and ER $\alpha^{+/+}$ mice.

Conclusions: The presented data clearly demonstrate that impaired balance between classical and non-classical ER α signaling pathways reduced cortical bone density. Moreover, the inactivation of classical ER α signaling (but not non-classical) may result in opposite responses to estrogen. Finally, these findings support the hypothesis that the balance between classical and non-classical estrogen signaling is crucial and that when the balance is changed, this may lead to aberrant tissue response to estrogen.

Involvement of estrogen signaling in the regulation of longitudinal bone growth is unequivocal. To investigate the involvement of genomic and non-genomic pathways in bone biology, mice heterozygous for a knock-in mutation abolishing ERE binding (non-classical ER α knock-in [NERKI]) were crossed with heterozygote ER α knockout mice and the resulting female ER $\alpha^{+/+}$, ER $\alpha^{+/NERKI}$, and ER $\alpha^{-/NERKI}$ mice were investigated. Both ER $\alpha^{+/NERKI}$ and ER $\alpha^{-/NERKI}$ mice were found to have shorter bone length as compared with control ER $\alpha^{+/+}$ mice. This decrease in bone length is especially striking as ER $\alpha^{-/-}$ as well as ER $\alpha^{-/-}\beta^{-/-}$ mice have normal bone length [3]. This observation demonstrates that the balance between classical ERE-mediated and non-classical estrogen pathways is important for normal bone growth. To date two strains of ER α knockout mice have been studied, one with complete ER α inactivation, and another with a remaining 46-kDa short form of ER α lacking the AF-1 domain but harboring DNA- and ligand-binding domains. Interestingly, bone length is decreased in 46-kDa ER $\alpha^{-/-}$ mice [4] but is not affected in complete ER α knockouts [3]. Based on the findings presented in the current paper, it can be speculated that the 46-kDa form of ER α has an imbalanced signaling between classical and non-classical ER-activated pathways. Moreover, the famous ER α knockout man [5] is believed to produce the 46-kDa form of ER α [6] and, thus, may have impaired balance between classical ERE-mediated and non-classical estrogen-activated pathways. Altogether, the present study clearly demonstrates that our understanding of the mechanisms of estrogen signaling is far from complete and further studies are needed to elucidate the precise actions of estrogens. For the big picture, let us keep in mind that endogenous estrogen and the delivered estrogen activate both ER α and ER β , and it is the unique ratio of these two that matters in terms of growth acceleration or arrest.

Contrasting skeletal phenotypes in mice with an identical mutation targeted to thyroid hormone receptor α_1 or β

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Background: The actions of thyroid hormone (T_3) are mediated via binding to nuclear T_3 receptors (TRs), which act as transcription factors. The TRs are encoded by two genes, TR α and TR β that are located on the human chromosomes 17 and 3, respectively. Alternative splicing gives rise to five major TR isoforms that are expressed in temporal-spatial patterns during development and in differing ratios depending on tissue. T_3 is essential for normal skeletal development and mineralization and for linear growth. Hypothyreosis causes delayed skeletal maturation and complete growth arrest if sufficiently severe during childhood. Hyperthyreosis, on the other hand, causes accelerated bone maturation and may induce short stature by premature epiphyseal fusion. Mutations in the TR β gene in man cause the syndrome of resistance to thyroid hormone (RTH) and the mutated receptor may interfere in a dominant negative way with the actions of normal wild-type TR α and TR β .

Methods: Transgenic mice carrying identical mutations in either TR- α_1 or β were created. The TR β mutation (TR β PV/PV) is known to cause gross RTH in both humans and mice. Bone lengths were determined from both embryonic (E17.5) and postnatal ages up to 50 days of age. Expression of mRNA was analyzed by in situ hybridization and activation of IGF-IR and GHR signaling was examined by immunohistochemical analysis in wild-type and mutant growth plates.

Results: The skeletal phenotype of the TR α_1 mutation showed signs of 'skeletal hypothyroidism' with delayed endochondral and intramembranous ossification, severe postnatal growth retardation and delayed closure of the skull sutures. No sex dimorphic difference in bone length was found. This was accompanied by impaired GH receptor as well as IGF-I receptor expression and signaling in the growth plate. In contrast, the TR β mutants showed signs of 'skeletal thyrotoxicosis' with increased GH receptor and IGF-I receptor signaling.

Conclusion: The authors conclude that the divergent phenotypes of the identical mutation in the TR α_1 and TR β genes arise because the pituitary gland is a TR β -responsive tissue whereas bone is TR α -responsive.

This work is an important extension of the work from the same group in 2003 [7] which showed the detailed skeletal phenotype of the TR β (PV/PV) mutation with advanced skeletal maturation and

increased mineralization and premature closure of the epiphyses. Together these papers have increased our understanding of the different tissue distributions of the TR receptors and the importance of a balanced thyroid hormone influence on the skeleton. The biological/developmental and teleological significance of the localization of the thyroid hormone receptor variants in different tissues as well as differences in the temporal expression is fascinating. Autosomal dominant resistance to thyroid hormone (RTH), which results from mutant TR β proteins, also impairs skeletal development causing short stature and bone dysplasias [8] when elevated T₄ and T₃ concentrations hyperstimulate TR α , resulting in skeletal thyrotoxicosis. See another twist on that concept in the next paper.

Thyroid hormones regulate fibroblast growth factor receptor signaling during chondrogenesis

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Background: A balanced thyroid hormone (T₃) activity is essential in skeletal development and a prerequisite for normal linear growth. Hypothyroidism causes growth retardation and epiphyseal dysgenesis whereas hyperthyroidism causes accelerated bone maturation and premature closure of skull bones – craniosynostosis. Certain effects of T₃ on bone and cartilage seem to be mediated through the fibroblast growth factor (FGF) family of membrane receptors (FGFR1–3, but not FGFR4) and ligands that, to a significant extent, are expressed in the growth plate. The aim of this study was to investigate whether T₃ influences chondrogenesis via the FGFR system in the chondrogenic cell line ATDC5 which undergoes a defined differentiation program in vitro.

Methods: ATDC5 cells were cultivated with or without the presence of 100 nM T₃ throughout the culture period (28 days). A short stimulation (0–30 min) with FGF2, FGF9, FGF18, EGF or PDGF was done just before harvesting the cells. Chondrocytes were also investigated from transgenic mice with a targeted inactivating mutation of thyroid hormone receptor (TR) β (TR β /PV mice) or α ; either by in situ hybridization (both α and β) or after establishing a primary chondrocyte culture (only α).

Results: T₃ caused a reduction of the number of cells in vitro but increased the chondrocyte differentiation markers alkaline phosphatase and collagen type X. RT-PCR analysis revealed several FGFR splice variants. T₃ stimulated expression of all FGFR mRNAs in ATDC5 cells and the stimulation was maximal after 6 days coinciding with the time at which T₃ inhibited cell proliferation and initiated the onset of hypertrophic chondrocyte differentiation. The greatest effect was seen on the expression of FGFR3 and the influence tapered during the chondrogenesis differentiation period. FGFR3 expression was markedly reduced in TR α mutants that show ‘skeletal hypothyroidism’ but was increased in TR β PV-mutant mice that exhibit ‘skeletal thyrotoxicosis’.

Conclusion: T₃ regulates chondrogenesis partly via stimulating expression of FGFR3, which inhibits cell proliferation and stimulates the onset of hypertrophic chondrocyte differentiation. T₃ furthermore exerts differential effects on the FGFR system during chondrogenesis.

The authors have previously shown T₃ to stimulate FGFR1 in osteoblasts in vitro and in vivo, whereas FGFR2 is not regulated by T₃. Yet, the major FGFR expressed in the growth plate cartilage is FGFR3. Accordingly, mutations of FGFRs 1 and 2 primarily affect osteoblast function and result in craniosynostosis syndromes, whereas gain-of-function FGFR3 mutations affect chondrocyte function resulting in, e.g., achondroplasia and hypochondroplasia. We can now learn about the biological interactions between T₃ and FGFR signaling. Hypothyroidism or deletions in the TR α 1 gene resulted in impaired hypertrophic chondrocyte differentiation and disturbed the interface of the growth plate with the underlying metaphysis. The TR β (PV; named after the affected PV kindred) mutant mouse is interesting as it harbors a resistance towards T₃ and still gives a skeletal phenotype as in thyrotoxicosis. In utero these mice have accelerated growth and advanced endochondral and intramembranous ossification. Postnatally, this advanced bone maturation results in premature closure of growth plates and

shortened bone length together with increased bone mineralization and craniosynostosis. In humans, the PV mutation similarly shows a complete loss of T₃ binding and exhibits a dominant negative effect on the TR. Individuals with this mutation exhibit clinical features including goiter, short stature, decreased weight, hypercholesterolemia, tachycardia, hearing loss, attention deficit–hyperactivity disorder, decreased IQ, and dyslexia. They have markedly increased thyroid hormone levels but normal or only slightly increased serum levels of TSH. It is thus obvious from the reviewed study and these examples that thyroid hormone is an important regulator of skeletal tissues and body growth.

New hope

Elevated expression of hypoxia inducible factor-2 α in terminally differentiating growth plate chondrocytes

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J Cell Physiol 2006;206:435–440

Background: Hypoxia-inducible factor-1 α (HIF-1 α) is essential for the survival of chondrocytes in a hypoxic environment of the growth plate due to well-known stimulation of vascular endothelial growth factor (VEGF) and glycolytic enzymes. However, the full chondrocyte response to hypoxia and the molecular control of VEGF expression in connection with growth plate differentiation and vascularization remains poorly understood. HIF-2 α is a homologue of the HIF-1 α transcription factor involved in the activation of a number of hypoxia responsive genes, but its role in chondrogenesis is less known.

Methods: Percoll density gradient centrifugation was employed in order to separate populations of chick chondrocytes according to their differentiation stages. A differential display analysis was employed for analysis of gene expression in chondrocytes at different stages of differentiation.

Results: Highly upregulated expression of HIF-2 α mRNA during chondrocyte differentiation was demonstrated. HIF-1 α mRNA was also expressed, although at the same level in all of the chondrocyte fractions. The elevated expression of HIF-2 α during chondrocyte differentiation was accompanied by increased VEGF expression. Supporting this observation, levels of HIF-2 α , VEGF, placental growth factor, and glucose transporter-1 proteins were elevated along with differentiation of the ATDC5 murine chondrocyte cell line. Moreover, immunohistochemistry showed the presence of HIF-2 α in the hypertrophic layer of the mouse growth plate. The expression of HIF-2 α was also detected in articular chondrocytes but was restricted to the superficial tangential zone.

Conclusions: HIF-2 α is likely to be involved in the regulation of blood vessel formation and cartilage resorption, processes crucial for endochondral ossification.

The growth plate of long bones is a constitutively a vascular and hypoxic tissue. The ability of cells to tolerate lowered oxygen levels entirely depends on blood vessel invasion in the growth plate and cartilage resorption. VEGF is a key regulator of this angiogenic process. Blocking of VEGF function in the growth plate results in impaired cartilage resorption and accumulation of hypertrophic chondrocytes [9], resulting in growth arrest, but the regulating mechanisms of VEGF expression in the growth plate remain an enigma. The current paper proposes that HIF-2 α , a member of the HIF family of hypoxia-induced transcription factors, regulates the expression of VEGF in the growth plate, and the level of expression increases with maturation. Coexpression of VEGF and HIF-2 α was demonstrated in different models, including chick primary chondrocytes, murine growth plates and in a chondrocyte cell line. Although VEGF stimulation by HIF-2 α has been shown in other tissues [10], the data presented here are associative and functional conformation of the observed coexpression required. VEGF expression has been demonstrated in human growth plates [11], and it can be hypothesized that proper vascularization is essential for pubertal closure of the epiphyseal growth plate. Thus, VEGF and genes regulating its expression can be potential targets for therapeutic modulations of longitudinal bone growth.

Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independent of PTHrP

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J Clin Invest 2005;115:1734–1742

Background: The length of the columnar region of proliferative growth plate chondrocytes is believed to be determined by the rate of differentiation of periarticular into columnar chondrocytes, the rate of columnar cell proliferation, and the rate of differentiation of columnar to hypertrophic chondrocytes. Parathyroid hormone-related (PTH-related) protein (PTHrP), regulated by Indian hedgehog (Ihh), prevents premature hypertrophic differentiation, thereby maintaining the length of columns. Ihh regulates cartilage development through PTHrP-independent pathways as well.

Methods: The authors used an array of mutant mice allowing them to further define the role of PTHrP and Ihh in the developing growth plate.

Results: Mosaic ablation of the PTH/PTHrP receptor in the growth plate caused upregulation of Ihh action, PTHrP upregulation, acceleration of periarticular chondrocyte differentiation, and elongation of the columnar region. Decreasing Ihh action in these mice reduced elongation of columns, whereas decreasing PTHrP showed only a modest effect on column length. Overexpression of Ihh caused PTHrP upregulation, elongation of columns, and acceleration of periarticular chondrocyte differentiation. PTHrP heterozygosity in this model had a minimal effect on the elongation of columns. Moreover, the elongation of columns and stimulation of periarticular chondrocyte differentiation in these models were still observed when PTHrP signaling was maintained so that it remained constant.

Conclusion: The data support a role for Ihh in stimulating the differentiation of periarticular chondrocytes to become columnar growth plate chondrocytes and thereby regulate the length of columns independent of PTHrP.

BMP signaling stimulates cellular differentiation at multiple steps during cartilage development

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Proc Natl Acad Sci USA 2005;102:18023–18027

Background: Bone morphogenetic proteins (BMPs) play important roles at multiple stages of endochondral bone formation. However, the roles of BMP signaling in chondrocytes *in vivo* are still controversial.

Methods: In the present study a constitutively active BMP receptor 1A (*caBmpr1a*), was overexpressed in chondrocytes using two systems. *CaBmpr1a* was directly driven by a rat type-II collagen promoter in a conventional transgenic system and indirectly driven in a UAS-Gal4 binary system.

Results: *CaBmpr1a* expression caused shortening of the columnar layer of proliferating chondrocytes and upregulation of maturation markers, suggesting acceleration of differentiation of proliferating chondrocytes toward hypertrophic chondrocytes. In addition to the acceleration of chondrocyte differentiation, conventional transgenic mice showed widening of cartilage elements and morphological alteration of perichondrial cells, possibly due to stimulation of differentiation of prechondrogenic cells. Moreover, bigenic expression of *caBmpr1a* rescued the differentiation defect of prechondrogenic cells in *Bmpr1b*-null phalanges.

Conclusion: The data indicate that BMP signaling is necessary for phalangeal prechondrogenic cells to differentiate into chondrocytes and that signaling of BMP receptor 1B in this context is replaceable by that of a constitutively active BMP receptor 1A. This suggests that BMP signaling in prechondrogenic cells and in growth plate chondrocytes stimulates their chondrocytic differentiation and maturation toward hypertrophy, respectively.

Fig. 1. Indian hedgehog (Ihh)/parathyroid hormone-related protein (PTHrP) negative-feedback loop. PTHrP is secreted from periarticular chondrocytes, diffuses through the growth plate and inhibits transition from proliferative to hypertrophic chondrocytes (a), thereby, delaying the production of Ihh. When the source of PTHrP production is sufficiently distant, chondrocytes undergo differentiation and produce Ihh. The Ihh, in turn, stimulates the production of PTHrP at the ends of bones by inhibiting Gli3 transcription factor (b), thereby rescuing PTHrP from Gli3-dependent downregulation (c). Additionally, Gli3 inhibits recruitment of stem-like chondrocytes into the proliferative layer in a PTHrP-independent manner (d). Finally, Ihh also stimulates the rate of chondrocyte proliferation (e) and the conversion of perichondrial cells into osteoblasts of the bone collar (f).

Parathyroid hormone-related (PTH-related) protein (PTHrP) and Indian hedgehog (Ihh) are important regulators of cartilage development. In their first paper, the authors propose a model for the regulation of growth plate chondrocyte differentiation by Ihh and PTHrP at distinct steps. The model states that in addition to regulating PTHrP expression and chondrocyte proliferation, Ihh directly acts on periarticular chondrocytes to stimulate their differentiation to columnar chondrocytes, which regulates the length of the columnar region. PTHrP, on the other hand, is required for prevention of premature differentiation of chondrocytes into prehypertrophic and hypertrophic chondrocytes, and thereby suppresses premature expression of Ihh (see also the paper below by Koziel et al., and fig. 1). This model is attractive and supported by a multitude of experimental data, although it is a simplification as chondrocytic differentiation is known to be affected by an array of proteins including the BMPs. Indeed in their second paper, the authors report that BMP signaling in perichondrial cells or their precursor cells stimulates their differentiation into chondrocytes and that the BMP pathway regulates hypertrophic chondrocyte differentiation independent of the PTHrP-signaling pathway. Thus, BMP signaling can stimulate both differentiation of prechondrogenic cells into chondrocytes and differentiation of proliferating chondrocytes toward hypertrophic chondrocytes. Although these two papers have increased our knowledge substantially, the precise mechanisms whereby chondrocytes form stacks of flat cells in orderly arrays are incompletely understood.

Gli3 acts as a repressor downstream of Ihh in regulating two distinct steps of chondrocyte differentiation

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Development 2005;132:5249–5260

Background: Indian hedgehog (Ihh) regulates endochondral ossification forming a negative feedback loop with parathyroid hormone-related protein (PTHrP). Together these paracrine factors balance the rate of proliferation and the onset of hypertrophic differentiation of chondrocytes in the growth plate. Moreover, Ihh directly stimulates chondrocyte proliferation and ossification of the perichondrium. However, downstream signaling of the Ihh during endochondral ossification is unknown. The zinc-finger transcription factor Gli3 is involved in downstream signaling of hedgehog proteins in other tissues.

Methods: The role of Gli3 in mediating Ihh response was analyzed during endochondral ossification in mouse long bones. Mice deficient in *Ihh*, *Gli3* or both, as well as mice overexpressing *Ihh* within the growth plate and deficient in *Gli3* were employed in order to reveal the genetic interaction between Ihh and Gli3. In situ hybridization was employed for analysis of gene expression.

Results: Knocking out *Gli3* in *Ihh*-deficient mice restored both chondrocyte proliferation and the accelerated onset of hypertrophic differentiation observed in *Ihh*^{-/-} knockout mice. The expression of the Ihh-regulated genes patched (*Ptc*) and *PTHrP* was also recovered in *Ihh*^{-/-}*Gli3*^{-/-} mutants. Reversibly, knocking out *Gli3* in mice that overexpress *Ihh* in chondrocytes accelerated hypertrophic differentiation by reducing the domain and possibly the level of *PTHrP* expression. Finally, analysis of chondrocyte differentiation in *Gli3*^{-/-}-deficient mice demonstrated that Gli3 inhibited the differentiation of distal, low-proliferating chondrocytes into columnar, high proliferating cells.

Conclusion: The recruitment of resting chondrocytes into the proliferative layer is inhibited by Gli3 independent of PTHrP, but the transition from proliferating into hypertrophic chondrocytes is regulated by Gli3-dependent expression of *PTHrP*. Moreover, by regulating distal chondrocyte differentiation, Gli3 seems to position the location of the domain of *PTHrP* expression.

The GLI3 gene was isolated by virtue of its cross-hybridization to the zinc finger gene GLI, which is amplified in certain glioblastomas. The present study demonstrates that Gli3 is an underlying transcription factor involved in several regulatory steps of chondrocyte differentiation. (I) Gli3 acts as a strong repressor of Ihh target genes and PTHrP. (II) Gli3 controls the switch from distal into columnar chondrocytes in a PTHrP-independent manner and the switch from columnar into hypertrophic chondrocytes by regulation of *PTHrP* expression. (III) *PTHrP* expression is regulated by Gli3 repressor, and possibly activator. Together with the above two cited papers by Kobayashi et al., a new model of Ihh-PTHrP interaction can be proposed, where Ihh inhibits Gli3, which in turn is a potent repressor of *PTHrP* gene expression as well as a repressor of the transition from resting to proliferative chondrocytes (fig. 1). A constitutively active PTH/PTHrP receptor leads to Jansen's metaphyseal chondrodysplasia, whereas loss of function in the PTH/PTHrP receptor results in Bloomstrand's chondrodysplasia. Thus theoretically an inactivating mutation of the *Gli3* gene would lead to PTHrP overexpression and result in a phenotype similar to Jansen's metaphyseal chondrodysplasia. In contrast, overproduction of Gli3 would result in a Bloomstrand's chondrodysplasia-similar phenotype. Indeed, mutations in the *Gli3* gene result in severe bone malformations and have been reported in patients with the Greig cephalopolysyndactyly [12] and Pallister-Hall syndromes [13]. With increasing bone length, the Ihh signal is not strong enough to release Gli3 repressor activity in the most distal cells. Continuous proliferation of the distal cells might consequently release more and more cells from the influence of Ihh and increase the population of future epiphyseal cells without disrupting the Ihh/PTHrP interaction.

Hypophosphatemia leads to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes

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Proc Natl Acad Sci USA 2005;102:9637–9642

Background: Rickets is seen in association with vitamin D deficiency and in several genetic disorders associated with abnormal mineral ion homeostasis. Studies in vitamin D receptor (VDR)-null mice have demonstrated that expansion of the late hypertrophic chondrocyte layer, characteristic of rickets, is secondary to impaired apoptosis of these cells. The observation that normalization of mineral ion homeostasis in the VDR-null mice prevents rachitic changes suggests that rickets is secondary to hypocalcemia, hypophosphatemia, or hyperparathyroidism, rather than impaired VDR action. The authors aimed to determine which of these abnormalities is responsible for impaired chondrocyte apoptosis and subsequent rachitic changes.

Methods: Two experimental models were used: diet-induced hypophosphatemia/hypercalcemia and hypophosphatemia secondary to mutations in the *PheX* gene. The former model is associated with suppressed parathyroid hormone levels as a consequence of hypercalcemia. The latter model demonstrates normal calcium and parathyroid hormone levels, but 1,25-dihydroxyvitamin D levels that are inappropriately low for the degree of hypophosphatemia.

Results: The data demonstrate that normal phosphorus levels are required for growth plate maturation and implicate a critical role for phosphate-regulated apoptosis of hypertrophic chondrocytes via activation of the caspase-9-mediated mitochondrial pathway.

Conclusion: Circulating phosphate is a key determinant of hypertrophic chondrocyte apoptosis.

Taken together, the models used in the current investigations support the hypothesis that circulating phosphate is a key determinant of hypertrophic chondrocyte apoptosis *in vivo*. Parallel studies in an *in vitro* culture model suggest that phosphate acts directly on hypertrophic chondrocytes rather than regulating the production of paracrine and/or endocrine factors that, in turn, promote apoptosis of these cells. Identification of a specific phosphate sensor or transporter expressed in hypertrophic chondrocytes that is responsible for these effects is a critical next step. Characterization of this sensor/transporter will identify the basis for the postulated threshold in circulating phosphate, below which impaired apoptosis is observed, as well as play a critical role in identifying potential therapeutic agents for the treatment for rachitic disorders.

New mechanisms

Smad4 is required for the normal organization of the cartilage growth plate

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Dev Biol 2005;284:311–322

Background: Members of the transforming growth factor- β (TGF- β) superfamily, including bone morphogenetic proteins (BMPs), play an important role in the regulation of bone development. However, the majority of BMP-mediated functions have been established using *in vitro* culture systems or overexpression systems due to the functional redundancy of a large number of ligands and receptors, as well as the critical role of BMPs during early embryonic development. Smad4 is the central intracellular mediator of TGF- β and BMP signaling.

Methods: To evaluate the role of *Smad4* in skeletal development, conditional mutation of the gene was employed using the Cre-loxP system in chondrocytes.

Results: *Smad4* was expressed strongly in prehypertrophic and hypertrophic chondrocytes. The knock-out of *Smad4* in chondrocytes caused a severely disorganized growth plate morphology characterized by expanded resting zone, reduced chondrocyte proliferation, accelerated hypertrophic differentiation, increased apoptosis and ectopic bone collars in the perichondrium. Additionally, knocking out *Smad4* decreased the expression of Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP). The cultured mutant metatarsal bones failed to respond to TGF- β 1, while the hypertrophic differentiation was largely inhibited by Sonic hedgehog (Shh), showing independence of Ihh/PTHrP and the *Smad4*-mediated TGF- β signals in the regulation of chondrocyte differentiation.

Conclusion: *Smad4* is a tumor suppressor that is critical for transmitting signals from TGF- β and related ligands. This is the first genetic evidence demonstrating that *Smad4*-mediated TGF- β signals inhibit the chondrocyte hypertrophic differentiation, and are required for the normal organization of chondrocytes in the growth plate.

The importance of bone morphogenetic proteins (BMPs) for normal bone development is unequivocal. Data presented in the current paper together with two recent reports [14, 15] indicate that unidirectional growth is, at least partly, determined by BMPs. *Smad4* is the comediator of BMP signaling. It forms heterodimers with receptor-regulated Smads, translocates to the nucleus and activates gene transcription. The present study is the first genetic evidence showing that conditional ablation of the *Smad4* gene results in disorganization of growth plate morphology and acceleration of hypertrophic differentiation. Moreover, the authors have demonstrated that Ihh/PTHrP inhibits differentiation of chondrocytes independent of the TGF β -*Smad4* signaling pathway, showing that the two fundamental systems of paracrine regulation of endochondral ossification, Ihh/PTHrP and BMPs, act independent of each other. Comparison of *Smad4* conditional knockouts with the recently described double *BMPR1a* and *BMPR1b* knockout mouse [14] reveals more abnormalities of the growth plate formation in case of BMP-receptor inactivation. This observation suggests that additional unknown signaling pathways are involved in BMP signaling in growth plate chondrocytes. Moreover, BMPs upregulate the expression of Sox9 and GADD45 β [16] both stimulating chondrocyte differentiation, whereas knocking out *Smad4* also stimulates differentiation of chondrocytes. These discrepancies demonstrate that our understanding of BMP-mediated regulation of bone development is far from complete. The next paper gives another hint on the importance of BMP/TGF β .

Maintenance of chondroitin sulfation balance by chondroitin-4-sulfotransferase-1 is required for chondrocyte development and growth factor signaling during cartilage morphogenesis

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Development 2005;132:3989–4003

Background: Proteoglycans are formed by attachment of polysaccharide chains of heparan sulfate or chondroitin sulfate (glycosaminoglycans) to core proteins. Glycosaminoglycan (GAG) synthesis includes the attachment of sulfate groups to specific positions of the polysaccharide chains by sulfotransferases. Chondroitin-4-sulfotransferase 1 (C4st1; also called carbohydrate sulfotransferase-11, *Chst11*) is an enzyme specific for the transfer of sulfate groups to the 4-O position in chondroitin. Proper GAG sulfation is essential for normal growth factor signaling and bone development. However, the biological significance of chondroitin sulfation remains unclear.

Methods: A gene trap mutation of the *C4st1* gene was created and mice harboring this loss-of-function mutation were generated. Bone development was analyzed in *C4st1* mutant mice by employing morphological and histochemical techniques. Gene expression was analyzed by in situ hybridization and immunofluorescence.

Results: *C4st1* gene ablation caused severe chondrodysplasia characterized by impaired hypertrophy of chondrocytes, elevated apoptosis and disorientation of chondrocyte columns. Moreover, a chondroitin

sulfation imbalance and mislocalization of chondroitin sulfate in the growth plate were demonstrated. Strong upregulation of TGF β signaling with concomitant downregulation of BMP signaling was observed in *C4st1* knockout mice. Interestingly, Indian hedgehog signaling was unaffected.

Conclusion: Chondroitin 4-O sulfation by *C4st1* is required for proper chondroitin sulfate localization, regulation of TGF β and BMP signaling and proper growth plate assembly. Overall, the data demonstrate an important role of proper chondroitin sulfation in normal bone development.

Over the last few years we have learned about the importance of sulfotransferases (STs) in steroid and thyroid hormone physiology. We now learn of another family of STs that affect the growth plate. Glycosaminoglycans (GAGs) such as heparan sulfate and chondroitin sulfate are long chains of repeating disaccharide subunits, which are covalently linked to core proteins to form proteoglycans. During their biosynthesis, a number of sulfotransferases modify the disaccharide through transfer of sulfate groups to specific positions on the sugar moieties. Mature proteoglycans constitute the cartilage extracellular matrix, which contributes significantly to the width of the growth plate, and are important in cell migration, proliferation and survival, as well as modulation of growth factor signaling. Ablation of chondroitin-4-sulfotransferase 1 (*C4st1*) in mice induces imbalanced TGF β /BMP signaling, increased apoptosis, impaired differentiation and, as a result, severe chondrodysplasia in the growth plates. Mutations in the *C4st1* gene have not yet been reported in humans, but mutations of chondroitin-6-sulfotransferase 1 (*C6st1*) are known to be associated with chondrodysplasia [17]. The constitutively active TGF β signaling observed in *C4st1*-deficient mice leads to impaired chondrocyte differentiation. Interestingly, constitutively active mutations of TGF β in humans are associated with Camurati-Engelmann disease [18] characterized by a thickening of the bone collar of the long bones. A similar increase in thickness of the bone collar has also been observed in *C4st1* knockout mice, suggesting that upregulation of TGF β signaling may resemble the bone phenotype observed in patients with Camurati-Engelmann disease. Furthermore, the authors report increased apoptosis in growth plate chondrocytes of *C4st1*-deficient mice. Increased chondrocyte apoptosis has previously been linked to diminished bone growth potential and bone malformations [19, 20]. Altogether, inappropriate GAG sulfation may cause severe growth abnormalities either due to elevated apoptosis, impaired TGF β /BMP signaling or both. The molecular mechanisms underlying these changes require further elucidation.

A novel role for GADD45 β as a mediator of MMP-13 gene expression during chondrocyte terminal differentiation

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Background: The growth arrest and DNA damage-inducible 45 β (*GADD45 β*) gene product has been implicated in stress response, cell cycle arrest, and apoptosis.

Methods: Mice deficient in *GADD45 β* were employed to reveal the functional role of *GADD45 β* in the regulation of longitudinal bone growth. Primary cultures of chondrocytes and stable cell lines were used to reveal the signaling pathways involved. Gene expression was analyzed by immunohistochemistry, in situ hybridization, micro-array and real-time PCR. Transient transfections were performed to study how *GADD45 β* affects the expression of MMP-13 and collagen type X.

Results: Expression of *GADD45 β* mRNA was demonstrated in the embryonic growth plate coincident with Runx2 protein in pre-hypertrophic chondrocytes, whereas *GADD45 β* protein was primarily detected in hypertrophic chondrocytes congruently with mRNA of the *Mmp-13* gene. In *Gadd45 β ^{-/-}* mouse embryos, impaired mineralization and decreased bone growth were observed as well as down-regulation of *Mmp-13* and *Col10a1* gene expression in chondrocytes of the hypertrophic zone. Small interfering RNA-*GADD45 β* blocked terminal differentiation of chondrocytes in vitro and abrogated expression of both *Mmp-13* and collagen type X. Finally, in synergy with Runx2, *GADD45 β* was able to stimulate *Mmp-13* promoter activity in chondrocytes through activation of the AP-1 transcription factor, which was activated presumably by JNK-mediated phosphorylation of JunD.

Conclusion: GADD45 β stimulates terminal chondrocyte differentiation through activation of the AP-1 transcription factor followed by upregulation of MMP-13 and collagen type X expression.

The growth arrest and DNA damage-inducible protein GADD45 β , otherwise implicated in the stress-response proteins that are associated with growth arrest, has been identified as a targeted protein for BMP-2, a well-known inducer of chondrocyte differentiation. The present work clearly demonstrates that GADD45 β is a crucial factor for proper hypertrophy of epiphyseal chondrocytes. The underlying mechanism involves stimulation of the expression of matrix metalloproteinase (MMP)-13, which is active against collagen II, and the expression of collagen type X. It has been reported that mutations of the *Col10a1* gene causes the Schmid type of chondrodysplasia [21], whereas mutations of the *Mmp-13* gene cause the Missouri variant of spondyloepimetaphyseal dysplasia [22]. Based on these clinical findings, it is likely that GADD45 β is involved in the regulation of longitudinal bone growth in man. However, so far, no mutation in this gene has been reported in humans.

New genes

Genetic linkage of human height is confirmed to 9q22 and Xq24

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Hum Genet 2006;119:295–304

Background: Human height is an important and heritable trait. Previous genome-wide linkage studies have suggested that 9q22 and Xq24 are linked to height.

Methods: A genome-wide linkage scan was performed in 3,726 Caucasians.

Results: A maximum LOD score of 4.34 was detected on 9q22 and a two-point LOD score of 5.63 was attained for Xq24. Interestingly, 9q22 harbors the ROR2 gene, which is required for growth plate development, and Xq24 was linked to short stature.

Conclusion: With the largest sample from a single population of the same ethnicity in the field of linkage studies for complex traits, this study provides overwhelming evidence substantiating 9q22 and Xq24 for height variation.

Several segregation analyses have suggested that, while generally under polygenic influence, human height is controlled by ‘major’ genes that contribute to a relatively large fraction of human height variation. Therefore, although there might be a large number of genes influencing height, only a limited number of them may have large effects that are detectable with feasible sample sizes, with the remaining genes contributing to the polygenic background. In all of the genome-wide linkage scans for height so far, among all the regions reported, only very few reached the significance threshold for linkage (LOD > 3.0) implicating the difficulty to detect height genes. The authors present the largest sample size and most likely, the highest statistical power in all the genome-wide linkage scans for height. The confirmation of 9q22 and Xq24 for their linkage to human height was supported by the significant linkage detected in both the total sample and in an independent sub-sample. Interestingly, an important gene for height development, receptor tyrosine kinase-like orphan receptor 2 (ROR2), exists on 9q22, and several syndromes of idiopathic short stature have been linked to Xq24. The ROR2 gene (MIM 602337) is selectively expressed in the chondrocytes of all developing cartilage anlagen and required for cartilage and growth plate development. Mutations of this gene cause Robinow syndrome (MIM 268310), a severe skeletal dysplasia with generalized shortening of limbs and segmental defects of the spine. Linked to the Xq24 region are several X-linked syndromes characterized by short stature, including the mental retardation with short stature (MRSS; MIM 300360) and the mental retardation with GH deficiency (MRGH; MIM 300123) syndromes. The above findings represent additional evidence suggesting the importance of 9q22 and Xq24 to human height variation. Unfortunately, this group of geneticists focus on LOD scores rather than giving us any information on the magnitude of the effect in terms of centimeters. If you have patients with

9q22 or Xq24 deletions, this is your turn to report them. We all have patients with Turner syndrome and isochromosome Xq, and thus 3 copies of the Xq24 locus.

Reviews

Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate

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Birth Defects Res C Embryo Today 2005;75:330–339

Background: This review describes the fate of the hypertrophic chondrocyte in the growth plate and the impact of the cartilage microenvironment on cell survival and apoptosis. The hypertrophic chondrocytes are directly involved in osteogenesis and terminally differentiated cells have been considered to undergo changes in shape, size, and phenotype, and assume the characteristics of an osteoblast. Some studies based on microscopic evaluation of cells that are present at the chondro-osseous junction have supported the notion of transdifferentiation. Although these investigations provided a novel perspective on endochondral bone formation, they were flawed by the failure to consider the importance of stem cells in osseous tissue formation. Subsequent studies indicated that many, if not all, of the cells of the cartilage plate die through the induction of apoptosis. With respect to agents that mediate apoptosis, solubilization of mineral and hydrolysis of organic matrix constituents generates high local concentrations of ions, peptides, glycans, and secreted matrix metalloproteins. A number of these agents serve as potent chondrocyte apoptogens.

Results/Conclusion: The authors present a new concept: hypertrophic cells die through the induction of autophagy. In the cartilage microenvironment, combinations of local factors cause chondrocytes to express an initial survival phenotype and oxidize their own structural macromolecules to generate ATP. While delaying death, autophagy leads to a state in which cells are further sensitized to changes in the local microenvironment. One such change is similar to ischemia reperfusion injury, a condition that leads to tissue damage and cell death. In the growth cartilage, an immediate effect of this type of injury is sensitization to local apoptogens. The authors emphasize the importance of the local microenvironment, in particular pO_2 , in directing chondrocyte survival and apoptosis.

Earlier investigations pointed to a direct role of the hypertrophic chondrocyte in the generation of endochondral bone. Although these experiments provided a novel perspective on endochondral bone formation, subsequent work indicated that many, if not all, of the cells of the cartilage plate die through the induction of apoptosis. In this review the authors focus on the control of apoptosis and they present a new concept: hypertrophic cells die through the induction of autophagy. Instead of immediately activating the cell death machinery, chondrocytes display an initial survival phenotype and oxidize their own structural macromolecules to generate ATP. The importance of oxidative stress as a stimulus for cell death has been studied using the ischemia reperfusion model. When this model is applied to the chondro-osseous junction, the stimulus for cell death is vascularization and oxygenation of the plate itself. From a clinical perspective, these new insights could be used to design therapies to modulate cell fate and bone growth. Therapeutic interventions that are now being utilized to modify the cells' reaction to reperfusion injury could potentially be applied to events that take place in the growth plate and thereby to control skeletal growth in children.

Retention of the matricellular protein SPARC in the endoplasmic reticulum of chondrocytes from patients with pseudoachondroplasia

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J Histochem Cytochem 2006;54:269–274

Background: Pseudoachondroplasia (PSACH) is a dominant disorder caused by a mutation in the large extracellular matrix protein COMP (cartilage oligomeric matrix protein) that is essential for normal cartilage formation. Chondrocytes from individuals with PSACH show distended rough endoplasmic reticulum organelles which have been shown to contain COMP, type-IX collagen and matrilin-3. The result is a reduced secretion into the extracellular matrix of these proteins as well as premature death of the chondrocytes which contributes to loss of linear growth potential. In this paper the role of the chaperone protein SPARC (secreted protein, acidic and rich in cysteine) in this process is investigated. A chaperone protein works by facilitating the proper folding of proteins to stabilize them and to allow, for example, export or degradation.

Methods: Costochondral chondrocytes were prepared and cultures of cartilage nodules were established from PSACH individuals with certain defined COMP mutations and from control individuals. The chondrocytes were immunostained with anti-SPARC.

Results: SPARC immunostaining gave a positive signal from the hypertrophic zone of the growth plate, specifically around and, in some cases, within the hypertrophic chondrocytes. In contrast, the secretion of SPARC from PSACH chondrocytes seemed altered by retaining SPARC intracellular with minimal levels of SPARC in the cartilage extracellular matrix.

Conclusion: Mutated COMP is retained within the endoplasmic reticulum together with its chaperone SPARC causing a severely diminished amount or quality of extracellular matrix in the growth plate and in articular cartilage. Unlike other misfolded proteins that are targeted for degradation, much of the retained COMP escapes degradation, compromises cell function, and causes cell death.

Expression of mutant cartilage oligomeric matrix protein in human chondrocytes induces the pseudoachondroplasia phenotype

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J Orthop Res 2006;24:700–707

Background: Over 70 mutations in the extracellular matrix protein COMP (cartilage oligomeric matrix protein) have been identified in skeletal dysplasias, pseudoachondroplasia (PSACH) and in one variant of multiple epiphyseal dysplasia (EDM1), which, to varying extents, give compromised linear growth as well as premature osteoarthritis. Misfolding of the COMP protein and its retaining within the rough endoplasmic reticulum (rER) of chondrocytes have been demonstrated in both PSACH and EDM1. These growth plates display characteristic large lamellar-appearing rER with sequestering of the normally secreted extracellular matrix protein matrilin-3 and type-IX collagen together with the mutated COMP. The paper reports an in vitro model that recapitulates the PSACH phenotype.

Methods: Costochondral chondrocytes were collected from patients undergoing pectus excavatum operations and the cells were cultivated for up to six passages. The cells were transfected with recombinant adenovirus containing wild-type or a mutant COMP gene together with a cytomegalovirus promoter to enhance the production of the green fluorescent reporter gene. Transfected and non-transfected cells were grown to form differentiated cartilage nodules that was maintained for 17 days.

Results: Mutant COMP expressed in normal cells was retained in enlarged rER cisternae, which also retained type-IX collagen and matrilin-3. Furthermore, a reduced secretion of these proteins from chondrocytes with mutated COMP was observed.

Conclusion: This should be a useful model for investigation of different COMP mutations compatible with either PSACH or EDM1.

Pseudoachondroplasia (PSACH) is a dominant disorder caused by a mutation in the large extracellular matrix protein COMP (cartilage oligomeric matrix protein) that is essential for normal cartilage formation. These two papers focus on the cellular effects of different COMP mutations known to cause PSACH. Mutated COMP is demonstrated to be retained within the endoplasmatic reticulum (ER) together with its chaperone SPARC causing a severely diminished amount or quality of extracellular matrix in the growth plate and in articular cartilage. From a clinical point it is worthwhile to recognize the unique growth pattern in PSACH: almost completely normal growth during the first year of life or so, followed by a continuous and uninterrupted deterioration in growth until final height that may be as short as 1 m. Contributing to this pattern is probably a continuous premature death of chondrocytes in the growth plate; a result of the increasing rough ER inclusions but also a diminished amount of extracellular cartilage matrix. These proteins were secreted normally into the extracellular matrix of wild-type COMP-transfected cells but in reduced quantities from cells transfected with mutated variant. The COMP mutations found in multiple epiphyseal dysplasia type-1, generally give only a slightly compromised growth but, similar to PSACH, go with premature osteoarthritis. The COMP molecule, being present both in the human growth plate and articular cartilage, may normally function as an integrator between the collagen-containing fibrils and the extrafibrillar matrix which comprises the cartilage-specific proteoglycan aggrecan and other molecules.

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