Bone, Growth Plate and Mineral Metabolism

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It has been an outstanding year of publications in the field of pediatric endocrinology. We have made a subjective selection of 19 papers and divided the chapter into 'bone', 'growth plate' and 'mineral metabolism' where each paper is discussed from a pediatric endocrine point of view. A clear breakthrough and **mechanism of the year** is the discovery of RANKL/RANK as key thermoregulators creating a link between bone physiology and central control of body temperature and fever. **A new paradigm** is represented by two papers discovering that serotonin regulates bone mass accrual in opposite directions depending on site of synthesis. A paper describing **a new mechanism** of IGF-1 to modulate thyroid hormone effects in the growth plate is also highlighted. We selected a few papers identifying **new genes** linked to important clinical conditions if mutated. These include *cyclophilin B* – linked to severe osteogenesis imperfecta, and *ENPP1* – linked to hypophosphatemic rickets. As **food for thought**, we included a paper disclosing how hibernating bears prevent bone loss.

New paradigms

OPG autoantibodies linked to bone loss in celiac disease

Osteoporosis associated with neutralizing autoantibodies against osteoprotegerin

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Background: Osteoporosis is a recognized complication of celiac disease and it is generally considered to be secondary to malabsorption and calcium and vitamin D deficiencies, rather than a consequence of an autoimmune process.

Methods: Serum samples were obtained from a 40-year-old male patient with celiac disease and autoimmune hypothyroidism, who developed high-turnover osteoporosis, as well as from 10 age-matched healthy male controls, 15 patients with celiac disease, and 14 patients with autoimmune hypothyroidism. Osteoprotegerin (OPG) immunoprecipitation assays and RANK signaling assays were performed.

Results: In Western blotting, OPG immunoprecipitated with patient's serum, while serum specimens from 10 healthy controls, 12 patients with celiac disease, and 14 patients with primary hypothyroidism were negative for the autoantibody. Three samples of 15 patients with celiac disease were positive, though at a lower intensity. RANK signaling assay demonstrated that NF-κB activation was inhibited by the addition of human recombinant OPG in the presence of the immunoglobulin fraction from control serum, but the inhibitory effect of OPG was lost in the presence of immunoglobulins purified from the patient's serum.

Conclusion: A high turnover osteoporosis in a patient with celiac disease was associated with the spontaneous development of autoantibodies against OPG.

This case report identifies a young man with autoimmune hypothyroidism, celiac disease and high bone turnover osteoporosis. A new pathophysiological mechanism of rapid bone loss was discovered: neutralizing autoantibodies against osteoprotegerin (OPG). The high-turnover osteoporosis, elevated alkaline phosphatase levels, and fragility fractures are consistent with the phenotype seen both in targeted OPG^{-/-} mice and humans with juvenile Paget's disease and OPG-inactivating mutations. His bone disease was very severe and despite the gluten-free diet, calcium and vitamin D supplementation, his osteoporosis worsened. However, a remarkable response to zoledronic acid treatment, which is a potent inhibitor of osteoclastic bone resorption, indicated that elevated bone turnover was the primary cause of this patient's osteoporosis. This strongly suggests that his acquired illness was due to the development of neutralizing autoantibodies against OPG. It is tempting to speculate that in this case, denosumab, i.e. RANKL antagonist, would have been an even more specifically targeted therapy. The mechanism by which OPG autoantibodies developed remains unclear, but presumably, endogenous OPG had become the target of an autoimmune response in this patient. They also demonstrated the presence of autoantibodies against osteoprotegerin in 3 of 15 patients with celiac disease. The 3 patients with the autoantibodies had lower bone mineral density values than those without the autoantibodies. Future studies should determine whether OPG autoantibodies are associated with the development or severity of osteoporosis in other patients with celiac disease or other autoimmune diseases.

New genes

Mutations in osteogenesis imperfecta go beyond collagen type I

Severe osteogenesis imperfecta in cyclophilin B-deficient mice

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Background: Osteogenesis imperfecta (OI) is an inherited disorder of collagen and majority of cases are caused by point mutations in the type I collagen genes COL1A1 and COL1A2. The syndrome is characterized by exquisitely fragile bones due to osteoporosis. OI also occurs in humans with homozygous mutations in prolyl-3-hydroxylase-1 (P3H1, encoded by the *LEPRE1* gene). P3H1 exists in a complex with CRTAP and cyclophilin B (CypB), encoded by the *Ppib* gene. Mutations in CRTAP cause OI in mice and humans, through an unknown mechanism, while the role of CypB in this complex has been unknown.

Methods: To study the role of mammalian CypB, the authors generated mice lacking this protein.

Results: In early life, *Ppib*^{-/-} mice developed kyphosis and severe osteoporosis. The morphology of collagen fibrils in *Ppib*^{-/-} mice was abnormal and in vitro studies revealed that procollagen did not localize properly to the Golgi in CypB-deficient fibroblasts. The authors found that the levels of P3H1 were substantially reduced in *Ppib*^{-/-} cells, while CRTAP was unaffected. Conversely, knockdown of either P3H1 or CRTAP did not affect cellular levels of CypB, but prevented its interaction with collagen in vitro. Furthermore, knockdown of CRTAP also caused depletion of cellular P3H1 and the post-translational prolyl-3-hydroxylation of type I collagen by P3H1 was essentially absent in CypB-deficient cells and tissues from CypB-knockout mice.

Conclusion: The data presented in this paper provide a significant new mechanistic insight into the pathophysiology of OI and reveal interactions of members of the P3H1/CRTAP/CypB complex in directing proper collagen formation.

Lack of cyclophilin B in osteogenesis imperfecta with normal collagen folding

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Background: OI is a heritable disorder causing bone fragility. Mutations in type I collagen result in autosomal dominant OI, whereas mutations in either of two components of the collagen prolyl 3-hydroxylation complex (CRTAP and P3H1) cause autosomal recessive OI with rhizomelia (shortening of proximal segments of upper and lower limbs) and delayed collagen folding.

Methods: The authors identified two siblings in a consanguineous Senegalese family. The siblings had recessive osteogenesis imperfecta without rhizomelia and their genomic DNA was screened.

Results: No mutations in *COL1A1*, *COL1A2*, *CRTAP* or *LEPRE1* were identified, but the siblings had a homozygous start-codon mutation in the *Ppib* gene resulting in a lack of CypB, the third component of the complex. The proband's collagen had normal collagen folding and normal prolyl 3-hydroxylation.

Conclusions: The data demonstrates that recessive OI can be caused by a homozygous start-codon mutation in the *Ppib* gene. The normal collagen folding and prolyl 3-hydroxylation however indicates that CypB is not the exclusive peptidyl-prolyl *cis-trans* isomerase that catalyzes the rate-limiting step in collagen folding, as is currently thought.

It is now clear that osteogenesis imperfecta (OI) is not caused only by mutations in collagen 1 α and 2 chain genes. Novel mutations associated with this disease have been found in the enzymatic complex which is needed to process collagen into mature fibrils. For example, *CRTAP*, reviewed in *Yearbook* 2007 [1], and *LEPRE1* [2] mutations have previously been shown to be linked to OI. The current study by Choi et al. demonstrates that mice lacking cyclophilin B, a third partner of the collagen hydroxylation enzyme complex, also show phenotypic features of OI. Interestingly, soon after the publication of this paper, Barnes et al. reported two OI siblings from a consanguineous family due to a homozygous start codon mutation in the peptidyl-prolyl isomerase B (*PPIB*) gene which results in a lack of cyclophilin B. They however observed a normal collagen folding and prolyl 3-hydroxylation in these patients, which is in contrast to the findings of Choi et al. showing that translational prolyl-3-hydroxylation of type I collagen by P3H1 was essentially absent in cells and tissues from CypB-deficient mice. Thus, the fundamental questions concerning the function of the 3-hydroxylase modifications, the role of CypB in the complex and the identity of the major collagen isomerase still need to be resolved. However, these data are useful in explaining the clinical and phenotypical variety of OI. Identifying novel mutations will furthermore improve the molecular diagnosis of OI in the future.

New hope

A novel role for TGF- β in bone remodeling

TGF- β_1 -induced migration of bone mesenchymal stem cells couples bone resorption with formation

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Background: Bone remodeling is an important biological process that depends on the precise coordination of bone resorption and subsequent bone formation. Disturbances in the balance of bone remodeling are associated with skeletal diseases, such as Camurati-Engelmann disease (CED).

Methods: This study used multiple methods, including in vitro cultures of osteoclasts and bone marrow stromal cells (BMSCs), as well as in vivo studies on transforming growth factor (TGF)- β_1 knockout mice and CED transgenic mice.

Results: Bone resorption released active TGF- β_1 , which induced the migration of BMSCs to the bone resorptive sites. The process was mediated through a SMAD signaling pathway. Analyzing mice carrying a CED-derived mutant TGFB1 (encoding TGF- β_1), which showed the typical progressive diaphyseal dysplasia seen in the human disease, high levels of active TGF- β_1 were found in the bone marrow. Treatment with a TGF- β type I receptor inhibitor partially rescued the uncoupled bone remodeling and prevented the fractures.

Conclusion: Since TGF- β_1 functions to couple bone resorption and formation, modulation of TGF- β_1 activity could be an effective treatment for diseases of bone remodeling.

Transforming growth factors- β (TGF- β) play an important role in bone metabolism and it has been hypothesized to function as a coupling factor that links bone resorption to bone formation. However, the molecular mechanisms have not been elucidated so far. This recent study by Tang et al. demonstrates that TGF- β_1 is the key chemoattractant that tightly couples bone degradation and formation in space and time. It induced the migration of bone marrow stromal cells to the bone remodeling area. In addition, the authors demonstrate that mice carrying a mutation in the TGF- β_1 gene develop Camurati-Engelmann disease, a disease characterized by progressive diaphyseal dysplasia, and express high levels of TGF- β_1 in the bone marrow. Interestingly, TGF- β receptor inhibition was shown to partially rescue uncoupled bone remodeling. This intriguing study provides evidence for the critical role of TGF- β in bone metabolism, but a few important points remain to be investigated. First, it should be clarified whether another important human TGF- β isotype (TGF- β_2) has a similar activity in bone marrow stromal cells as TGF- β_1 . Second, the potential impact of TGF- β inhibitors and stimulators in osteoblast differentiation should be investigated using in vivo systems. Even though this work reveals a novel role for TGF- β_1 in bone remodeling and suggests TGF- β_1 as a potential therapeutic target for bone diseases, we do not know how molecules affecting TGF- β could be delivered specifically and efficiently to target cells. These questions should be answered before considering potential clinical applications of TGF- β in the treatment of bone disease.

New hope

Embryonal stem (ES) and induced pluripotent stem (iPS) cells – powerful tools in bone biology

Directed differentiation of hematopoietic precursors and functional osteoclasts from human ES and iPS cells

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Background: The differentiation of human embryonic stem (ES) cells into multiple hematopoietic lineages is well established. In addition, recent discoveries in generating induced pluripotent stem (iPS) cells has further enabled understanding of specific embryonic lineages and opened new opportunities for patient-specific stem cells, drug discovery and studying disease mechanisms.

Methods: In this study, the stepwise generation of bone-resorbing osteoclasts from ES and iPS cells is reported. Human ES and iPS cells were maintained and differentiated towards monocyte-macrophage lineage and finally to functional osteoclasts.

Results: A precursor population enriched for cells expressing the monocyte-macrophage lineage markers CD14, CD18, CD11b, and CD115 was obtained by generation of a primitive streak-like population in embryoid bodies, followed by specification to hematopoiesis and myelopoiesis by vascular endothelial growth factor (VEGF) and hematopoietic cytokines in serum-free media. When cultured in the presence of essential osteoclast differentiation factors, i.e. macrophage colony-stimulating factor (M-CSF) and

receptor activator of nuclear factor- κ B ligand (RANKL), precursor cells formed large, multinucleated osteoclasts that expressed tartrate-resistant acid phosphatase and were capable of resorption. Molecular analyses confirmed the RANKL-dependent expression of the osteoclastic marker genes NFATc1, cathepsin K, and calcitonin receptor.

Conclusion: This study reports the possibility to generate large numbers of osteoclasts from human ES and iPS cells in a consistent, reproducible, and defined manner.

Osteoblasts are derived from mesenchymal stem cells, whereas osteoclasts are highly specialized, multinucleated cells that are derived from hematopoietic stem cells. Induced pluripotency as a tool for biological discovery was selected as the Method of the Year 2009 by *Nature Methods* [3]. Since the first reports of such a form of cellular reprogramming a few years ago, research on iPS cells has progressed on a vast speed. Although the potential of mouse ES cells to differentiate into osteoclasts has been reported previously, the current study by Grigoriadis et al. for the first time demonstrates the generation of osteoclasts from human ES and iPS cells. Since human osteoclasts are traditionally obtained by a time-consuming process of isolating and differentiating peripheral blood monocytes, the new method can be utilized to more efficiently identify novel antiresorptive compounds for osteoporosis, as well as for other conditions of accelerated bone resorption. In addition, the derivation of iPS cells from patients harboring cell-autonomous genetic mutations enables studying disease pathology. This can be done by evaluating how the individual mutations affect osteoclast differentiation and function in vitro. This study furthermore establishes a basis for genetic rescue and autologous cell-based therapies for disorders characterized by increased bone loss.

New mechanisms

Oxidative stress and osteogenesis

FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts

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Background: Osteoporosis, a disease characterized by low bone mass, is associated with decreased number of osteoblasts and increased levels of oxidative stress within osteoblasts.

Methods: Since transcription factors of the FoxO family confer stress resistance, the authors investigated their potential impact on skeletal integrity. They employed osteoblast-specific deletion of FoxO proteins in mice (*FoxO1^{ob-/-}*) and performed a vast range of histological, cellular, biochemical and molecular analyses.

Results: Among the three FoxO proteins, only FoxO1 was required for proliferation and redox balance in osteoblasts and thereby controlling bone formation. FoxO1 regulation of osteoblast proliferation occurred through its interaction with ATF4, a transcription factor regulating amino acid import, as well as through its regulation of a stress-dependent pathway influencing p53 signaling. Bone formation and bone mass in *FoxO1^{ob-/-}* mice was normalized by decreasing oxidative stress levels or increasing protein intake.

Conclusion: These results identify FoxO1 as a crucial regulator of osteoblast physiology and provide a direct mechanistic link between oxidative stress and the regulation of bone remodeling.

Forkhead box (Fox) proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity. The O subclass of human Fox proteins (FoxO) include FoxO1, FoxO3, FoxO4 and FoxO6. Oxidative stress enhances the activation of FoxO transcription factors that defend against oxidative stress by activating genes involved in free radical scavenging and apoptosis. In this report, the authors utilized osteo-

blast-specific deletion of FoxO proteins, and showed that only FoxO1 was required for proliferation and redox balance in osteoblasts. They also demonstrated that FoxO1 regulated osteoblast proliferation via interacting with activating transcription factor 4 (ATF4), as well as via influencing p53 signaling. Bone formation in mice lacking FoxO1 in osteoblasts returned to normal by a decrease in oxidative stress or an increase in protein intake. Interestingly, in another paper in the very same volume of *Cell Metabolism*, Ambrogini et al. [4] performed a simultaneous conditional deletion of all three FoxOs in mice and observed an increase in bone oxidative stress and osteoblast apoptosis, as well as decrease in osteoblast number, bone formation and bone mass. They furthermore demonstrated that overexpression of a FoxO3 transgene in mature osteoblasts decreased oxidative stress and osteoblast apoptosis, with an increase in osteoblast number, bone formation rate, and vertebral bone mass. Despite some inconsistency in the role of individual FoxOs, these papers nevertheless identify FoxO proteins as crucial regulators of bone formation and bone mass homeostasis in mice. They provide evidence for FoxO-dependent oxidative defense as a mechanism to protect against oxidative stress in osteoblasts. An increasing amount of evidence has linked oxidative stress with aging and the development of age-related diseases. Thereby it is tempting to speculate that impaired function or deletion of FoxO proteins could be involved in bone loss observed in pediatric conditions of early aging, such as Werner syndrome or Hutchinson-Gilford progeria.

Food for thought **How do hibernating bears prevent bone loss?**

Grizzly bears (*Ursus arctos horribilis*) and black bears (*Ursus americanus*) prevent trabecular bone loss during disuse (hibernation)

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Background: Reduced skeletal loading (disuse) causes an imbalance in bone formation and bone resorption, thereby leading to loss of cortical and trabecular bone. In contrast, bears completely prevent cortical bone loss by balancing intracortical bone remodeling during disuse (hibernation). Trabecular bone, however, is more detrimentally affected than cortical bone in other animal models of disuse.

Methods: The effects of hibernation on bone remodeling, bone architectural properties and bone mineral density (BMD) of grizzly bear (*Ursus arctos horribilis*) and black bear (*Ursus americanus*) trabecular bone were studied. Bones were obtained from hibernating (16–18 weeks after hibernation) and active (at least 14 weeks of physical activity following hibernation) grizzly bears. In addition, bones from black bears killed by hunters in the fall or in the spring were collected.

Results: No differences were observed in bone volume fraction or BMD between hibernating and active bears or between pre- and post-hibernation bears in the ilium, distal femur, or calcaneus. Even though indicators of cellular activity (mineral apposition rate, osteoid thickness) decreased, trabecular bone resorption and formation remained balanced in hibernating grizzly bears.

Conclusion: Bears appear to prevent bone loss during disuse by maintaining a balance between bone formation and bone resorption, which consequently preserves bone structure and strength. Further investigation on the mechanisms of preventing disuse-induced bone loss in bears may contribute to development of novel treatments for osteoporosis.

Bears hibernate for around 6 months of the year, and thereby experience annual periods of bone disuse and remobilization that are approximately equal in length. However, the material and structural properties of black bear (*Ursus americanus*) and grizzly bear (*Ursus arctos*) bone are not compromised with age or during hibernation. The important concept based on the data presented here and in previous reports from the same group is that hibernating bears possess a mechanism by which they do not lose bone (both trabecular and cortical) associated with disuse. No increase in cortical

porosity and no difference in trabecular bone mass were found after hibernation compared to before. In addition, trabecular bone showed no differences in eroded surface or osteoid surface, suggesting that the bone remodeling rate was not altered during hibernation. Balanced bone formation and resorption occurs likely to preserve calcium homeostasis during hibernation while the bears recycle (instead of excrete) catabolic waste products. The possible mechanisms could involve neuroendocrine control of bone remodeling and energy regulation, e.g. via PTH and serotonin. This is a fascinating study on an exceptional material and could lead to an expanded understanding of how to reduce the negative bone balance associated with disuse or age-related bone loss.

Important for clinical practice **A new disease gene for brachydactyly**

Deletion and point mutations of PTHLH cause brachydactyly type E

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Background: Brachydactyly type E (BDE [MIM 113300]) is characterized by a general shortening of metacarpals and metatarsals and/or phalanges. The genetic cause of the great majority of BDE cases is unknown.

Methods: As submicroscopic aberrations are a known cause for congenital limb malformations and brachydactylies, the authors performed array-based comparative genomic hybridization (array CGH) via a whole-genome oligonucleotide array (244K, Agilent Technologies).

Results: In a large pedigree with BDE, short stature, and learning disabilities, the authors detected a microdeletion of approximately 900 kb encompassing *PTHLH*, the gene coding for parathyroid hormone-related protein (PTHrP). Mutation analysis performed in these individuals identified two missense (L44P and L60P), a nonstop (X178WextX*54), and a nonsense (K120X) mutation in *PTHLH*. One of the missense mutations was tested in chicken micromass culture with a retroviral expression system and was shown to result in a loss of function.

Conclusion: Loss-of-function mutations in *PTHLH* cause BDE with short stature.

Brachydactylies are a family of limb malformations characterized by short hands and/or feet due to aplastic or hypoplastic skeletal elements, including Turner syndrome, or Albright hereditary osteodystrophy. The phenotype is variable even within families, ranging from moderate shortening of individual metacarpals to a shortening of all bones in the hands and/or feet. Isolated brachydactyly type E (BDE) is characterized by a general shortening of metacarpals and metatarsals and/or phalanges. BDE has been associated in sporadic cases with microdeletions of 2q37, as well as with mutations in *HOXD13* [MIM 142989] [5, 6]. However, the genetic cause of the great majority of BDE cases has remained unexplained. In a large pedigree with BDE, the authors detected a microdeletion encompassing *PTHLH*, the gene coding for parathyroid hormone-related protein (PTHrP). PTHrP is known to regulate the balance between chondrocyte proliferation and the onset of hypertrophic differentiation during endochondral bone development. Inactivation of PTHrP in mice results in short-limbed dwarfism due to premature differentiation of chondrocyte. The primary mediator of PTHrP/PTH receptor signaling in chondrocytes is *GNAS*. Haploinsufficiency of *GNAS* in mice results in premature differentiation of growth plate chondrocytes, thus resembling the phenotype described for PTHrP/PTH receptor as well as for *PTHLH*-deficient mice [7]. The here characterized pedigree with BDE adds another condition to the PTHrP-pathway disease family. The observation that heterozygous loss-of-function mutations in *PTHLH* result in a growth defect that is very similar to that observed in *GNAS* mutations suggests a particular importance for this component of the pathway in the growth of bones of the hands and feet.

Thyroid hormone-mediated growth and differentiation of growth plate chondrocytes involves IGF-1 modulation of β -catenin signaling

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Background: Terminal differentiation of growth plate chondrocytes is in part regulated by thyroid hormone through modulation of the Wnt/ β -catenin signaling pathway. Thyroid hormone is a known stimulator of IGF-1 receptor expression and IGF-1 has been described as a stabilizer of β -catenin.

Methods: Chondrocytes were isolated from the resting zone of the distal femoral growth plate of 2-day-old neonatal Sprague-Dawley rats and cultured in monolayer or 3D pellets.

Results: The authors show that IGF-1 and IGF1R (adenovirus transfection) stimulate Wnt-4 expression and β -catenin activation in growth plate chondrocytes. The positive effects of IGF-1/IGF1R on chondrocyte proliferation and terminal differentiation was partially inhibited by Wnt antagonists. T_3 activated IGF-1/IGF1R signaling and IGF-1-dependent PI3K/Akt/GSK3 β signaling in proliferative and prehypertrophic growth plate chondrocytes. T_3 -mediated Wnt-4 expression, β -catenin activation, cell proliferation and terminal differentiation of growth plate chondrocytes were partially prevented by inhibitors of the IGF1R and PI3K/Akt signaling.

Conclusion: The authors conclude that interactions between thyroid hormone and β -catenin signaling in regulating growth plate chondrocyte proliferation and terminal differentiation are modulated by IGF-1/IGF1R signaling through both the Wnt and PI3K/Akt signaling pathways.

Fig. 1. Schematic diagram of the proposed interactions between thyroid hormone, IGF-1/IGF1R, and β -catenin signaling pathways in regulating cell proliferation and terminal differentiation of growth plate chondrocytes.

Thyroid hormone is a systemic factor that potently regulates skeletal maturation in the growth plate and thyroid hormone receptor α (TR- α) and is essential for regulating the process of endochondral ossification. IGF-1 signals via the type 1 IGF receptor (IGF1R), both are expressed in the proliferating and prehypertrophic zone chondrocytes of the growth plate [8]. TR α 1 deleted mice have impaired *Igf1r* expression and IGF-1 signaling in the growth plate, suggesting that the IGF1R is a physiologic target for thyroid hormone action in the growth plate [9]. The investigators behind this report reported previously that thyroid hormone interacts with Wnt/ β -catenin signaling pathway in the terminal differentiation of growth plate chondrocytes [10]. They now present data supporting that IGF-1 is actively involved in the crosstalk between the Wnt/ β -catenin and PI3K/Akt pathways in the regulation of growth plate chondrocyte proliferation and differentiation by thyroid hormone. Thyroid hormone initially may stimulate IGF-1/IGF1R signaling in growth plate chondrocytes, activating the β -catenin signaling pathway through either the PI3K/Akt pathway or the Wnt pathway, in turn regulating the transcription of β -catenin responsive genes and subsequently promoting cell proliferation in the growth plate (fig. 1). Clinicians who initiate thyroxine treatment in hypothyroid children often observe a prominent catch-up growth soon after treatment start. At least partly this can now be explained by thyroid hormone interaction with IGF-1/IGF1R signaling in the growth plate.

New genes

A novel activator of *Ihh* that paces bone growth

Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating *Ihh* transcription

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Background: Ablation of activating transcription factor 4 (Atf4) in mice leads to severe skeletal defects, including delayed ossification and low bone mass, short stature and short limbs. Atf4 is expressed in proliferative and prehypertrophic growth plate chondrocytes, suggesting an autonomous function of Atf4 in chondrocytes during endochondral ossification.

Methods: Wild-type (WT) and *Atf4*^{-/-} embryos and mice were obtained by crossing *Atf4*^{+/-} mice. The authors also established Atf4-overexpressing chondrocytes.

Results: In *Atf4*^{-/-} growth plate, the typical columnar structure of proliferative chondrocytes was found to be disturbed. The proliferative zone was found to be shortened, whereas the hypertrophic zone transiently expanded. The expression of Indian hedgehog (*Ihh*) was markedly decreased, whereas the expression of other chondrocyte marker genes, such as type II collagen (*Col2a1*), PTH/PTHrP receptor (*Pth1r*) and type X collagen (*Col10a1*), was found to be normal. Furthermore, forced expression of *Atf4* in chondrocytes induced endogenous *Ihh* mRNA and *Atf4* was found to directly bind to the *Ihh* promoter and activate its transcription. Supporting these findings, reactivation of hedgehog signaling pharmacologically in mouse limb explants corrected the *Atf4*^{-/-} chondrocyte proliferation and short limb phenotypes.

Conclusion: The authors conclude that Atf4 acts as a novel transcriptional activator of *Ihh* in chondrocytes that paces longitudinal bone growth by controlling growth plate chondrocyte proliferation and differentiation.

The investigators behind this paper have previously shown that inactivation of the transcription factor *Atf4* in mice results in severe osteopenia, which is caused by a failure of *Atf4*^{-/-} osteoblasts to achieve terminal differentiation and to synthesize type I collagen, the main constituent of bone matrix [11]. This study reveals Atf4 as a crucial regulator of chondrogenesis and identifies *Ihh* as a transcriptional target of Atf4 in chondrocytes. Mice lacking Atf4 exhibit dwarfism and are characterized by markedly reduced growth plates, decreased chondrocyte proliferation and an abnormally

expanded hypertrophic zone. These phenotypic abnormalities are similar to those of *Ihh*^{-/-} mice [12], which, together with the dramatic decrease in *Ihh* expression observed in *Atf4*^{-/-} growth plates, indicate that *Atf4* and *Ihh* lie in the same genetic pathway regulating chondrogenesis during skeletal development. This study reveals a novel mechanism by which *Atf4* regulates chondrocyte proliferation and differentiation via upregulating *Ihh* expression. It remains unclear, however, what upstream signals regulate *Atf4* expression and activity and what downstream effector molecules of hedgehog signaling are controlling chondrocyte proliferation and differentiation. Many extracellular regulators, including members of the FGF, BMP, Igf1, Wnt and PTHrP families, are reported to regulate chondrocyte proliferation and differentiation. Whether they act on chondrocytes by modifying *Atf4* activity remains to be determined. It is also of great interest to study whether the same mechanisms are responsible for the bone defects seen in *Atf4*^{-/-} mice. No human disease has so far been identified linked to a mutated *Aft4*. Based on the phenotypic similarities between *Aft4* and *Ihh* mice, a mutated *Aft4* should be considered when you see a patient with features of brachydactyly A1 (MIM #112500), a disorder where a mutated *ihh* is found in some, but not all patients. The features to look for are short stature associated with middle phalanges of all the digits that are rudimentary or fused with the terminal phalanges.

New concerns

Loop diuretics may affect growth plate chondrocytes and bone growth

A key role for membrane transporter NKCC1 in mediating chondrocyte volume increase in the mammalian growth plate

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Background: The underlying mechanisms of growth plate chondrocyte volume increase and bone lengthening are poorly understood. The Na-K-Cl co-transporter (NKCC) is well known to be activated in many cell types leading to cell volume increase. The authors hypothesised that NKCC may be responsible for the volume expansion normally occurring in hypertrophic growth plate chondrocytes.

Methods: Metatarsals/metacarpals from 7-day-old rat pups (P7) were incubated in the presence/absence of the specific NKCC inhibitor bumetanide and whole bone lengths and histological analysis of growth plate measured after 24 h. Fluorescent NKCC was visualised by immunohistochemistry applying confocal laser scanning microscopy on rat (P7) tibial growth plates. Microarray analysis was performed on mRNA isolated from proliferative and hypertrophic zone cells of tibial growth plates from P49, P53, and P58 rats.

Results: Bumetanide resulted in a dose-dependent suppression of bone growth. Histological analysis showed that this was linked to a reduction in hypertrophic zone height. Quantification of fluorescence immunohistochemistry demonstrated a significant transition of NKCC from the intracellular space of proliferative cells to the cytosolic membrane of hypertrophic cells. In addition, microarray analysis showed an increase in NKCC1 mRNA between proliferative and hypertrophic cells.

Conclusion: The authors conclude that NKCC contributes to the volume increase normally occurring in hypertrophic growth plate chondrocytes. This statement was supported by data showing an increase of NKCC1 mRNA in hypertrophic zone cells, its cellular localisation, and reduced bone growth in the presence of the NKCC inhibitor bumetanide.

The growth plate is highly organized, with a 'reserve' zone preceding proliferative zone (PZ) chondrocytes. After a regulated period of time a marked differentiation occurs, signified by a dramatic increase in volume, resulting in the formation of hypertrophic zone (HZ) chondrocytes. The volume of a typical cell increases 15-fold in the HZ and it has been postulated that this cell volume increase occurs through a combination of classical hypertrophy and regulated volume expansion [13]. However, the mechanisms which drive growth plate HZ chondrocyte volume expansion are unknown.

The intracellular sodium concentration ($[Na^+]_i$) plays a key role in determining cell volume, and Na^+ is moved across the cytoplasmic membrane through a range of transport proteins. One of these, the Na-K-2Cl co-transporter (NKCC), hitherto known to act in neuronal tissues, is an obvious candidate for HZ chondrocyte expansion due to its ability to increase cell volume in other cell types [14]. NKCC mediates electro-neutral ion transport, and is characterized by its sensitivity to loop diuretics (e.g. bumetanide, furosemide). Two NKCC isoforms are known, the near ubiquitous NKCC1 and KCC2. Although considered to be present in all tissues only mRNA for NKCC1 has been reported in growth plates, where it demonstrates increased expression from the PZ to HZ [15]. In the present study, the authors tested the hypothesis that NKCC1 is involved with HZ chondrocyte volume increase and longitudinal bone growth. Whole metatarsal/metacarpal rudiments were incubated with the loop diuretic bumetanide, a specific NKCC inhibitor, which inhibited bone elongation by approximately 35%. If NKCC plays a major role in HZ chondrocyte volume increase, it would be expected to be under the same hormonal control that regulates bone growth. Indeed, NKCC has been shown to be regulated by cortisol in other tissue [16] but if this is the case also in the growth plate remains to be elucidated. It is important to point out that there are limited reports of skeletal growth retardation in children exposed to loop diuretics. Reduced growth might however go unreported due to the conditions which required prescription of loop diuretics. Further investigation is warranted to determine the regulatory control of NKCC in the mammalian growth plate and to the possible detrimental effect on bone growth with chronic exposure to loop diuretics in children.

New concerns

Aromatase inhibition may cause osteoporosis and polycystic ovaries; at least in female rats

Marginal growth increase, altered bone quality and polycystic ovaries in female prepubertal rats after treatment with the aromatase inhibitor exemestane

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Background: As a potential approach for growth enhancement in children with short stature aromatase inhibition has been proposed. The authors aimed to assess the effect and potential adverse effects of aromatase inhibition on growth in female rats.

Methods: Prepubertal Wistar rats were given intramuscular injections with placebo or the aromatase inhibitor exemestane (10, 30 or 100 mg/kg/week) for 3 weeks. A control group was ovariectomized (OVX). Weight and length gain, tibia and femur length, growth plate width, organ weights, and insulin-like growth factor I (IGF-I) levels were measured. In addition, the histology of ovaries, uterus and brain were analyzed and X-ray microtomography was applied to the femur.

Results: At 100 mg/kg/week (E100), exemestane significantly increased weight gain and growth plate width, but less prominently than OVX. In the femur, trabecular number and thickness were decreased in the metaphysis and epiphysis in both E100 and OVX rats. Importantly, E100 significantly decreased ovarian weight and multiple cysts were seen upon histological evaluation. No significant effects were found on IGF-I levels and brain morphology.

Conclusion: The authors conclude that at a high dose, exemestane marginally increases growth in female rats. However this is achieved at the expense of osteopenia and polycystic ovaries.

In the present article, the authors report the results of a study, in which sexually immature female rats were treated with the steroidal, irreversible aromatase inhibitor exemestane. The main conclusion is that exemestane partially inhibits aromatase activity, which caused only a marginal length gain and appendicular growth, but also resulted in multiple ovarian cysts and changes in bone archi-

texture consistent with early-stage osteopenia. Although aromatase inhibition was probably incomplete, pronounced adverse effects were found in ovarian histology and bone architecture, and it remains unclear whether these would have long-term permanent consequences for reproduction and fracture risk, respectively. Species differences in dependency on estrogen signaling probably underlie the discrepancies found in growth phenotype between these aromatase inhibitor treated rats and estrogen-deficient patients described in the literature. However, considering the potential side effects on bone physiology and morphology of the ovaries, treatment with aromatase inhibitors in girls in clinical practice should not be advised.

Follow-up on a YB 2009 paper Time for new nutritional recommendations for pregnant women?

Maternal vitamin D status determines bone variables in the newborn

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Background: Maternal vitamin D status may program neonatal skeletal development. The objective of this paper was to determine the association of mothers' vitamin D status with bone variables of their newborns.

Methods: 125 pregnant women participated in a longitudinal follow-up. The mean values for age, BMI before pregnancy, pregnancy weight gain, and total daily vitamin D intake in mothers were 31 years, 23.5 kg/m², 13.1 kg, and 14.3 µg, respectively. Blood was collected from mothers during the first trimester and 2 days postpartum, and from umbilical cords at birth for analysis of 25-hydroxyvitamin D (25-OHD), PTH, and bone remodeling markers. Bone variables were measured by pQCT on average 10 days postpartum. Bone contour was analyzed for the detection of total bone mineral density (BMD), bone mineral content (BMC), and cross-sectional area (CSA).

Results: The median value of the individual means for first trimester and 2-day postpartum 25-OHD level, 42.6 nmol/l, was used as cutoff to define two equal-sized groups (below or above median). Newborns of mothers with levels below median were heavier ($p = 0.05$), and 60% were boys. However, tibia BMC was 0.047 g/cm higher ($p = 0.01$), and CSA was 12.3 mm² larger ($p = 0.02$), but no difference in BMD was observed above median compared with below median group.

Conclusions: The data showed that although the mean total intake of vitamin D among mothers met current Nordic recommendations, 71% of women and 15% of newborns were vitamin D deficient during the pregnancy. Moreover, the results suggest that maternal vitamin D status affects bone mineral accrual during the intrauterine period and influences bone size.

Vitamin D regulates 3% of the human genome, including effects on bone health throughout life. Maternal vitamin D status during pregnancy may program skeletal development [17] and body composition in the offspring [18], by influencing the interaction between osteoblasts and adipocytes. Moreover, low maternal 25-OHD may be responsible for prematurity and in late pregnancy is associated with reduced intrauterine long bone growth and slightly shorter gestation [19]. This study examined the effect of maternal vitamin D status on skeletal variables of newborns. It confirmed that newborns whose mothers had mean 25-OHD levels above 42.6 nmol/l had higher tibial bone mineral content and higher cross-sectional area but not bone mineral density (BMD). It is important to emphasize that the average maternal intake of vitamin D in the study group was 14.3 µg, which meets current Nordic recommendations for pregnant women (10 µg or 400 IU). However, despite this, most of the mothers were vitamin D deficient throughout pregnancy. It is obvious that these recommendations need to be updated and adapted to modern lifestyle (indoors) and desired serum levels. The fact that BMD was not affected by maternal vitamin D status implies that in utero other hormones, such as PTHrP, which is secreted from the mammary glands and the placenta, promote adequate calcium supplementation to the fetus.

Loss-of-function ENPP1 mutations cause both generalized arterial calcification of infancy and autosomal-recessive hypophosphatemic rickets

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Background: The analysis of rare genetic disorders affecting phosphate homeostasis has led to the identification of several proteins essential for the renal regulation of phosphate homeostasis as FGF23. The authors considered ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) as a causal factor of hypophosphatemia, since it has been reported to occur in some patients with generalized arterial calcification of infancy (GACI).

Methods: The cohort comprised 60 index cases with hypophosphatemia who were found negative for *PHEX*, *FGF23* and *DMP1* mutations. Using homozygosity mapping, the authors identified a candidate region on chromosome 6q23, comprising approximately 35 genes, among them the *ENPP1* gene.

Results: A loss-of-function mutation in the *ENPP1* gene was found in members of four families affected with hypophosphatemic rickets.

Conclusion: *ENPP1* was identified as the fourth gene, following *PHEX*, *FGF23*, and *DMP1*, to cause, if mutated, hypophosphatemic rickets resulting from elevated FGF23 levels.

Autosomal-recessive hypophosphatemic rickets is associated with an inactivation mutation in the ENPP1 gene

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Background: Human disorders of phosphate handling and hypophosphatemic rickets have been shown to result from mutations in *PHEX*, *FGF23*, and *DMP1*, presenting as X-linked recessive, autosomal-dominant, and autosomal-recessive patterns, respectively.

Methods: An extended Bedouin family with hypophosphatemic rickets and 236 Bedouin control individuals of the same geographic region were investigated by homozygous mapping.

Results: The authors identified an inactivating mutation in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) gene causing autosomal-recessive hypophosphatemic rickets (ARHR).

Conclusion: These results suggest a different pathway involved in the generation of ARHR, and possible additional functions for *ENPP1*.

These two papers extend our knowledge of hypophosphatemic rickets, while evoking unresolved questions. Last year we discussed hypophosphatemia as the common denominator of all rickets [20]. The three main causes for hypophosphatemia are high PTH, elevation in FGF23 and renal P loss. Now we learn that mutations in the *ENPP1* gene can cause either of two diseases – generalized arterial calcification of infancy (GACI) or hypophosphatemic rickets. Interestingly, the same mutation seems responsible for these two entities, as discussed in the article by Lorentz-Depiereux et al. *ENPP1* encodes the NPPI enzyme that generates pyrophosphate from ATP in the external cellular matrix. Pyrophosphate is the major hydroxyapatite inhibitor. In the absence of pyrophosphate, hydroxyapatite production is accelerated, and is accompanied by excessive secretion of collagen type I to produce the primary bone elements. In children with GACI who survived, reductions in phosphorous levels and in Tmp/GFR were observed during the second year of life. In some cases, FGF23 was ele-

vated. It is still not clear why in some patients the main phenotype is GACI, and in others hypophosphatemic rickets.

Mechanism of the year

RANKL/RANK as key thermoregulators – link between bone physiology and central control of body temperature and fever

Central control of fever and female body temperature by RANKL/RANK

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Background: Receptor-activator of NF- κ B ligand (TNFSF11, also known as RANKL, OPGL, TRANCE and ODF) and its tumor necrosis factor (TNF)-family receptor RANK are essential regulators of bone remodeling, lymph node organogenesis and formation of a lactating mammary gland. RANKL and RANK are also expressed in the central nervous system.

Methods: In this study tissue-specific Nestin-Cre and GFAP-Cre rank(floxed) deleter mice were used. Two patients with autosomal-recessive osteopetrosis were also included in the study.

Results: In both mice and rats, central RANKL injections triggered severe fever. The function of RANK in the fever response was genetically mapped to astrocytes. Importantly, Nestin-Cre and GFAP-Cre rank(floxed) deleter mice were resistant to lipopolysaccharide-induced fever as well as to fever in response to the key inflammatory cytokines IL-1 β and TNF- α . RANKL activated brain regions involved in thermoregulation and induced fever via the COX2-PGE₂/EP3R pathway. Moreover, female Nestin-Cre and GFAP-Cre rank(floxed) mice exhibited increased basal body temperatures, suggesting that RANKL and RANK control thermoregulation during normal female physiology. In addition, 2 patients with RANK mutations were found to exhibit impaired fever response during pneumonia.

Conclusion: The data identify a novel function for the important osteoclast differentiation factors RANKL/RANK in female thermoregulation.

In the last years we have learned that RANKL and RANK play a major role in the activation of osteoclasts; in the absence of RANK activity, osteopetrosis develops. Surprisingly, it was shown that the RANKL/RANK regulate formation of lactating mammary gland during pregnancy [21]. Now we learn that RANK protein is also specifically expressed in the preoptic area (POA) and the medial septal nucleus (MSn). It directly connects bone physiology to the central control of female body temperature, as well as to fever in inflammation. In male mice loss of RANK in neurons had no significant effect on basal circadian body temperatures while genetic inactivation of RANK in female mice brain resulted in altered physiological thermoregulation that is regulated also by ovarian sex hormones. In this elegant study the authors have shown that RANK is a critical mediator of fever in response to inflammation in mice. However, the clinical observation in 2 siblings with homozygous RANK mutation and severe osteopetrosis, exhibiting an impaired fever response to pneumonia, suggests that RANKL/RANK regulates fever also in humans.

New paradigms

Serotonin regulates bone mass accrual in opposite directions depending on the site of synthesis

A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure

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Background: Leptin inhibition of bone mass requires the integrity of specific hypothalamic neurons but not expression of its receptor on these neurons. The same is true for its regulation of appetite and energy expenditure. This suggests that leptin acts elsewhere in the brain to achieve these three functions.

Methods: In this study, mice were generated by embryonic stem cell manipulation to obtain Tph2^{+/-} (tryptophan hydroxylase-1), and thereafter were intercrossed. For further analyses, immunohistochemistry, in situ hybridization, axonal tracing, and microcomputed tomography as well as measurement of serotonin levels in the brain, deoxypyridinoline in the serum and urinary elimination of catecholamines were performed.

Results: The authors showed that brainstem-derived serotonin (BDS) favors bone mass accrual following its binding to Htr2c receptors on ventromedial hypothalamic neurons, and appetite via Htr1a and 2b receptors on arcuate neurons. Leptin inhibits these functions and increases energy expenditure because it reduces serotonin synthesis and firing of serotonergic neurons.

Conclusion: The authors conclude that their study modifies the map of leptin signaling in the brain and identifies a molecular basis for the common regulation of bone and energy metabolism.

Pharmacological inhibition of gut-derived serotonin synthesis is a potential bone anabolic treatment for osteoporosis

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Background: As gut-derived serotonin (GDS) inhibits bone formation, the authors asked whether hampering its biosynthesis could treat osteoporosis through an anabolic mechanism.

Methods: They synthesized an inhibitor of tryptophan hydroxylase-1, the initial enzyme in GDS biosynthesis, and this small molecule was orally administered once daily for up to 6 weeks in ovariectomized rodents.

Results: Oral administration of this small molecule once daily for up to 6 weeks acted as a bone metabolic agent fully rescuing gonadectomy-induced bone loss. The rescue was due to an increase in osteoblasts number and bone formation rate, whereas the osteoclast surface per bone surface was unaffected.

Conclusion: The authors concluded that these results provide a proof of principle that inhibiting gut-derived serotonin biosynthesis could become a new bone anabolic treatment for osteoporosis.

These two papers demonstrate the complexity of bone mass regulation by serotonin. Serotonin is probably the first example of a molecule whose influence on bone mass accrual depends on the site of synthesis: Gut-derived serotonin inhibits bone mass while brainstem-derived serotonin (BDS) favors bone density. We emphasize that although BDS, acting as a neurotransmitter, accounts for only about 5% of the total pool of serotonin in the body, its influence on bone remodeling is dominant over gut-derived serotonin (GDS). It has been shown in the past that leptin inhibits bone mass accrual by the activation of the sympathetic activity. Just the opposite, BDS decreases sympathetic activity. In this paper Yadav et al. demonstrated that leptin inhibits serotonin synthesis in Thp2 (tryptophan

Fig. 2. Depending on site of synthesis, serotonin regulates bone mass in opposite directions: duodenum-derived serotonin acts on osteoblasts through the Htr1b receptor and Creb (cAMP response element binding; transcription factor) response to inhibit their proliferation while brainstem-derived serotonin favors bone mass accrual following its binding to Htr2c receptors on ventromedial hypothalamic neurons and appetite via Htr1a and 2b receptors on arcuate neurons [23].

hydroxylase 2) expression neurons. The net effect is activation of the sympathetic system to reduce bone mineral density. In the second paper, the authors show that an inhibitor of GDS synthesis acts as a bone anabolic agent, and can fully rescue gonadectomy-induced bone loss. Considering the lower rate of bone remodeling in rodents, as compared to humans, as well as the lack of haversian remodeling in the former, these results need be confirmed in humans. Rodents grow throughout their lives and do not remodel their cortical bone. This biology is now harnessed with an orally available inhibitor of gut serotonin synthesis that promotes bone formation in rodents without altering brain serotonin [22] which highlights the significance of serotonin as a modulator of bone metabolism (fig. 2).

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