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Review

Mechanistic and therapeutic insights gained from studying rare skeletal diseases

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ABSTRACT

Rare bone diseases account for 5% of all birth defects and can cause significant morbidity throughout patients' lives. Significant progress is being made to elucidate the pathophysiological mechanisms underlying these diseases. This paper summarizes presentation highlights of a workshop on Rare Skeletal Diseases convened to explore how the study of rare diseases has influenced the field's understanding of bone anabolism and catabolism and directed the search for new therapies benefiting patients with rare conditions as well as patients with common skeletal disorders.

Contents

Introduction

Genetic bone diseases are an important cause of disability in the US and remain difficult to diagnose and treat owing to variability in disease

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expression and symptom severity [1]. The interconnection between the different components of bone—cells, vasculature, and matrix—makes it challenging to dissect the biological mechanisms affected by these rare disorders. New methods for imaging the skeleton, performing massively parallel sequencing of DNA and RNA, creating animal models of human skeletal disease, and studying the consequences of mutation at the single cell level, have facilitated our understanding of fundamental mechanisms responsible for skeletal growth and homeostasis. Many new discoveries that are relevant to persons affected by common skeletal disorders such as osteoporosis and osteoarthritis have their origins in the study of patients with rare bone diseases. Thus, studying rare skeletal diseases has improved our understanding of skeletal biology and contributed to the development of new approaches for improving bone health.

This manuscript summarizes twelve lectures delivered by invited speakers at an NIH-supported workshop on Rare Bone Diseases held on September 11, 2014, in Houston, TX, and attended by more than 250 clinicians and scientists.

The nosology of rare bone diseases — Krakow

Dr. Deborah Krakow provided examples of clinical, radiologic, and biochemical characterizations of rare skeletal diseases facilitating the discovery of pathways and processes involved in skeletal patterning, growth, and homeostasis.

Heritable skeletal disorders form a heterogeneous group of more than 450 well-defined diseases, resultant from mutations in more than 200 genes. Initial attempts at classification relied primarily on radiographic and clinical findings and distinguished between skeletal dysplasias, a general condition affecting bone and cartilage, and dysostoses, a disorder manifested by abnormalities in an individual or group of bones [2]. Despite variability in the clinical manifestations and severity of these pathological states, significant phenotypic overlap posed a challenge in disease recognition and risk assessment, creating the need for a comprehensive, systematic classification that could guide diagnosis.

Spranger first proposed the concept of 'bone families' in the 1980s as disorders with common phenotypic, radiographic, and histologic features sharing the same underlying pathophysiological mechanisms and/or molecular pathways [3]. With the advent of molecular biology, diseases once thought to be distinct entities are now grouped according to the gene(s) and pathway(s) affected. For example, mutations in filamin A account for conditions displaying wide variability such as Melnick–Needles syndrome, otopalatodigital syndromes and frontometaphyseal dysplasia [2]. By contrast, similar clinical features may be due to mutations in different genes, as in the case of the multiple epiphyseal dysplasia group of diseases, associated with defects in cartilage oligomeric matrix protein, collagen type IX, and matrilin 3, all thought to similarly participate in the assembly of the extracellular matrix (ECM) [2]. Polygenic disease entities may in fact share the same mechanistic basis. Osteogenesis imperfecta (OI), for instance, is attributed to the action of several dysfunctional proteins, previously deemed unrelated and now recognized to be involved in common processes in mineralization and signaling in the matrix. The study of OI has allowed biologists to identify interactions between gene products that would have never been predicted. Further, new pathological components have been identified as a result of recognizing that a gene associated with a specific bone disorder is also responsible for symptoms affecting other organs and systems. Gain-of-function mutations in transient receptor potential cation channel subfamily V member 4 (TRPV4), a calcium channel, result in distinctive phenotypes: mild brachyolmia, spondylometaphyseal dysplasia, Kozlowski type, and the more severe metatropic dysplasia [2]. Notably, Charcot–Marie–Tooth disease type 2C is also caused by defects in this receptor [4], an observation that led to the identification of a pathological neuromuscular component in the TRPV4 family of disorders [5].

The most recent revision of the Nosology and Classification of Genetic Skeletal Disorders combines pathological, histologic, and biochemical information, as well as molecular and developmental aspects, to categorize recognized disease entities into 40 distinct groups [2]. The disease classification scheme goes beyond its role in assisting diagnosis and informing treatment and counseling, by suggesting links between molecules and pathways. Serpentine fibula-polycystic kidney syndrome, thought to be a filamin-related disorder based on clinical and radiographic phenotype, clearly illustrates this concept. However serpentine fibula-polycystic kidney syndrome and the rare Hajdu–Cheney syndrome are caused by truncating mutations in neurogenic locus notch homolog 2 (NOTCH2) [6]. This raises the interesting hypothesis of filamin involvement in the NOTCH signaling pathway.

The classification of rare bone diseases has undoubtedly delineated important elements of normal and diseased bone physiology and contributed to improved diagnosis. In the age of molecular medicine, and despite some unsolved entities, nosological schemes enable clinicians to quickly recognize signs and symptoms, establish otherwise unforeseen mechanistic relationships, and potentially identify new therapeutic targets.

Skeletal elements affected in rare bone disorders

Drs. Lynda Bonewald, Stuart Ralston, Maurizio Pacifici, Bjorn Olsen, and Brendan Lee gave examples of rare bone diseases yielding insights about independent and coordinated functions of bone cells, vasculature, and matrix on skeletal health.

Osteoblasts and osteocytes — Bonewald

Osteoblasts have a vital role in bone homeostasis and undergo a tightly regulated differentiation process. Runt-related transcription factor 2 (RUNX2) is an early mediator of osteoblast specification and direct regulator of OSTERIX (OSX), a member of the Sp zinc-finger transcription factor family that further specifies the osteoblastic lineage [7]. Deficiencies in osteoblast-specific proteins such as type I collagen and tissue non-specific alkaline phosphatase (TNSALP) have been associated with OI and hypophosphatasia, respectively [2]. Osteocytes, which constitute over 95% of all bone cells in the adult skeleton, originate from the terminal differentiation of osteoid-producing osteoblasts that become embedded in the bone matrix. They have endocrine and mechanosensory functions in bone remodeling and are able to establish and direct communication between themselves and the bone surface by extending and retracting their cellular processes into the bone marrow and vascular spaces. Osteocytes also regulate osteoclasts through receptor activator of nuclear factor kappa-B (NFκB) ligand (RANKL) and produce osteoblast-modulating factors such as sclerostin, encoded by the sclerostin gene (*SOST*) [8]. Deleterious mutations in SOST, a strong inhibitor of bone formation responsive to mechanical load via the wingless-related integration site (Wnt)/β-catenin signaling pathway, result in sclerosing bone syndromes, whereas activating mutations in fibroblast growth factor 23 (FGF23), also highly expressed in osteocytes, cause autosomal dominant hypophosphatemic rickets [2]. Autosomal recessive hypophosphatemic rickets is due to defects in dentin matrix acidic phosphoprotein 1 (DMP1), and a sex-linked form of the disease is caused by mutations in phosphate-regulating neutral endopeptidase on chromosome X (PHEX) [9]. DMP1 and PHEX downregulate FGF23 signaling, which is actively involved in the systemic regulation of phosphate metabolism, essential for bone mineralization [10]. Elevated levels of circulating FGF23 also exert pathological effects in the heart by inducing vascular calcification in patients with chronic kidney disease [11].

Although therapeutic approaches to bone disease have traditionally focused on osteoblasts, current research supports the use of agents targeting osteocyte-specific proteins such as sclerostin. Anti-sclerostin antibodies reduce bone loss and promote fracture healing in animal

models of OI, and monoclonal antibodies (mAbs) romosozumab and blosozumab are currently undergoing clinical evaluation in the context of osteoporosis [12]. Likewise, treatment with an antibody targeting FGF23 restores serum phosphate levels and bone defects in mice and patients with X-linked hypophosphatemic rickets [12,13]. Finally, denosumab, an inhibitor of RANKL, greatly reduces the risk of fractures in post-menopausal women with osteoporosis through inhibition of osteoclast differentiation [14]. Data from this and other phase III studies supported the marketing authorization of the agent for the treatment of osteoporosis.

While many aspects of the important conversion of osteoblasts into osteocytes remain unknown, including the putative role of mineralization and the action of Wnt/β-catenin signaling in triggering this transition, future research studies addressing these questions will certainly reveal new osteoblastic and osteocytic therapeutic targets in the context of bone diseases.

Osteoclasts — Ralston

The study of hereditary rare bone diseases has contributed to the understanding of the osteoblastic lineage, but also of monocyte-derived osteoclasts, known to be involved in disease states affecting bone mass. Oligogenic Paget's disease of bone (PDB), the result of increased osteoclastic activity or number, is characterized by osteolytic lesions with high bone turnover, pain and fractures, whereas in osteopetrosis impaired differentiation and function of the bone-resorptive cells result in increased bone mass associated with bone marrow failure, pathological fractures, osteoarthritis, and osteomyelitis. Osteoclast-rich and -poor osteopetrosis subtypes present distinct genetic causes. Loss-of-function mutations in the RANKL/RANK system deplete the osteoclastic cellular reserves, and deficiencies in carbonic anhydrase and in bone-matrix-degrading protein cathepsin K hamper osteoclast function [15]. Not surprisingly, odanacatib, an inhibitor of cathepsin K, induced impressive improvements in bone mass density in phase III clinical trials [16]. Likewise, predisposing genes for PDB are involved in important osteoclastic processes and constitute feasible therapeutic targets. In opposition to what occurs in osteopetrosis, gain-of-function mutations in RANK explain the clinical manifestation in PDB-like syndromes such as familial expansile osteolysis and early-onset PDB, whereas loss-of-function mutations in osteoprotegerin (OPG), an inhibitor of osteoclastogenesis, underlie the pathological features of juvenile PDB. Classical PDB, by contrast, may result from alterations in genes coding for macrophage colony-stimulating factor 1 [17], which promotes osteoclast differentiation from mesenchymal stem cells, and p62, a protein involved in RANKL/NFκB signaling [18]. New genes identified by genome-wide association studies include those coding for optineurin [17], a negative regulator of osteoclast differentiation, and for Ras and Rab interactor 3, with unknown function. Based on past findings, it is not unreasonable to expect that these new factors may constitute relevant targets in the treatment of bone diseases, but the role of the products of these genes transcends therapeutic intervention. The Zoledronate In the Prevention of Paget's disease (ZIPP) study (ISRCTN11616770) is currently testing the use of genetic markers to target treatment. Individuals with a family history of PDB are screened for mutations in *SQSTM1*, the gene coding for p62, and are subsequently randomized to receive zoledronic acid or placebo and evaluated for bone lesions for five years. This approach aims to identify undiagnosed, asymptomatic participants who may benefit from early treatment in order to reduce the risk of irreversible complications associated with the disease.

The knowledge acquired from the study of diseases associated with osteoclast dysfunction has greatly improved our understanding of normal osteoclast biology. The continuing dissection of their molecular mechanisms enables the identification of therapeutic targets and potentially of genetic markers that can be used to inform patient treatment plans.

Chondrocytes — Pacifici

Most skeletal elements, including long bones, vertebrae and cranial base, are endochondral structures and thus chondrocytes are key players in their embryonic formation and postnatal growth and repair. Chondrocytes originate from condensed preskeletogenic mesenchymal cells, and quickly organize in the growth plate where they undergo proliferation and hypertrophy and are largely replaced by bone and marrow cells. Several transcription factors and signaling pathways, such as sex-determining region Y-box 9, 5 and 6 (SOX9, SOX5 and SOX6) and RUNX2 and fibroblast growth factor (FGF), Bone morphogenetic protein (BMP) and WNT signaling proteins, are involved in this intricate process which, when disrupted, leads to aberrant growth plate development and function [19]. Rare skeletal disorders have provided critical clues into the functioning of growth plate chondrocytes, and following are two important examples.

Hereditary multiple exostoses (HME) syndrome, characterized by the formation of benign cartilage tumors (exostoses), illustrates the detrimental impact of growth plate disruption. In 2–3% of the cases, exostoses may lead to chondrosarcoma, a particularly aggressive bone cancer with poor prognosis. Interestingly, exostoses always form next to, but never within, the growth plate, unlike other types of benign cartilage tumors such as endochondromas. Most HME cases are caused by a deficiency in glycosyl synthases exostosin (EXT) 1 and EXT2, which results in systemic deficiency in heparan sulfate (HS) [20]. HS, a component of cell-surface and matrix-associated proteoglycans, binds and stores regulatory factors such as FGFs, WNTs, BMPs, and vascular endothelial growth factor (VEGF), and regulates their distribution and activity in the growth plate [21], thus maintaining the integrity of the growth plate itself as well as of the growth plate–perichondrial border. Functional defects in this important border result in exostosis formation. Also, ectopic activation of prochondrogenic BMP signaling along the perichondrium occurs prior to exostosis formation, as observed in a perichondrium-specific *Ext1* knockout mouse [22]. Moreover, inhibition of HS synthesis and function in vitro with heparanase or the small molecule Surfen stimulates chondrogenesis and is accompanied by increased SMAD phosphorylation and BMP receptor (BMPR) expression. Indeed, cells obtained from patients with HME show higher levels of heparanase, suggesting that the inhibition of this endoglycosidase in vivo may represent a therapeutic strategy to prevent the formation of exostoses [23].

In disorders affecting the autopod skeleton, inactivating mutations in growth differentiation factor 5 (GDF5), a member of the transforming growth factor-beta (TGF-β) superfamily, have recently been identified in patients with specific forms of brachydactyly, and active forms of the protein cause multiple synostoses syndrome 2 [2]. A unique mutant form of GDF5 harboring a tryptophan-to-arginine change in amino acid 414 (W414R) found in a family has recently been shown to possess concurrent gain- and loss-of-function properties; it is resistant to Noggin—a natural antagonist of GDF5—and hence more active, but it also has reduced capacity to activate BMPR1A [24]. The gain-of-function property likely caused multiple synostoses traits in the patients, while the loss-of-function led to brachydactyly. GDF5 is thus a key determinant of the shape, size and growth of skeletal elements directly or indirectly affecting growth plate activity, and the multiple joint fusions observed in patients with multiple synostoses syndrome also likely reflect a failure to maintain the functionality of tissue–tissue borders in mutant skeletal elements.

The above examples reiterate the fact that the activity of the growth plate determines the pace and extent of overall skeletal growth and skeletal morphogenesis, and research focusing on rare conditions affecting its function has provided critical insights on skeletal development and growth and synovial joint formation. How complex signaling networks maintain the integrity and functionality of tissue–tissue borders in the skeletal system remains, however, one of the most intriguing mysteries in skeletal biology.

Vasculature — Olsen

Osteoblast differentiation is closely connected to vascular development. During early bone formation, osteoblast progenitor cells in the perichondrium migrate into primary ossification centers and respond to VEGF signaling originated in hypertrophic chondrocytes. VEGF is also produced by bone progenitor cells and osteoblasts to stimulate perichondrial angiogenesis and osteoblast differentiation. A third participant in this signaling network, the endothelium, stimulates osteoblast activity and VEGF secretion through hypoxia-inducible factor 1 alpha (HIF1α) [25,26]. It is not surprising then that abnormalities in angiogenesis can cause significant skeletal malformations. In fact, germline inactivating mutations in phosphatase and tensin homolog (PTEN) in Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome manifest as hamartomatous growths [27], which may be in fact an indirect effect of vascular disfunction or a direct effect of bone disfunction. A direct effect on bone cells in humans is supported by the presence of increased numbers of osteoblasts and bone progenitors in a conditional *Pten* null mouse [28].

In Klippel–Trenaunay syndrome, vessel defects go hand in hand with bone hypertrophy. Mutations in phosphatidylinositol 4,5-bisphosphatase 3-kinase catalytic subunit alpha (PIK3CA) have been identified in most of these patients, as well as in patients with congenital, lipomatous, overgrowth, vascular malformations, epidermal nevi and spinal/skeletal anomalies and/or scoliosis (CLOVES) syndrome [29], in which bone overgrowth may be the result of a paracrine signaling defect involving VEGF. A similar mechanism may be at work in the rare Proteus syndrome, caused by somatic mosaic mutations in RAC-alpha serine/threonine protein kinase (AKT1), also an active participant in VEGF signaling [30]. Other rare syndromes such as Sturge–Weber, the result of pernicious mosaic gain-of-function mutations in guanine-nucleotide-binding protein G(q) subunit alpha (GNAQ), substantiate a possible defect in paracrine signaling from surrounding tissues [31]. In Gorham–Stout disease, characterized by massive osteolysis and hemangiomatosis, VEGF secreted by osteoblasts may induce vascularization, while absence of nuclear VEGF most likely affects osteoblast differentiation [32]. Recent data demonstrated the role of tumor endothelial marker 8 (TEM8) in the pathophysiology of hemangiomas [33] and growth retardation, alopecia, pseudoanodontia, and optic atrophy (GAPO) syndrome [34] through regulation of VEGF signaling in endothelial cells. TEM8 forms a complex with VEGFR2 in endothelial cells and inhibits transcription of VEGFR1, thus protecting the cells from excessive signaling. In hemangiomas, the TEM8 complex becomes inactive, leading to hyperactive VEGF-dependent VEGFR2 signaling.

As expected, the dynamic bone–blood tissue boundary is paramount in the growth of skeletal elements. Pathologies disrupting this boundary reflect the cross-talk in signaling pathways essential for cellular differentiation and maintenance processes in the vascular and skeletal systems. Further dissection of the genetic mechanisms behind these diseases is warranted in order to identify new players in the maintenance of vascular, skeletal, and ECM homeostasis.

Matrix — Lee

The primary structural component of the ECM, type I collagen, has long been implicated in OI. Mutations in the alpha 1 chain of type I collagen, the most common cause of OI, lead to low bone mass, bone fragility and deformities, and increased risk of fractures [35]. The ability of bone to resist fractures depends on the intrinsic properties of the materials that form the bone matrix, the amount of bone (i.e. mass), and the spatial distribution of the bone mass (i.e. microarchitecture). Type I collagenopathies first hinted toward defects beyond alterations in the structure of the ECM and prompted evaluation of bone properties at the organ, tissue, material, and molecular levels. Recessive forms of OI comprise defects in enzymes responsible for collagen post-translational modifications, such as the prolyl 3-hydroxylase-1 (P3H1) complex, as well as in collagen chaperones, which recruit other modifying proteins [35]. Homozygous *P3h1* null mice show overall decreased bone density, delayed ossification, and defects in collagen-rich tissues such as tendons and skin [36]. However, mice harboring a mutation that abolishes the hydroxylase activity of P3H1 (H662A) show decreased trabecular bone mass and abnormal collagen fibers, but no generalized defects in connective tissue. Despite the abrogation of enzymatic function in this mouse model, the stability of the P3H1 complex is maintained and no post-translational over-modification of other amino acid residues occurs, enabling normal assembly and folding of the collagen fibers [37]. As in the *P3h1* knockout mouse, complete ablation of cartilage-associated protein (CRTAP), a component of the P3H1 enzymatic complex, affects collagen fibril assembly and collagen–protein interactions, important for matrix–cell signaling [38]. *Crtap* null mice show increased TGF-β signaling, also observed in the dominant form of OI due to the G610C mutation found in a large Amish kindred, owing to changes in binding of the proteoglycan decorin to type I collagen, which disrupt TGF-β binding to collagen fibers and protein–protein interactions [39]. Overall, animal studies combined with genetic analysis of human OI mutations confirm that alterations in the biochemistry of collagen proteins can significantly affect folding and intracellular trafficking through the endoplasmic reticulum (ER) and the Golgi apparatus. These altered structural and distribution patterns, together with defects in collagen extracellular fiber assembly and cross-linking, result in defective matrix–bone cell communication.

The underlying deficiencies in OI are not merely structural; disruption of the collagenous reticulate can negatively affect cell signaling within the matrix, with profound effects in bone architecture and strength. In the future, additional signaling pathways affected in collagenopathies will certainly be identified, and the relative contributions of normal homeostatic responses such as ER stress will be determined. The main challenge consists in modeling all the pathophysiological components of these diseases in order to target them in an optimal way and personalize treatment.

The transcriptional landscape in the skeleton — McMahon

Dr. Andrew McMahon described experiments in which DNA bound to the chondrocyte transcription factor SOX9 or the osteoblast transcription factor OSX was immunoprecipitated from genetically engineered mice, massively parallel-sequenced, and compared to massively parallel mRNA sequence in order to understand how these transcription factors coordinately regulate gene expression in cells.

Defects in cellular differentiation programs and the signaling pathways that govern them during endochondral ossification undeniably compromise skeletal development. At the intracellular level, the balance between proliferation and differentiation requires the concerted action of master regulatory signals at different steps. While RUNX2 is essential to determine the fate of osteoblasts early on in the differentiation process, it also switches chondrocyte proliferation into hypertrophic differentiation at a later stage. SOX9 and other members of the SOX family of transcription factors are essential for chondrocyte specification and differentiation [40]. *SOX9* haploinsufficiency causes campomelic dysplasia [2], and differing degrees of severity in skeletal malformations in animal models, ranging from complete absence of ossification to impaired chondrocyte differentiation, according to the stage of development of SOX9 removal [41]. Transcriptional profiling of a neonatal chondrocytic sample obtained from mouse ribs revealed that this transcription factor acts as a focal point in the gene regulatory network of the cell, binding to about 27,500 regions in the genome. SOX9 is usually associated with highly conserved regions corresponding to regulatory regions, namely promoters, but is also active in sites distant from the transcription start. In the first case, SOX9 engagement is not through enrichment of bona fide SOX9-binding sites; association to these regions correlates with the strength of transcription of housekeeping genes without a strong chondrogenic signature. In the second type of

interaction, SOX9 binds directly to clusters of active enhancer elements. These super enhancer regions are flanked by acetylated nucleosomes, a hallmark of open chromatin, and transcriptional coactivators such as p300 to regulate cartilage- and chondrocyte-specific genes.

Despite the pivotal action of SOX9 at promoter and other regulatory sites, other factors engage with the transcription factor and affect its transcriptional program. Motifs for activation protein 1 (AP-1) are highly enriched in SOX9-recovered regions, and the distribution of c-Jun, an AP-1 subunit, clearly overlaps with that of SOX9 around the gene for collagen type II (*Col2a1*). In vitro studies suggest that c-Jun interacts with SOX9 at chondrocyte enhancers, possibly inhibiting one another, but its role in chondrogenesis is currently unknown. Therefore, subtle changes in levels of SOX9 may have important implications in the context of rare genetic diseases in which there is overexpression of this transcription factor.

Similar procedures applied to calvarial samples showed the presence of about 2,000 binding sites for OSX (*Sp7*), predominantly located more than 10 kb away from the transcription start site. As expected, these OSX-binding motifs were recovered near genes that have previously been implicated in skeletogenesis and ossification. OSX binds AT-rich regions within these motifs through partners such as DLX5, which is capable of binding to a novel enhancer at the *Notch2* locus, a known inhibitor of osteoblast differentiation [42].

The coordinated activation/inactivation of genes involved in chondrocyte and osteoblast differentiation and proliferation ensures the continuous elongation of the growth plate. These complex regulatory networks are expected to undergo dynamic changes during bone remodeling and development and may be involved in bone disease through (de)stabilization of interactions and (in)activation of specific DNA binding sites.

How rare bone diseases inform the search for agents to improve skeletal health

Drs. Graham Russell, Michael Whyte, Matthew Warman, Denise Adams, and Laurence Legeai-Mallet provided examples of rare bone diseases that suggested new strategies for improving skeletal health, or provided animal models or patient populations for which initial proof of principle validations could be performed.

Treating osteoclastic overactivity — Russell

The use of drugs that inhibit bone resorption ('anti-resorptives') dominates the therapy of bone diseases, many of which are characterized by enhanced bone destruction. These disorders include many common diseases such as osteoporosis, PDB, myeloma, and bone metastases secondary to breast, prostate and other cancers, but also many less common acquired or inherited diseases such as OI.

The term 'anti-resorptive' is used to refer to drugs that inhibit bone resorption, usually via direct or indirect actions on osteoclast development and activity; alternative terms such as 'anti-catabolic' are sometimes used to contrast with 'anabolic' agents that stimulate bone formation. For osteoporosis, treatments have historically included hormones such as estrogens as well as calcitonins, which have been replaced by more effective treatments. Selective Estrogen Receptor Modulators (SERMs) display the benefits of estrogens without all their adverse effects and offered much promise, but many failed during clinical development. Raloxifene and bazedoxifene are SERMs that continue to be used in selected patients. Currently, the mainstay of treatment worldwide is still with bisphosphonates, which have been used clinically for more than 40 years, and which can reduce fracture occurrence at vertebral and non-vertebral sites, including the hips. The most recently introduced new drug is the anti-RANKL antibody denosumab, also effective against these fractures. Several cathepsin K inhibitors have also been studied, but among these only odanacatib is close to being registered for clinical use. Strontium salts (e.g. ranelate) have been introduced for treating osteoporosis in some but not all countries, but their mode of action remains unclear [43].

It is fascinating to note how the study of rare diseases has led to many of the drugs now used or being developed for the treatment of skeletal diseases. Even bisphosphonates, as stable chemical analogs of inorganic pyrophosphate (PPi), can be traced back to studies on the inherited disorder hypophosphatasia (HPP). In HPP, the enzyme alkaline phosphatase (ALP) is deficient, and the resulting increased levels of PPi, the body's natural water softener, contribute to the defective skeletal mineralization. Altered pyrophosphate metabolism also occurs in other calcification disorders, such as chondrocalcinosis and infantile vascular calcification.

Bisphosphonates were first studied for their inhibitory effects on mineralization. Only when it was realized that they could also affect mineral dissolution were their effects on bone resorption evaluated. The development of both denosumab and cathepsin K inhibitors can also be traced back to the study of rare diseases of bone. Among other potential anti-resorptives derived from studies of various osteopetrotic disorders are Src inhibitors, chloride channel blockers, and adenosine triphosphate (ATP) proton pump inhibitors, but to date only denosumab has been registered for clinical use. It is evident that the pharmacological basis for the action of each of these agents is different, and these properties need to be considered when determining the optimal ways they can be used in clinical practice.

At the cellular level, bisphosphonates impair osteoclast activity via inhibition of farnesyl pyrophosphate synthase, which is involved in the prenylation of proteins required for the formation, function and survival of osteoclasts (e.g. Ras and Rho) [44]. Denosumab is effective in the prevention of fractures of the hip and spine, but shows differences in duration of action [45]. While the effects of zoledronate stabilize after three years of use [46], denosumab induces sustained increases in bone mass, even after six years [47]; however, biomarkers of bone turnover increase rapidly following discontinuation of treatment with denosumab while they are sustained after three years of interruption of zoledronate [48]. Moreover, several studies in patients with different types of cancer showed that denosumab is more effective than zoledronate in delaying the onset of first skeletal-related events [49,50].

Owing to their involvement in the mevalonate pathway, bisphosphonates may also have beneficial effects in other non-skeletal systems. Studies in humans and animal models suggest a positive effect on lifespan [51]. Moreover, alendronate reduces the risk of myocardial infarction in patients with rheumatoid arthritis [52], and zoledronate in combination with statins increases the lifespan of a mouse model of Hutchinson–Gilford progeria syndrome [53]. Bisphosphonates have an excellent safety profile, and new and even more potent compounds with lower bone affinity are under development for these novel applications.

Targeting enzymes and other proteins to bone — Whyte

The osteoblast/osteoclast dyad is not the only culprit in low bone mineral density, which can in fact result from defective skeletal bone mineralization in diseases such as HPP. The biochemical hallmark of the disease is low serum ALP activity [54]. In HPP, the tissue non-specific isoenzyme of ALP (TNSALP) is hypo-active [2]. TNSALP is a cell-surface enzyme expressed especially in the liver, bone, and kidney and plays a role in skeletal mineralization by regulating the extracellular level of PPi, a potent inhibitor of mineralization and the main pathological driver in HPP. Vesicles in the skeletal matrix rupture, releasing hydroxyapatite, but excessive PPi blocks the growth of these crystals for deposition into bone. Phosphoethanolamine (PEA) and pyridoxal 5′-phosphate (PLP) are additional natural substrates for the isoenzyme [55].

A total of 275 mutations in *TNSALP* have been identified in HPP across a wide spectrum of symptom severity [56]. The most severe form of the disease leads to extreme hypomineralization at birth, and

almost always causes death due to respiratory failure. In infantile HPP, patients appear normal at birth but develop rickets before six months-of-age, and respiratory failure with a fatal outcome occurs in approximately 50% of cases. During childhood, HPP can manifest as rickets with short stature, tooth loss, and craniosynostosis, whereas adults with HPP can present with metatarsal stress fractures, femoral pseudo-fractures, chondrocalcinosis, and PPi arthropathy. The mildest form of the disease ('odonto') is characterized by premature tooth loss with no radiological or skeletal abnormalities [55]. The disease remains without a medical treatment.

Initial studies showed that infusions of ALP-rich plasma obtained from patients with PDB and administered intravenously did not significantly improve clinical or radiographic findings in infantile HPP [57]. However, bone marrow cell transplantation and implantation of bone fragments and cultured osteoblasts improved the rickets [58,59]. Teriparatide, a recombinant form of parathyroid hormone (PTH) and PTH itself have been administered off-label to adults with HPP with the goal of stimulating TNSALP production in osteoblasts [60]. The partial success of these approaches led to the search for new bone-targeted therapies such as directed enzyme replacement therapy (ERT). Asfotase alfa is a recombinant protein that comprises the TNSALP homodimer and a terminal deca-aspartate motif responsible for targeting this biologic to mineral. Asfotase alfa rescues the metabolic and skeletal defects in the *Akp2 null* mouse, which recapitulates infantile HPP, including the dental abnormalities [61,62]. In an ongoing phase II extension study of life-threatening perinatal and infantile HPP, experimental asfotase alfa induced sustained improvements in bone mineralization and pulmonary and cognitive function. In these patients, changes in the radiographic features of severe HPP were observed as early as three months, with significant improvement in rickets and survival at one year. This agent may potentially be used in other conditions leading to abnormal accumulation of PPi levels, such as neurofibromatosis type-1. Overall, asfotase alfa showed a favorable tolerability profile, supporting the use of tissue-directed ERT in the treatment of mineralization defects.

Antibody-based modulation of extracellular signaling — Warman

Since Jenner scientifically demonstrated that cowpox inoculation could lessen disease severity caused by smallpox, germinal discoveries in the fields of immunology, cell biology, and molecular biology are culminating in an unprecedented ability to produce humanized monoclonal antibodies (mAbs) that have potent, sensitive, and selective biologic effects. The strategy of using mAbs targeting sclerostin, a Wnt signaling antagonist, to improve bone mass and strength has its origins in the study of four rare skeletal diseases: sclerosteosis and van Buchem disease, which are caused by mutations affecting *SOST* [63], and osteoporosis-pseudoglioma syndrome (OPPG) and dominant high bone mass (HBM) syndrome, which are caused by mutations affecting the Wnt co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) [64–66].

Studies with cultured cells and genetically modified mice suggest that sclerostin deficiency and LRP5 HBM mutations enhance Wnt signaling in the bone [67,68]. Although patients lacking sclerostin or having LRP5 HBM-causing mutations can develop complications from excessive bone formation [69]; importantly, these patients do not appear to be at increased risk for non-skeletal complications of deranged Wnt signaling, such as neoplasia and cognitive impairment. Therefore, mAbs that neutralize sclerostin activity have been developed with the goal of selectively enhancing Wnt signaling in the bone to increase bone formation [70].

Animal models of human rare disease phenotypes have been studied to support the use of antibodies to target Wnt signaling with the objective of increasing bone mass and strength. A mouse model harboring the missense mutation responsible for HBM recapitulates the human phenotype [71] and induces LRP5 resistance to the inhibitory effects of sclerostin on bone formation [68]. Therapies that employ mAb-mediated sclerostin inhibition in human populations are currently in phases II and III clinical trials for patients with common forms of low bone mass and strength (www.clinicaltrials.gov). However, these same therapies may benefit patients with rare skeletal fragility syndromes as well. For example, sclerostin depletion improved skeletal properties in *Lrp5* null mouse models of human OPPG [72,73]. Moreover, the benefit of enhancing Wnt signaling in patients with OI is being tested in mouse models for mild, moderate, and severe OI by crossing OI mice with Lrp5 HBM mice [74], and by administering anti-sclerostin neutralizing antibodies to OI mice [74–77]. Mice with a $Col1a2^{G610C}$ OI mutation and an *Lrp5* HBM-causing allele had increased bone mass and strength relative to mice with OI alone, as did $Col1a^{G610C}$ OI mice and *Brtl* mice that received anti-sclerostin antibodies [74,76,77], although not the more severe *Jrt* model of OI [75]. Therapeutic mAbs targeting other biologically active proteins in bone may also benefit patients with OI. Anti-TGFß antibodies have recently been shown to improve bone mass and strength in the $Col1a2^{G610C}$ knockin and *Crtap* null models of human OI [39].

In summary, the study of rare diseases sharing defects in key members of the Wnt signaling has definitely helped unravel the role of this pathway in the process of bone formation, pointing to the feasibility of using inhibitory antibodies in the treatment of rare bone diseases. It is likely that other human skeletal disease phenotypes caused by loss-of-function or gain-of-function mutations in secreted or outer cell membrane proteins will provide new targets for therapeutic antibodies that can enhance bone health.

Inhibition of intracellular signaling by small molecules — Adams

The mammalian target of rapamycin (mTOR)/PI3K/AKT signaling pathway regulates cell growth, proliferation, and survival, and interacts with several other pathways, including the angiogenic HIF pathway [78]. Consequentially, small molecule inhibitors of mTOR/PI3K/AKT signaling constitute promising agents in the treatment of cancer and vascular anomalies. Several case studies report the successful use of sirolimus, an mTOR inhibitor, in the treatment of kaposiform hemangioendothelioma (KHE), a low-malignancy tumor with a lymphatic component, without significant side effects [79,80]. Preliminary data from an ongoing open-label, phase II trial (NCT00975819) conducted in children and young adults with complicated vascular anomalies showed the efficacy of sirolimus in specific subgroups of patients, with an overall partial response of approximately 85% after one year. Remarkably, quality of life, also a primary outcome measure of the trial, improved significantly or remained stable with treatment with sirolimus. In this trial, mucositis was the most frequent side effect, and increased risk for interstitial disease was observed, as well as pulmonary, metabolic, and immune adverse events. The long-term effects of sirolimus are unknown, and low doses are currently being used to prevent complications. mTOR inhibitors also show anti-tumor activity and acceptable tolerability in mouse models predisposed to cancer, but have produced somewhat poor outcomes in clinical trials as monotherapy, with the exception of renal cell carcinoma [78]. Dual mTOR/PI3K agents are currently being tested in combination with cytotoxic therapy to improve efficacy and reduce the possibility of acquired drug resistance.

In the context of bone pathologies, animal studies showed that inhibition of the mTOR pathway decreases bone resorption through interference with the RANKL/RANK/OPG system. The mTOR inhibitor everolimus inhibited the proliferation of osteosarcoma-derived cells and improved tumor response when used in combination with zoledronate, an approach that may overcome development of resistance to the small molecule [81]. The phase III BOLERO-2 study (NCT00863655) evaluated a combination of everolimus and an aromatase inhibitor (exemestane) in postmenopausal women with metastatic breast cancer; significant survival benefits were observed, with reduced bone resorption and incidence of malignant progression in bone.

The positive outcomes obtained with mTOR inhibition in the control of vascular and oncologic disease and their complications paved the way to new therapeutic applications for dual small molecule inhibitors of mTOR/PI3K. Ongoing studies are comparing chemotherapy with vincristine with sirolimus in KHE and investigating the potential additional benefits of combining sirolimus with bisphosphonates in bone cancer settings. Unfortunately not much is known about the effects of these small molecules in patients with both bone and vascular abnormalities, such as Gorham-Stout and Generalized Lymphatic Anomaly, and the next few years will certainly reveal new treatment approaches.

Use of synthetic polypeptides to promote skeletal growth — Legeai-Mallet

Peptides have been successfully used in the treatment of several diseases, insulin being one of the first notable synthetic polypeptides used in therapy. Several peptide therapeutics are now under development or available for indications as diverse as prostate cancer, diabetes, and Cushing disease, but they present limitations, such as susceptibility to enzymatic degradation, immunogenicity, and the possibility of aggregation, adsorption, and denaturation once inside the cells. Bone-related disorders have also been targeted with synthetic peptides, in particular acromegaly, short stature, osteoarthritis, and osteoporosis. Recombinant insulin-like growth factor-1 (mecasermin) is now available in the US and EU for the treatment of patients with growth failure due to insensitivity to growth hormone (GH) [82]. Another synthetic peptide, teriparatide, increases bone formation and is also chondroprotective [83,84]; intermittent teriparatide administration rescues the abnormal skeletal development in a mouse model of achondroplasia [85], the most common form of dwarfism. GH is minimally effective for the treatment of achondroplasia, and current management of the disease involves surgical limb lengthening, which is associated with high risk of infection and joint and soft tissue damage [86]. Activating mutations in the FGF Receptor 3 (FGFR3) cause achondroplasia by dysregulating downstream pathways, such as mitogen-activated protein kinases (MAPKs) [87].

The importance of C-type natriuretic peptide (CNP) and its receptor, natriuretic peptide receptor 2 (NPR2), for endochondral skeletal growth was suggested by mice overexpressing brain natriuretic peptide [88], or mice with loss-of-function mutations in *Cnp* [89], and in humans who have acromesomelic dysplasia, type Maroteaux [90], due to loss of NPR2 or skeletal overgrowth due to with chromosomal translocations involving CNP [91]. Transgene-mediated overexpression of CNP improved the skeletal phenotype in an achondroplasia mouse model [92]. A new CNP analog, designated BMN-111, is currently being investigated for the treatment of achondroplasia. CNP, a 22-amino acid peptide, has a short half-life due to neutral endopeptidase degradation, but its analog under development shows similar selectivity and potency and is resistant to enzymatic proteolytic activity. The peptide is a known negative regulator of FGFR3, and its administration improves the phenotype in a mouse model of achondroplasia in a MAPK-dependent manner [93]. In a six-month study of wild-type cynomolgus monkeys, BMN-111 increased tibial length and growth rate and did not raise safety concerns. In cultures of primary chondrocytes obtained from patients, BMN-111 abrogates the constitutive activation of FGFR3 through inhibition of ERK1/2 phosphorylation. Ex vivo, the peptide promotes proliferation and differentiation of murine chondrocytes heterozygous for the Fgfr3 Y367C mutation and rescues the size and architecture of the growth plate in embryonic femur cultures. Daily sub-cutaneous injections of BMN-111 for 20 days resulted in significant increases in the growth of the axial and appendicular skeleton in the $Fgfr3^{Y367C/+}$ mouse model mimicking achondroplasia [93]. A phase I study (NCT01590446) in healthy volunteers was completed in 2012, and phase II evaluation is underway in patients 5–14 years old (NCT02055157). The objectives of the trial are to evaluate the safety of BMN-111 and changes in absolute growth parameters and in body proportions, as well as functional aspects of skeletal growth.

The phase II trial of BMN-111 in children with achondroplasia will explore whether the success obtained in preclinical animal studies can be extended to humans. As in OI, it is important to assess not only the 'quantity' but also the 'quality' of the new extended bones, as measured by several biomechanical parameters.

Conclusions

The new knowledge we have gained from patients with rare skeletal diseases is enormous and impossible to summarize in a single article. Using a tiny subset of examples, the speakers at the Rare Bone Disease symposium conveyed the importance of learning from patients affected with rare skeletal diseases. The study of these patients yield unexpected insights about the normal physiology of the skeletal system and suggest new targets and strategies for improving skeletal health.

Conflicts of interest

None.

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