

MEETING REPORTS

Meeting Report from the 26th Annual Meeting of the American Society for Bone and Mineral Research

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SEX HORMONES AND BONE

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Both estrogens and androgens are important for skeletal homeostasis, but their relative importance for bone modeling and remodeling and specific bone cells is not fully understood. Osteoblasts or stromal osteoblast precursor cells are considered to be the primary target of sex steroid hormone action; these cells signal the message to other cells, including osteoclasts. Osteoclast-specific deficiency of the androgen receptor (AR), however, generated by cre-lox methodology using cathepsin K gene Cre knockin mice, increases osteoclast number and function, as demonstrated by histology and bone markers. This clearly demonstrates a specific and direct role for androgen-AR action on osteoclasts (1). Both androgens and estrogens can increase trabecular bone mass and strength in both male or female animals, and many published studies indicate that AR can function

independently from the estrogen receptor (ER) in trabecular bone cells. This is confirmed by direct demonstration of additive stimulatory effects of ER and AR activation on trabecular bone in ovariectomized rats (2).

The main objective of selective ER and AR modulators is to improve bone mass and quality without major side effects (and preferably, even with beneficial effects) on other target tissues. Although the existing selective estrogen receptive modulators are fairly equivalent in action (lasofoxifene being slightly more potent than raloxifene) (3), researchers are actively searching for new structural compounds. Estrens may be one such new class of sex hormone receptor modulators, because they seem to selectively activate several receptors (e.g., AR and ER), with a high degree of selectivity for bone (4). Kousteni *et al.* (5) now provide further details with regard to the second signaling pathway activated by estrens, not by dihydrotestosterone or estradiol. The authors suggest that the phosphorylation of Smad 1/5/8 may explain the bone anabolic activity of these activators of nongenotropic estrogen-like

signals (ANGELS). Moreover, several agents known to block estren action also block smad phosphorylation, indicating the essential role of this signaling pathway (5).

A major concern that estrens are nonselective ligands for AR, because they clearly generate bone protective effects in male orchidectomized ER α knockout mice, was raised in a report by Windahl *et al.* (6). Further strengthening this conclusion was the fact that the effect of estren could be blocked by an antiandrogen. Moreover, estrens were found to be much less selective for bone than previously assumed, because their effect on bone was associated with similar effects on the uterus. The reasons for such different conclusions coming from two renowned labs are mysterious and will require further experiments, preferably in a different setting, to prove or disprove the unique biological profile of estrens as activators of nongenomic pathways with great selectivity for bone.

Selective steroid receptor knockout models are also useful in analyzing the mode of action of well-known compounds, such as tibolone, a bone protective agent with mixed androgen-, estrogen-, and progesterone-like effects. Indeed, the actions of tibolone on bone cells were clearly mediated by ER α (7), because ER α -deficient cells were no longer responsive to tibolone. Progress was also reported with regard to selective AR modulators: the nonsteroidal compound CMP1 increased periosteal bone formation and decreased endocortical bone resorption in orchidectomized rats and completely prevented orchidectomy-induced bone loss, while having minimal effects on prostate weight (8).

The cellular and molecular mechanisms responsible for the typical osteoclast expansion following ovariectomy involve first an expansion of hematopoietic stem cells, with subsequent expansion of T cells, leading to tumor necrosis factor α -mediated osteoclastogenesis (9). This early step of hematopoietic stem cell expansion is positively interleukin 7 (IL-7)-dependent, as demonstrated by anti-IL-7 experiments, and negatively dependent on transforming growth factor β (10). Moreover, T-cell expansion preceding osteoclast generation is dependent on insulin-like growth factor 1 (IGF-1) from

liver, as demonstrated by selective liver IGF-1 gene knockout (11).

Other new data with regard to the molecular events following ER or AR activation were described at the Seattle meeting. Continuous periosteal bone growth during late puberty and throughout adult life distinguishes and causes sexual bone dimorphism. However, estrogens are also important for periosteal growth expansion, as demonstrated in ER α - and aromatase-deficient mice, but also in an adolescent male patient with congenital aromatase deficiency (12). The stimulatory (rather than expected) inhibitory effect of low estrogen concentration on periosteal growth could be explained by growth hormone receptor-independent (and thus direct) stimulation of liver IGF-1 synthesis and secretion (13), as demonstrated by marked periosteal bone growth in growth hormone receptor-deficient mice treated with low-dose estrogens. The role of estrogens in cortical bone was addressed using a totally different approach of generating mice with a mutated ER that can only mediate actions that do not require an estrogen response element, thus only via protein-protein interactions. In such mice, in whom estrogens can signal only through nonclassical pathways, estrogen deficiency increases and estrogen replacement decreases cortical bone. This implies that in gonad-intact mice, estrogens have dual and opposite signaling -- via estrogen response element-mediated gene regulation to increase bone mass and ligand-activated ER-protein-protein interaction, creating an opposite effect (14). Estrogen receptor-related receptors (ERRs) are a subclass of orphan nuclear receptors that resemble ERs. Osteoblast-targeted overexpression of ERR α decreases bone mass at both trabecular and cortical compartments. This indicates that ERR or ERR-mediated signaling negatively regulates bone mass (15).

Several groups reported preliminary data on gene (array) analysis following AR or ER manipulation, thus more data can be expected with regard to post-AR or ER signaling (16-18).

GENETICS OF BONE AND MINERAL DISORDERS

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Following a recent report implicating 12/15-lipoxygenase as a negative regulator of peak BMD in mice (19), two groups (20,21) examined the contribution of this candidate gene to BMD variation in humans. Unlike mice, humans have two separate enzymes (12-lipoxygenase and 15-lipoxygenase), which are encoded by the genes *ALOX12* and *ALOX15*, respectively. Functionally, mouse 12/15-lipoxygenase is more similar to human 15-lipoxygenase than to human 12-lipoxygenase. A minor genetic association between hip BMD and a single nucleotide polymorphism (SNP) in the 5'-flanking region of *ALOX15* was identified in postmenopausal female residents of Anhui Province in China (20). In contrast, a genetic association study of young white sib pairs in the United States (21) found no association between *ALOX15* SNPs and peak BMD variation, but did identify a significant association between allelic variation in the 5' region of *ALOX12* and spine BMD. The investigators noted that genetic variation in *ALOX12* accounted for only 3% of BMD population variation, which explains why this genomic region (17p13.3) was not initially detected in a whole genome scan for linkage association and illustrates the limited contribution that variation at a single genetic locus can exert on a complex trait, such as BMD (22).

One of the major challenges of quantitative trait loci (QTL) mapping by linkage analysis in both animal models and human populations is that the identified region generally extends over a large stretch of the genome, typically 20–30 centimorgans (cM). Within this region may reside hundreds of genes. In mouse models, it is possible to take advantage of the inherent relatedness of many inbred strains and compare previously determined haplotype maps to exclude large genomic regions within a QTL where no allelic differences exist between the two progenitor strains. This approach successfully reduced a 25-cM QTL on distal chromosome 6, known to influence

serum insulin-like growth factor 1 levels and femoral BMD, to two haplotype blocks (< 2 cM) (23). Gene expression studies of osteoblastic mRNA from the two progenitor strains further narrowed the search to only two potentially important candidate genes within the highlighted region. Another approach to fine-map a QTL region in mice for further characterization was the creation of nested interval-specific congenic sublines that allow for genetic decomposition of a QTL region. This approach was successfully used in mice derived from C57BL/6 and C3H progenitors to discover the presence of two separate genetic loci on mouse chromosome 1 -- a proximal region (25-62 cM from the centromere) associated with reduced vertebral trabecular bone mass (BV/TV) in both female and male mice and a distal region (71-109 cM) that was associated with increased BV/TV in female congenic mice, and curiously, reduced BV/TV in their male counterparts (24). Other investigators reported a similar outcome in the analysis of QTL influence on femoral volumetric BMD in mice derived from a cross of C57BL/6 and *Mus musculus castaneus* strains, also on chromosome 1 (25). Comparison of interval-specific congenic lines indicated the presence of at least two, if not three, distinct genetic loci within the original 30-cM QTL region that seem to act via different mechanisms to regulate femoral BMD. These results clearly highlight the underlying complexity (*i.e.*, multiple genes, sex-dependent expression, etc.) that can exist within a given skeletal QTL.

The genomes of four mammals (human, dog, mouse and rat), two worms, and several yeasts have been fully sequenced, and more genomes will be completed in the near future. Analysis of the individual genome sequence provides much insight into genome structure, but less into genome function. A major challenge for genomics research is to identify functional DNA and assign a role to it. Comparative genomics can serve an important role in the functional annotation of the genome. As a complement to ongoing studies in human and mouse populations, it was therefore gratifying to see continued work in pedigreed baboons, providing cross-species replication of BMD QTL on human chromosome 11q12-13 (where low-density lipoprotein receptor-related protein 5

[LRP5] is known to reside), as well as new work with intercrosses between divergent strains of laboratory rats (26,27) and chickens (28), all of which promise to facilitate the identification of genes affecting bone density and bone strength.

Skeletal phenotyping of mice with spontaneous or genetically engineered mutations has proven to be a useful method for discovering (or confirming) how genes function to regulate bone growth and repair. Sex is known to exert a profound effect on the skeleton, with the male phenotype providing considerable fracture risk reduction. In an experiment that could only be performed with genetically engineered mice, the impact of the Y chromosome on bone development was assessed (29). Peak bone mass, geometry, and strength were examined in normal 20-week-old adult male and female mice, along with mice in which the testis-determining gene (*Sry*) had been deleted from the Y chromosome to produce XY *Sry*⁻ animals with ovaries or inserted as a transgene on an autosome to produce XX *Sry*⁺ animals with testes. The sex differences in skeletal development observed in this model were found to be independent of sex chromosome complement and wholly dependent on gonadal hormones. Moreover, the absence of gonadal steroids eliminated any sex-specific skeletal advantage. This report echoed the findings of many other groups that sex-specific differences in bone geometry and microarchitecture need to be considered in the design of studies intended to discover and characterize genes affecting bone biology.

The skeletal phenotyping of cathepsin K null mice was also presented (30). Cathepsin K is a cysteine protease that degrades type I collagen. Not unexpectedly, cathepsin K null mice exhibited increased bone mass and bone strength. However, histomorphometric analysis of these mice revealed a surprisingly high rate of bone formation, despite the reduced osteoclast activity that accompanies the absence of cathepsin K. The uncoupling of bone formation from bone resorption in these mutant mice suggests that cathepsin K may serve an important function in the process of bone remodeling to balance bone formation with resorption rate. The pathophysiologic and therapeutic implications of this observation

are likely to generate considerable future interest in this enzyme system.

It is now well recognized that the Wnt coreceptor LRP5 plays an important role in bone mass accrual. However, the intracellular signaling mechanisms responsible for Wnt action in bone are unclear. Using Cre-mediated recombination technology, strong evidence was presented supporting a central role of canonical Wnt signaling via β -catenin in osteogenesis (31). Mice with an osteoblast-specific deficiency in β -catenin exhibited reduced bone mass, whereas mice deficient in APC (an intracellular protein that binds β -catenin and is necessary for its downregulation) exhibited increased bone mass. In another report, lithium chloride treatment was shown to exert a potent bone anabolic effect in laboratory mice; lithium chloride acts downstream of the Wnt receptor to augment β -catenin signaling (32). Combined, these experimental findings raise the possibility that modulation of Wnt signaling may provide a new pharmacologic avenue to positively affect bone mass.

IMAGING ASSESSMENT OF BONE STATUS

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Noninvasive/nondestructive measurement of bone mineral and structure using imaging techniques is essential to the diagnosis and management of osteoporosis and other metabolic bone disorders. BMD alone is limited in differentiating between patients with and without vertebral fracture, which necessitates that clinical studies enroll hundreds and even thousands of patients and follow them for many years to develop sufficient statistical power for analysis of disease and treatment effects on fracture reduction. Quantitative computed tomography (QCT) has also been used, but QCT-based estimates of BMD have generally only shown modest correlations to *in vitro* measurements of vertebral strength.

Trabecular structure analysis from multidetector row CT images can better

discriminate postmenopausal women with vertebral fracture than can DXA (33). High-resolution spiral CT assessment of the trabecular structure of the vertebral body, in combination with BMD, improves prediction of biomechanical properties (34). An independent association of sex steroid levels with cortical and trabecular area and QCT volumetric BMD is found in elderly men, but such association is lacking in young men (35).

Finite element models derived from QCT scans may improve predictions of vertebral strength, because they mechanically integrate all of the geometrical and material property data within the scans. QCT BMD values of each bone voxel can be converted into elastic modulus values using predetermined correlations between the elastic modulus and QCT-derived BMD. Finite element models integrate mechanically all of the anisotropic, homogeneous, and complex geometry of the bone structure examined. It has been demonstrated that voxel-based finite element model-derived estimates of strength are better predictors of *in vitro* vertebral compressive strength than are clinical measures of bone density derived from QCT, with or without geometry (36). Although imaging resolution is not critical in cross-sectional studies using clinical CT scanners, longitudinal studies that seek to track more subtle changes in stiffness over time should account for the small (but highly significant) effect of voxel size (37). However, these data were generated in the laboratory with excised bone specimens without soft tissue; their practical value remains to be established from clinical and epidemiological studies. In addition, compressive loading experiments used in the laboratory differ from *in vivo* conditions.

The QCT three-dimensional (3-D) finite element model of the proximal femur shows moderate reproducibility in postmenopausal women (38). Finite element analysis of DXA images, by converting DXA image pixel of the porcine proximal femur to Young's modulus value, can predict mechanical integrity (39).

DXA assessment of the proximal femur of 697 women (age range, 20-87 years) and QCT evaluation of 26 cadavers have shown that the mineral mass and external

dimensions of the femoral neck are dissociated because of the differing behavior of the periosteal and endocortical envelopes. Apparent volumetric BMD is size dependent; it is lower in bigger bones and higher in tubular bones, such as the femoral neck (40). Direct and DXA measurements of 26 white female cadavers have shown that the femoral neck is ellipsoid and that the assumption of circularity introduces errors in volume and volumetric BMD (41).

Many studies have used microCT, from technique development to its various applications. An *in vivo* human microCT scanner has been reported to examine distal radius and tibia with isotropic resolution of 120 μm (42). In microCT assessment, the increase of rotation step leads to a decrease in the signal-to-noise ratio of the image, and the error of measurement caused by a decrease in the signal-to-noise ratio is more critical than threshold for trabecular structural assessment, because of artifactual disconnection of trabeculae (43). MicroCT can assess 3-D human cortical porosity (44), which has found successful application in the assessment of the effect of PTH treatment on cortical bone (45). Fractal analysis of calcaneus radiographs has shown that teriparatide treatment in postmenopausal osteoporotic women improves trabecular microstructure (46). Using synchrotron radiation, microCT examination of sequential triple iliac biopsies showed that five-year risedronate treatment did not cause significant hypermineralization (47). MicroCT and histomorphometric assessment of iliac crest bone biopsies from postmenopausal women treated with alendronate for 10 years showed normal microarchitecture (48). In animal studies, microCT can find applications in the assessment of skeletal phenotype in gene knockout and knockin mice (49,50) and osteoporotic (51, 52) and arthritic (53) rodents.

Peripheral QCT (pQCT) assessment of tibial cortical geometry and muscles in children at pre-, peri-, and postpubertal stages demonstrates that sex differences in bone structure and muscle strength influence the muscle-bone relationship (54). Prepubertal boys and girls have small differences, but at postpubertal stages, cortical bone area increases more in boys than in girls. However, pQCT

application to thin cortical bone in children can underestimate volumetric BMD, because of limited resolution and partial volume effects (55).

Relatively fewer studies use magnetic resonance imaging (MRI) than CT, possibly because of its high cost and complexity. The scaling index method has been shown to extract texture measures of trabecular bone structure in high-resolution MRI of the distal radius and to provide structural information independent of DXA BMD (56). MRI can detect early osteoporotic vertebral fracture in postmenopausal women, in whom conventional radiographs seem normal (57). Measurement of ^{31}P nuclear magnetic resonance (NMR) signals of bone mineral and some fraction of ^1H NMR signals of bone matrix by solid state projection MRI can potentially yield MRI mapping of the degree of bone mineralization (58).

A BIG YEAR FOR PEDIATRICS

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It was a big year for pediatric bone research at the 2004 American Society for Bone and Mineral Research meeting. In 2000, the search word "children" appeared in only 55 abstracts, and no oral or poster sessions were dedicated to pediatrics. The first oral session occurred in 2001 and was on the final day of the meeting in a small (but well attended) room. This year, the search word "children" received 112 hits, two oral sessions were dedicated to the topics of bone acquisition and pediatric bone disease, and there was one minisymposium on pediatric bone biology. It is a time of growth for pediatric bone researchers and clinicians. We've come a long way in a few years.

Despite the high interest, there is still much confusion in the field, particularly about what bone parameters to measure; which technology to use; how to describe what is "normal" or healthy; and how to measure and understand clinical populations. Like the adult field (and as Ego Seeman so eloquently reported in the BoneKEy report from last year's meeting

[59]), we are struggling with how to properly use terminology related to bone geometry and material properties and the elusive topic of bone "strength." In addition, pediatric bone researchers and clinicians also have to deal with how to best control for growth and individual variability in different stages of maturity, when describing bone in healthy or clinical populations, and understanding how bone changes in response to treatment or lifestyle intervention. Until recently, instruments designed for adult use, and standards designed to define adult bone fragility, have been often applied directly to children. Although much confusion remains, the growing number of abstracts and researchers/clinicians interested in pediatric bone shows that the field is moving in the right direction.

Normative Data

Clinicians have been complaining for years that normative data provided by DXA manufacturers is inadequate for children and adolescents. Several abstracts at this year's meeting reported new normative DXA, ultrasound, and peripheral quantitative computed tomography (pQCT) data for various ethnic groups, including ultrasound data in a large population of more than 9000 Japanese children (60). The Bone Mineral Density and Childhood Study and the initiative to achieve pediatric reference data, funded by the National Institutes of Health, reported results from its first year ($n = \sim 1550$ children; age range, 6-15 years; five study sites) for DXA (61,62), pQCT, and DXA vs. CT (63). The initiative will provide excellent normative data not only for DXA, but also for pQCT and CT, over the next several years. A series of longitudinal growth models that predict bone mineral content (BMC), accounting for sex, body size, and ethnicity (and their interactions), were created from existing combined longitudinal DXA data from four centers in the United States and Canada in more than 1800 children who were followed for four to 10 years (64). Once publicly available, these models should help clinicians to better interpret DXA data in children.

The debate over the age of "peak bone mass" continues. Data from the University of Saskatchewan longitudinal study showed that although total body bone

mass values level off at age 20 years (on average), there is tremendous individual variability. About 40% of children reach their "peak" value either before or after age 20 years, and there is also tremendous site-specific variability (65,66).

The Muscle-Bone Relationship

The late Harold Frost preached for decades that bone should adapt to the forces imposed on it and that the largest forces on bone come from muscle. We are finally starting to listen and understand -- sort of. Frank Rauch, Michael Parfitt, and others have helped interpret Frost from a pediatric perspective and from the cellular to whole bone level. At the level of whole bone, Rauch stated that bone was not designed to be as *heavy* as possible, but rather as *stable* as necessary (67). Thus, growing bone should be interpreted not only as the amount of material present, but also as the amount of material relative to the primary forces imposed on it. Several abstracts about the development of the muscle-bone functional unit were presented at this year's meeting.

It has been proposed in published work that the muscle-bone relationship digresses in puberty, such that girls have more bone mass for their muscle relative to boys, possibly because of a "packing" effect of estrogen (68). This theory has yet to be proven. Data presented at the meeting showed conflicting evidence. Cross-sectional studies using DXA data support the theory that around the time of puberty, girls have a greater bone mass to muscle mass ratio than do boys (69); however, longitudinal pQCT data at the midshaft of the tibia showed no sex difference in the muscle-bone relationship or the change in this relationship over time (70). Are these differences the result of study design -- cross-sectional vs. longitudinal? To technology (DXA vs. pQCT)? Or to real site or population-specific differences? Further longitudinal studies using multiple technologies will help to answer these questions.

Published work suggests that overweight children are at increased risk of fracture, and abstracts at this year's meeting support that work: children who fracture have a higher body mass index than do controls (71). Overweight and obese children have high bone mass and bone

strength, so why should they experience fracture? It seems that higher bone strength is simply adapted to higher absolute lean mass (*i.e.*, more muscle is needed to move greater body weight), but may be low for body weight (72,73). Future work using multiple technologies needs to further define the relationship between body composition, weight change, and bone strength during childhood and adolescence.

Clinical Populations: Bone Geometry and Intervention

Understanding how to describe bone in healthy children has been challenging. Even more difficult is understanding bone in children with chronic disease, who may have low bone mass because of small size, reduced muscle mass or loading, a tendency to mature late (or early), disease state, or pharmaceutical intervention. Some presentations at this year's meeting begin to give us insight.

Assessment of the muscle-bone relationship may be helpful in clinical settings (74). Children with Crohn's disease seem to have narrow bones and reduced resistance to bending (measured by DXA and the Hip Structure Analysis software), but also low lean mass. Lower bone strength seems adequate for low lean mass (75). Bone deficit in these children (measured by pQCT) was not related to steroid exposure (76). Children with sickle cell disease (n = 90; age range, four to 20 years) had narrow bones (based on measures of whole body bone area for height) and reduced bone mass (whole body BMC for height), compared with controls (n = 198). Researchers interpreted this to mean that children with sickle cell disease had less cortical bone strength, which may increase risk of fracture (77). But do they have less cortical bone strength? What do whole body BMC and bone area measurements from DXA really tell us about "cortical bone strength?" These data are important to get us thinking about how to interpret DXA measurements in clinical pediatric populations and what measures best tell us about bone "strength."

What happens with treatment? In children with osteogenesis imperfecta (types I-IV), treatment with intravenous pamidronate seemed to positively affect bone growth,

bone age, bone turnover, BMD by DXA, and bone parameters by pQCT, even in those who started treatment at age two years or younger (78); however, treatment did not seem to affect bone at the material level in a small sample ($n = 6$) (79). A well-designed randomized controlled trial of patients with osteogenesis imperfecta (types I-IV) showed that oral alendronate reduced bone turnover, increased spine BMD by more than 30% over placebo, increased BMD z-scores, and tended to reduce bone pain and increase physical activity. Although this treatment had a dramatic effect on bone, no difference in fracture incidence between treatment and placebo groups was found. Histomorphometry showed that cortical bone width was increased in patients treated with alendronate (80).

Although the newer technologies, such as pQCT and magnetic resonance imaging, are already used for research purposes, and at some enlightened institutions, for clinical work, the fact remains that DXA isn't going away. It is widely available to clinicians, and the low radiation dose and fast scan time make it appealing for pediatric use. There is, however, much work to be done before we know how to best interpret the meaning of these measurements -- using multiple technologies should help us get there. We've come a long way in the quantity and quality of our work in pediatric bone research in recent years, but confusion remains, and much work remains to be done from the cellular to whole bone level and from public health to clinical intervention.

TREATMENT OF OSTEOPOROSIS

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Only about 20% of fractures in the community occur in women younger than age 60 years. It is unlikely that treating the large number of women in this age group with osteopenia, but no prevalent fractures, is cost effective, because without treatment, most will not experience fracture (81). Should we treat women older than age 60 years with osteopenia and no fractures? This is also a difficult question.

There is evidence that raloxifene and strontium ranelate reduce fracture rates in women with osteopenia. New analyses suggest that risedronate reduces the risk of vertebral and nonvertebral fracture by 75% in women with osteopenia (82). Events were too few to analyze spine and nonspine fracture separately. Efficacy is essential, but so is compliance. Individuals who comply with therapy because of reinforcement seem to have fewer fractures with risedronate therapy than do those receiving no reinforcement (83). Fracture rates were 1.2% in the former group and 2.7% ($p = 0.049$) in the group with no reinforcement and poorer compliance.

Calcium supplements may reduce remodeling rates and modify BMD, but is there evidence of antifracture efficacy? The results of a five-year randomized double-blind controlled trial failed to support the notion that supplementation reduces vertebral and nonvertebral fracture (relative risk [RR] = 0.85; 95% confidence interval [CI], 0.66-1.1). The inference made by the authors that lower fracture rates in compliers (RR = 0.66; 95% CI, 0.46-0.95) were the result of calcium supplementation is questionable, because of potential biases introduced by the 40% dropout rate (84).

Correcting vitamin D deficiency in nursing home residents reduces the risk of fracture by about 30%. Whether this benefit is seen in the community is less clear. In one study, annual intramuscular injection of 300,000 units of vitamin D in 9440 community-dwelling people increased the risk of hip fracture (RR = 1.48; 95% CI, 1.01-2.17) (85). In two studies, the risk of falls was reduced (86,87). Fewer falls does not mean fewer fractures, because most falls do not result in fracture.

Long-term use of bisphosphonates may increase tissue mineral content, thus increasing material stiffness. After treatment with risedronate (5 mg/day) for five years, tissue mineral content was restored to the premenopausal range, but not above it (47). Similar observations were reported after 10 years of alendronate treatment (48). The results of a five-year randomized trial using alendronate in 1099 participants with previous treatment with alendronate who were randomized to placebo ($n = 437$) or

alendronate (5 or 10 mg/day) suggest that clinical spinal fracture was reduced in the alendronate group (RR = 0.45, 95% CI, 0.23-0.84), but no reduction was seen in nonspine or morphometric fracture (88).

Whether differences exist in antifracture efficacy among bisphosphonates, selective estrogen receptor modulators (SERMs), PTH, or combinations of these treatments is uncertain, because comparator trials using antifracture efficacy as an endpoint have not been done. Weekly treatment with alendronate seems to increase BMD by 1% to 2% more than such treatment with risedronate; in addition, the former suppresses remodeling by about 15% more than the latter (89). Inferences regarding superior fracture risk reduction based on greater BMD increment or greater bone marker decrement are difficult to make, because the proportion of fracture risk reduction accounted for by changes in these surrogates is small.

Lasofexifene may increase BMD more than raloxifene and suppress remodeling to a greater extent, (3) but the information needed is antifracture efficacy, especially evidence of hip and nonvertebral fracture risk reduction. In this regard, the new SERM PSK3471 does seem to *restore* trabecular structure and increase breaking strength of bone more effectively than raloxifene (90).

The Continuing Outcomes Relevant to Evista (CORE) trial, a four-year extension of the Multiple Outcomes of Raloxifene trial, did not provide evidence for nonvertebral fracture risk reduction (91). In patients with a baseline prevalent vertebral fracture in CORE, there was a reduction in nonvertebral fractures (RR = 0.78, 95%CI, 0.63-0.96), and in the combined eight years, nonvertebral fracture risk was reduced (RR = 0.64, 95%CI, 0.44-0.92). Should SERMs be used as first-line therapy for nonvertebral fracture risk reduction? I don't think so.

Anabolic agents like PTH reduce the risk of spine and nonspine fracture. Evidence of hip fracture risk reduction is not available. PTH seems to reduce the increase in risk associated with increasing numbers of prevalent fractures and more severe prevalent fractures (92). For example, one, two, and more than three

baseline fractures predict an incidence of 6.8%, 15.7%, and 22.6%, respectively, over 18 months. PTH reduced the incidence to 3.4%, 5.8%, and 7.2%, respectively. Similar figures apply to the effects of increasingly severe baseline fracture and further vertebral and nonvertebral fracture.

With the advent of anabolic therapy, the question of combined treatment has emerged. It seems that the benefits of PTH may be lost after discontinuation of the drug (93), and it is likely that antiresorptive therapy will be needed after treatment. Prior antiresorptive therapy with alendronate may blunt the effect of PTH (94), whereas drugs like raloxifene, which modestly reduce remodeling, may allow the anabolic effects of PTH to be realized (95). Whether combining PTH with an antiresorptive drug is more efficacious in preventing fracture remains to be seen. Combined therapy might reduce fracture rates more than either drug alone, but an argument can be made that the combined therapy may have a lesser antifracture efficacy than either drug alone. From animal studies, it seems that anabolic agents may reduce fragility by improving structural strength, whereas antiresorptives improve material stiffness (96).

Results of several newer agents were reported. Strontium ranelate reduces vertebral and nonvertebral fracture, and evidence now supports these benefits in women older than age 80 years, a subgroup of women with the highest risk of fracture (97). Whether strontium ranelate is an anabolic agent remains to be determined. A drug that inhibits glycogen synthase kinase-3 β seems to have anabolic properties, increasing periosteal and endosteal bone formation (98). OPG-Fc, a receptor activator of NF- κ B ligand (RANKL) antagonist, increases cortical bone area, bone mineral content, and BMD in monkeys (99). Cortical thickness increased by 251% of the distal radius and 39% of the proximal tibia. The radius in treated animals had an increase in periosteal circumference of 20% and a reduction of 40% in endosteal circumference. AMG 162, a humanized monoclonal antibody to RANKL, increased BMD at the spine within one month, similarly to alendronate (100).

OSTEOBLASTS: GONE WITH THE Wnt. . .

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Without the rise of a pivotal newcomer in the area of osteoblast-specific transcriptional regulation or the disclosure of a novel mouse/human genetics surprise, osteoblast biology at the 26th American Society for Bone and Mineral Research meeting was largely dominated by studies related to Wnt signaling. In particular, several groups reported comprehensive analyses of the expression of Wnt, Wnt receptors, and Wnt pathway modulators in osteoblasts (101-103). Although a few differences existed between the studies, all showed that most Wnt ligands are expressed by osteoblastic cells, with only Wnt3a, Wnt7a, and Wnt8a systematically reported absent in all studies, and Wnt1, Wnt2, Wnt3, and Wnt8b also reported absent in two of three studies. Likewise, most Wnt receptors were found to be expressed by osteoblasts. Adding to this complexity, both low-density lipoprotein receptor-related protein 5 (Lrp5) and Lrp6 coreceptors were also detected, as was Mesd, a chaperone protein required for their transport to the cell surface (104). Lastly, among Wnt signaling regulators, all five secreted frizzled-related protein Wnt antagonists were observed in bone; Dickkopf 1-3 (Dkk1-3) and the Dkk receptor Kremen1 (105), but not Kremen2, were expressed in osteoblasts.

In an effort to better understand the role of Wnt signaling in osteoblasts, while bypassing this molecular complexity, two studies (31,106) reported distinct conditional deletions of β -catenin, the central effector of canonical Wnt signalling (107). In the first case, a Dermo1-Cre mouse (108) was used to delete β -catenin in all skeletal progenitor cells at the initial stage of mesenchymal cell condensation (106). This very early inactivation led to a dual chondro/osteoblastic phenotype marked by severe skeletal deformities and pre/perinatal death of the mutant mice. Mutant embryos showed marked delay in chondrocyte maturation caused by decreased cyclin D1 and D2 expression.

Additionally, osteoblast differentiation was impaired, as illustrated by very low type I collagen and alkaline phosphatase expression and complete absence of osteocalcin expression. Neither the collar of long bones nor the parietal bones seemed to be mineralized by von Kossa staining, indicating that β -catenin is necessary for both endochondral and intramembranous ossification. The second model of β -catenin inactivation used a human osteocalcin-Cre strain (31), thus it resulted in loss of β -catenin function much later in development, and the mutant mice did not die before three to four weeks of age. The mouse bones showed thin cortices and an absence of trabeculae, consistent with a decrease in osteoblast number. *Ex vivo* culture of the mutant osteoblasts confirmed their inability to mineralize and express osteocalcin. Less expected was the finding of increased osteoclast number in the mutant mice. A role for canonical Wnt signaling during osteoclast differentiation was confirmed, however, in another mouse model generated by the same group. In this case, human osteocalcin-Cre mice were used to inactivate APC (31), a giant intracytoplasmic molecule mediating β -catenin degradation (107). Considering this function, APC deficiency should produce a phenotype opposite of that produced by β -catenin inactivation. Indeed, these APC mutant mice died at about two weeks of age, displaying thin osteosclerotic bones and anemia. This phenotype was essentially the result of a dramatic reduction in osteoclast number in the face of normal bone formation.

Another point of focus was the role and regulation of expression of the secreted Lrp antagonist Dkk1 in osteoblasts (109). In agreement with last year's finding that increased Dkk1 expression is associated with osteolytic lesions in patients with multiple myeloma (110), mouse models overexpressing Dkk1 in osteoblasts were shown to develop severe osteopenia associated with limb shortness and digit deletion at high levels of transgene expression (111,112). The transgenic mice displayed thin and discontinuous calvarial bones; both trabecular and cortical thickness were decreased. This phenotype was linked to a decrease in osteoblast number, whereas osteoclast number was unchanged. Of interest, considering the negative effect of Dkk1 on bone formation,

two studies reported that glucocorticoids induce *Dkk1* gene expression both in adult rat diaphysis and cultured osteoblasts (113,114), suggesting that Wnt signaling may be impaired in glucocorticoid-induced osteoporosis. *Dkk1* mRNA levels were also shown to be downregulated by PTH treatment of cultured embryonic tibiae (112), whereas *Wnt7b* expression was increased (115). Along with the fact that PTH treatment of osteoblastic cells can activate a reporter gene specific for a canonical Wnt response via β -catenin (116), and that transgenic mice harboring the *Lrp5* G171V gain-of-function mutation are more responsive to PTH anabolic effects than are wild-type mice (115), these data support the hypothesis that PTH and Wnt signaling may functionally concur to regulate bone formation positively. Considering the proven importance of these two pathways in human bone biology, it is predictable that this novel concept will induce much further attention in the future from both the basic science and medical communities.

RANK SIGNALING IN THE OSTEOCLAST

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Receptor activator of NF- κ B (RANK) signaling, combined with that of the macrophage colony-stimulating factor (M-CSF) receptor c-fms, is sufficient and necessary for stimulating osteoclastic differentiation, activation, and survival. At the most proximal level, RANK ligand (RANKL) binding to its cognate receptor induces the interaction of tumor necrosis factor receptor-associated factors (TRAFs) (especially TRAF2 and TRAF6) with RANK. These form a complex with TAB1 and TAB2 (directly or via interaction with RIP), which recruits the mitogen-activated protein kinase (MAPK) kinase kinase TAK1 to the complex. TAK1 can both feed into the p38 and Jnk cascades, and like NIK, phosphorylate I κ B kinase β (IKK β), which in turn, phosphorylates I κ B. Phosphorylated I κ B is degraded through the proteasome pathway, which derepresses NF- κ B, thus initiating the transcription of a variety of essential genes. Over the several years that RANK

signaling has been studied, the intracellular pathways leading to osteoclast differentiation have expanded beyond signaling to activate NF- κ B to include MAPK activation, signaling through phosphatidylinositol 3-kinase (PI-3K) to Akt and mTOR, and activation of the nuclear factor of activated T cells (NFAT).

The 2004 meeting included several presentations that expand our understanding of RANKL signaling in the osteoclast, with a broadening interest in other intracellular pathways that are meaningfully activated by or interact with the downstream elements of the RANK receptor. A large body of evidence that was presented pointed toward the importance of signaling through the NFAT transcription factor, which comprises the better part of this review, with an emphasis on its importance for controlling differentiation.

NFAT as a Key Regulator of Osteoclast Differentiation

NFAT activity has been described in a number of recent studies as both sufficient and essential for osteoclast differentiation (117-120). NFAT activation and translocation into the nucleus involve a dephosphorylation step that is mediated by the phosphatase calcineurin. Overexpression of c-Jun and c-Fos in osteoclasts induces NFAT expression, providing a possible mechanism for the induction of osteoclastogenesis. Consistent with a number of studies showing that NFAT is induced by RANKL treatment and that two calcineurin inhibitors, cyclosporine A and FK506, inhibit osteoclastogenesis, it was indeed shown that constitutively active NFATc1 and NFATc2 can induce osteoclastogenesis in the absence of RANKL treatment and can rescue the osteoclast differentiation defect when introduced into c-Fos(-/-) osteoclasts. Of interest, osteoclast differentiation induced by NFAT overexpression can also be blocked by expression of dominant-negative forms of c-Jun or c-Fos, suggesting that communication runs both ways.

As reported at the meeting, several laboratories have made a concerted effort to explore the role of NFAT in osteoclast differentiation, and a variety of signaling

pathways have been shown to induce its expression. That NFAT is sufficient to induce osteoclastogenesis was highlighted in work describing the phenotype of transgenic mice overexpressing constitutively active NFATc2 (121). NFATc2 can be placed upstream of NFATc1, based in part on the observation that although NFATc2 protein is expressed in undifferentiated osteoclast precursors, NFATc1 protein is not. Furthermore, overexpression of NFATc2 in M-CSF-dependent spleenocytes induces NFATc1 expression, and overexpression of NFATc1 is effective in inducing the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleate osteoclast-like cells. NFATc1(-/-) mice do not survive to birth, and deletion of the other NFAT isoforms does not result in a distinctive skeletal phenotype. However, transgenic mice with TRAP promoter-driven, osteoclast-specific expression of NFATc2 were found to exhibit a roughly 50% decline in bone volume, as measured by microcomputed tomography. Osteoclast-specific NFATc2 transgenics were viable and fertile, although they were much smaller than their wild-type littermates. Transgenics displayed a higher rate of osteoclast formation in long bones, with a substantially thinner growth plate. *In vitro* analyses showed a heightened sensitivity to M-CSF- and RANKL-induced osteoclastogenesis, which generated an increased number of osteoclasts with a greater average size. Consistent with its role as a more selective prodifferentiation transcription factor, this effect was not associated with any change in osteoclast survival after RANKL withdrawal.

Immediately upstream of NFAT lies the phosphatase calcineurin, and inhibitors of calcineurin do suppress osteoclastogenesis, as described above. Greater specificity for this effect was reported with calcineurin overexpression in RAW-C3 cells, which led to induction of NFATc1 and NFATc2 (but not NFATc4) expression (122). To further solidify the role of calcineurin in osteoclastogenesis, suppression of calcineurin isoforms using U1 antitarget vectors suppressed NFATc1 expression, with the following rank order for the four different isoforms targeted: $A\alpha > \beta 1 > A\beta = A\gamma$. Using stem cells from calcineurin $A\alpha(+/-)$ and $A\alpha(-/-)$ mice, up to a 50% reduction in the *in vitro* formation of

TRAP-positive osteoclast-like cells was observed in response to treatment with RANKL and M-CSF. These data are consistent with previous findings from pharmacological studies reporting that calcineurin is a key player in regulating NFAT activity.

Interplay Between NFAT and Other Transcription Factors

Several studies reported findings on how NFAT expression is controlled by other signaling intermediates downstream of RANK. These studies demonstrate the interplay between NFAT signaling and that of NF- κ B, as well as the induction of NFAT by inflammatory cytokines and novel signaling pathways involved in cross-talk between osteoclasts and osteoblasts, as discussed below.

Downstream of the RANK receptor, NFATc1 was shown to rescue the differentiation defect seen in NF- κ B p50/p52 double knockout (DKO) osteoclast precursors (123). DKO mice showed only a mild three-fold induction of NFATc1, compared with a 12-fold induction in wild-type mice, in response to RANKL treatment of M-CSF-dependent spleenocytes. Similarly, RANKL was severely impaired in inducing NFATc1 expression and osteoclastogenesis in IKK α -deficient liver-derived precursors (124). In relation to the NF- κ B p52 subunit, p100 processing was defective in IKK α (-/-) cells. In a follow-up study, the proteasome inhibitor MG132 was used to block both RANKL-induced p100 processing to form p52 and induction of NFATc1 in RAW264.7 cells. Together, these studies clearly place NFATc1 downstream of NF- κ B in RANKL signaling.

Consistent with the interplay between NFAT and c-Fos, overexpression of c-Fos in NF- κ B p50/p52 DKO spleenocytes was sufficient to restore partially both NFATc1 expression and the formation of osteoclasts *in vitro* (123). Introduction of a constitutively active nuclear form of NFAT3 (Δ NFAT) could restore RANKL-induced osteoclast differentiation, with lesser effects seen if RANKL was replaced with either tumor necrosis factor α (TNF α) or interleukin 1 (IL-1). Of interest, Δ NFAT was not sufficient to induce osteoclastogenesis in the p50/p52 DKO spleenocytes in the absence of RANKL,

which differs from the response seen in wild-type precursors overexpressing NFATc1 or NFATc2, as discussed above. With regard to other cytokines, the same study showed that although IL-1 failed to induce either NFATc1 expression or osteoclast formation in the wild-type osteoclast, it was effective if c-Fos was overexpressed. Furthermore, the study showed that TNF α could independently induce both osteoclast formation and NFATc1 expression in wild-type spleenocytes, and yet in the I κ B kinase α (IKK α)(-/-) background, TNF α could rescue RANKL-induced osteoclastogenesis without p100 processing or induction of NFATc1 (124). This finding suggests that under the right circumstances, signals originating at the TNF α and IL-1 receptors can mimic the RANKL effect. Meanwhile, the effect of combined RANKL and TNF α to bypass the IKK α (-/-) defect in the absence of p100 processing and NFATc1 induction suggests that NF- κ B and NFAT signaling are not always essential. The discovery that Δ NFAT cannot induce osteoclastogenesis in the p50/p52 DKO background without RANKL stimulation further suggests that NFAT might not always act as the "master switch" for differentiation.

Novel Pathways to NFAT in the Osteoclast

In an exhaustive study of immunoreceptor tyrosine-based activation motif (ITAM) signaling in osteoclasts, one study provided clear evidence that RANKL/TNF α /IL-1 signaling is not the only pathway leading to induction of NFAT in the differentiating osteoclast (125). The ITAM-encoding adapter proteins, DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcR γ), were shown to be crucial for osteoclast differentiation *in vitro* (126). Using DAP12(-/-) mice, which show a mild increase in bone mass vs. wild-type littermates, *in vitro* osteoclastogenesis was found to be normal in coculture, but was severely suppressed in isolated bone marrow macrophages stimulated with RANKL and M-CSF. This finding suggests that an osteoblast-mediated signal could overcome the defect and led to the identification of the osteoclast-associated receptor and its adaptor FcR γ as providers

of that compensatory signal. DKO mice lacking both DAP12 and FcR γ displayed an osteopetrotic phenotype and suffered an osteoclastogenesis defect *in vitro*, using the coculture system. This defect could be restored with the reintroduction of FcR γ by retrovirus. One can link the activity of these receptors to NFAT via their encoding of ITAMs within their cytoplasmic domains. ITAMs induce calcium signaling via phospholipase C γ (PLC γ), and indeed PLC γ was not phosphorylated in DKO cells, nor was there an intracellular calcium signal after stimulation with RANKL and M-CSF. Calcium signaling was restored if either DAP12 or FcR γ were reintroduced into respective knockout bone marrow macrophages, but no calcium signaling was restored if ITAM was mutated in the reintroduced adaptors. Osteoclast transcripts were then profiled in RANKL-stimulated cells by gene chip analysis for putative prodifferentiation factors that could possibly be disrupted in DKO cells. In these arrays, NFATc1 expression was substantially suppressed, compared with that seen in wild-type osteoclasts. To provide the final link, reintroduction of NFATc1 into DAP12(-/-) osteoclasts rescued the differentiation defect. The findings of this study therefore place NFATc1 downstream of DAP12 and FcR γ in osteoclastogenic signaling pathway(s). These adapters add to a growing list of NFAT inducers that includes RANK and TNF and IL-1 receptors. It seems likely that cross-talk between DAP12/FcR γ and RANK also occurs upstream of NFAT, and we await further information on this subject.

RANK Signaling Through PI-3K

In view of certain data presented at the meeting suggesting that NFAT signaling is not always necessary for osteoclast differentiation, it is worthwhile to explore other signaling paths that contribute to the differentiation and lifespan of the osteoclast. A relatively novel signaling pathway involves RANK (TNF α and IL-1) signaling through TRAF6 and c-src to activate the PI-3K pathway (127,128). In particular, one study very nicely illustrated the importance of this pathway for both differentiation and maintenance of osteoclast survival (129). Downstream of PI-3K, signaling through Akt to mTOR was previously implicated in maintaining

osteoclast survival, putatively through the regulation of protein translation, which itself was shown to be critical for osteoclast differentiation and survival. This new study helps to refine our understanding of the importance of RANKL-induced phosphorylation and activation of Akt and mTOR by showing distinct functions for each of these kinases. With the use of small interfering RNAs (siRNAs), the authors showed that Akt is required for osteoclast differentiation, and yet its absence does not sensitize the osteoclast to induction of apoptosis. In contrast to the Akt response, siRNA for mTOR activated caspase 3 activity with consequent induction of osteoclast apoptosis. Downstream, mTOR activity was tied to the downregulation of Bim expression, and siRNA to Bim protected osteoclasts from induction of apoptosis via M-CSF withdrawal. Although mTOR activation was previously shown to require both PI-3K and Akt activity in the osteoclast (127), suppression of Akt 1 and Akt2 with siRNA did not block signaling to mTOR, although PI-3K was still required. Of interest, Akt siRNA caused a reduction in the activation of IKK and the phosphorylation of I κ B in response to RANKL. This correlated with suppressed nuclear accumulation of the NF- κ B p50 subunit, providing a likely mechanism for the antidifferentiative effect. Ultimately, the biological relevance of separating Akt from mTOR signaling in the osteoclast explains the divergent effects of each respective kinase to control differentiation versus cell survival.

Summary

Presentations at the 2004 annual meeting showed substantial progress in unraveling the complex signaling pathways that extend from liganded RANK to the induction of osteoclast differentiation and protection from apoptosis. The large and growing body of evidence linking NFAT to the control of differentiation suggests that this transcription factor may be a key regulator, if not the master regulator, in osteoclasts. With the discovery that not only RANK, but also TNF α and IL-1 receptors, DAP12, and FcR γ can induce NFAT expression and/or activity, one can begin to understand how NFAT can serve as an integrator of diverse signals that influences osteoclast formation. This is evidenced not only by the number of

receptors/adaptors that induce NFAT, but also by the varied signaling pathways that seem to cross through NFAT in their control of osteoclast differentiation. At upcoming meetings, we also hope to see clarified exactly which transcriptional products downstream of c-Fos/c-Jun and NF- κ B create the link to NFAT induction and exactly how their activity might feed back into the transduction of signals (presumably through calcineurin) to NFAT activation. These questions are no doubt under investigation, and we may hear more in Nashville in 2005.

VITAMIN D

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Nutrition

Serum level of 25 hydroxyvitamin D (25OHD) remains the primary determinant of vitamin D sufficiency and insufficiency. The general consensus is that the "normal" range for 25OHD quoted by most laboratories is too low; however, there is no consensus as to what the "normal" range should be. Reliance is often placed on PTH measurement. A number of abstracts have demonstrated that 25OHD correlates negatively with PTH in a given population, although the scatter of points around the line of correlation is wide, reducing the value of this correlation for a given individual. However, studies reported at this meeting confirmed earlier observations that administration of vitamin D to subjects with a low 25OHD level increases 25OHD and reduces PTH. This relationship can be affected by a number of factors. For example, Tsugawa *et al.* (130) showed that elderly individuals have a higher PTH level, for any given 25OHD level. In addition, 1,25(OH) $_2$ D therapy may also alter this relationship (131). Another problem is that commercial assays for 25OHD use a variety of methods that do not necessarily provide congruent results, as shown in studies by Gemar *et al.* (132) and Holick *et al.* (133); furthermore, results may vary widely in the same individual (134). Therefore, it is not clear what level of 25OHD is appropriate.

If one accepts that a 25OHD level around 25-30 ng/mL (62-75 nmol/L) represents vitamin D repletion, and a level below 20 ng/mL, vitamin D insufficiency, the amount of vitamin D insufficiency in this world would be staggering. For example, Arabi *et al.* (135) reported that only 5% of elderly Lebanese people had a 25OHD level above 20 ng/mL; Harinarayan *et al.* (136) found that 80% of postmenopausal females in India had a 25OHD level below 20 ng/mL; and Berry *et al.* (137) noted that 72% of young girls in Manchester, England had a 25OHD level below 12 ng/mL. Even in sunny southern California, Blau *et al.* (138) noted that 29% of postmenopausal females had a 25OHD level below 20 ng/mL. Skin color matters. A number of abstracts confirmed published studies showing that African Americans and Hispanics have lower 25OHD levels than do whites. Moving from a sunny to a less sunny climate was found to have an adverse effect on 25OHD level, as demonstrated by Erkal *et al.* (139) in their study of Turks in Germany vs. Turks in Turkey. Disease matters, too. The results of other studies showed that children with cystic fibrosis (140), chronic inflammatory disease (141), and HIV (142) had lower 25OHD level than did healthy children. Furthermore, Looker (143) reported a negative correlation between body fat and 25OHD levels, especially in younger white females.

How great a clinical problem is a low 25OHD level? Casado *et al.* (144) showed a negative correlation between the incidence of hip fracture and 25OHD level, as well as earlier exposure to sunlight. Simonelli *et al.* (145) found that 82% of patients admitted with a hip or extremity fracture had a 25OHD level below 20 ng/mL. Al-Oanzi *et al.* (146) did not observe a difference in total 25OHD and 1,25(OH)₂D in patients with osteoporosis, but did see lower free concentrations of these metabolites, if calculations were based on vitamin D binding protein (DBP) and albumin levels (DBP levels were higher in osteoporotic subjects). Quality of life was negatively correlated with low 25OHD level (147).

Clinical Utility of Vitamin D Administration

Given that vitamin D insufficiency is widespread, how much and what type of

benefit can be derived by attempting to normalize vitamin D level? This question has been the subject of many publications that will not be reviewed here. However, a number of abstracts addressed aspects of the question. Several studies from different groups (86,87,148-151) showed a reduction in falls and sway with increased lower extremity strength in elderly subjects treated with vitamin D. However, fracture reduction was more difficult to demonstrate (148). In several studies, improvement in BMD was not observed, even with vitamin D or 1 α hydroxyvitamin D treatment (152-157), and such treatment seemed inferior to bisphosphonate therapy for this purpose (155,158). The general premise is that vitamin D supplementation works best in a vitamin D-deficient individual, with little gain once normalization of 25OHD level is obtained. However, vitamin D analogs may change this belief. For example, Matsumoto *et al.* (159) showed a dose-dependent increase in BMD in osteoporotic subjects given the Chugai analog ED-71. Although this was the only clinical report about ED-71 at the meeting, ED-71 was shown in a number of animal models to have beneficial effects on BMD (160-164), and the newer analog ED-120 is showing promise (165).

Mechanisms of Action

It is generally accepted that 1,25(OH)₂D₃ exhibits both genomic and nongenomic actions, but there is no agreement as to their relative physiologic importance. With respect to nongenomic actions, the field is divided as to whether 1,25(OH)₂D₃ has a unique membrane receptor or works via the nuclear vitamin D receptor (nVDR) that has been modified in some way to account for its activation by analogs that do not exert genomic actions. Nemere *et al.* (166) reported on their recently sequenced 1,25(OH)₂D membrane binding protein MARRS and showed that a ribozyme directed against it reduced 1,25(OH)₂D binding to the protein and protein kinase C stimulation of 1,25(OH)₂D₃. Boyan *et al.* (167) proposed that a phospholipase A2-activating PLAA protein linked MARRS to downstream signaling, although it was not clear whether 1,25(OH)₂D regulated PLAA. In contrast, Mizwicki *et al.* (168) used computer modeling of nVDR to suggest an alternative pocket where vitamin D analogs known to stimulate nongenomic actions could reside, suggesting that the

differential binding of these analogs to nVDR could mediate nonnuclear events. Barletta *et al.* (169) made the interesting observation that the previously described scaffolding protein MNAR, which plays a role in the nongenomic actions of the estrogen receptor, may do likewise for nVDR. Phosphatidylinositol 3-kinase (PI-3K) may play a role in 1,25(OH)₂D signaling, such that inhibition of PI-3K blocks the ability of 1,25(OH)₂D to increase vascular endothelial growth factor and hypoxia-inducible factor 1 α levels (170).

Most attention at the meeting focused on the nuclear hormone receptor and its genomic actions. Because moving nVDR in and out of the nucleus is likely of importance for its genomic (and nongenomic) actions, demonstration by Miyauchi *et al.* (171) of regions in nVDR required for nuclear localization and the role of importin 4 in this process was an important advance. Peleg *et al.* (172) pointed out the impact of the ubiquitination system in nVDR degradation and suggested that nuclear localization protects nVDR from this process. Although the DRIP and SRC complexes are well-known coactivators of nVDR, and an increase in their levels may partially explain the increased sensitivity of osteoclast precursors to 1,25(OH)₂D₃ in patients with Paget's disease (173), the mechanism by which 1,25(OH)₂D suppresses some genes is less clear. Okazaki *et al.* (174) found that both estrogen and 1,25(OH)₂D inhibited parathyroid hormone-related protein (PTHrP) expression in MCF-7 breast cancer cells, which is associated with recruitment of histone deacetylase 2 (HDAC2) and DNA-dependent protein kinase (DNAPK) to the DR3 half site nVDRE_m in the PTHrP promoter. Inhibitors of HDAC2 and DNAPK blocked the inhibition of PTHrP expression.

Hairless is emerging as an important corepressor of nVDR. Mutations in hairless cause alopecia in humans and mice with the same phenotype as seen in VDR mutations. Slater *et al.* (175) identified the sites in hairless and nVDR where interaction takes place. Although hairless can interfere with 1,25(OH)₂D-stimulated genomic actions, not all mutations in hairless that cause alopecia or disrupt the ability of hairless to block

nVDR function block VDR/hairless interaction (176). Furthermore, although hairless blocks 1,25(OH)₂D-stimulated gene expression, alopecia seen with VDR mutations is not dependent on 1,25(OH)₂D (animals null for 25OHD 1 α -hydroxylase do not lose hair), suggesting there is more to the story. One possibility is that a ligand in the hair follicle, other than 1,25(OH)₂D, is involved with regulating the effect of nVDR on hair follicle cycling. Jurutka *et al.* (177) showed that a number of unsaturated fatty acids, as well as lithocholic acid, could stimulate the expression of vitamin D responsive element containing promoter constructs, indicating the plausibility of this concept. Alternatively, nVDR may interact with a different signaling partner not requiring a ligand. The authors also provided evidence for this theory by demonstrating 1,25(OH)₂D-independent β -catenin binding to nVDR, with a reduction in T-cell factor/lymphoid-enhancing factor (TCF/LEF) promoter activity. Shi *et al.* (178) found 1,25(OH)₂D inhibition of TCF/LEF promoter activity in MC3T3-E1 cells transfected with both nVDR and the TCF/LEF promoter construct, suggesting that this interaction is ligand dependent, at least in these cells. β -catenin is important in hair follicle cycling, as well as in osteoblast function, so this interaction needs to be further investigated by those interested in bone and skin.

Metabolism

A number of abstracts focused on the regulation of vitamin D metabolism, in particular the production of 1,25(OH)₂D. Horwitz *et al.* (179) observed that PTH(1-34) caused a greater increase in serum levels of 1,25(OH)₂D than did PTHrP(1-36), despite comparable effects on serum and urine levels of calcium and phosphate. Yuan *et al.* (180) compared the effect of phosphate diets on 1 α -hydroxylase expression in mice overexpressing Npt2 (a renal phosphate transporter) with that in normal controls. The authors concluded that serum phosphate was not the sole determinant of 1 α -hydroxylase expression, because the high-phosphate diet in normal mice that was required to match the serum phosphate level in the Npt2 overexpressors reduced 1 α -hydroxylase expression below that seen in the Npt2 overexpressors. Fibroblast growth factor 23 (FGF-23) is a

known inhibitor of 1α -hydroxylase expression. Normal mice respond to a high-phosphate diet with increased FGF-23 expression. These responses were blunted in Npt2 null mice (181). The exact mechanism by which any of these hormones or ions controls 1α -hydroxylase expression remains controversial. Iyer *et al.* (182) introduced a new player, by noting that if GATA-1 (an activator of globin transcription) is overexpressed in renal cells, VDR and 1α -hydroxylase expression are both reduced. The control of substrate (*i.e.*, 25OHD) flow to mitochondrial 1α -hydroxylase was the subject of several abstracts from the Adams laboratory. Hsc 70 was found to be capable of binding 25OHD and $1,25(\text{OH})_2\text{D}$, and along with its mitochondrial homolog grp75, also seems to interact with 1α -hydroxylase (183,184), suggesting a direct delivery service by these proteins. To date, there is only one known 1α -hydroxylase, but it is more widely distributed than previously thought. In contrast, a number of enzymes have 25-hydroxylase activity. The mitochondrial form (*i.e.*, CYP27A1) is the dominant form in liver, but a number of microsomal forms have been described, including CYP2R1, which is expressed in prostate (185), kidney (186), and liver (186), and CYP2C11, which is found specifically in male rat liver (187). These vary somewhat in substrate specificity, and their relative contributions to overall vitamin D metabolism remain unclear.

Polymorphisms

Equating polymorphisms in VDR to function remains of interest. Kaczmariska *et al.* (188) focused on the Fok polymorphism (F/f) in exon 2, which determines the translation start site and the long/short (L/S) (*i.e.*, variable lengths) of A in the 3' untranslated region (3'-UTR). The authors concluded that the combination of F and L is additive on VDR function, perhaps because F increases VDR transcriptional activity and L increases VDR level. In a study of 78 females, Rapuri *et al.* (189) noted that those with the SS genotype lost bone more rapidly than did those with the LL genotype. Other investigators (190,191) have not observed striking correlations between known polymorphisms in the 3'-UTR and BMD or response to therapy. Fang *et al.* (192) scanned the DNA around

the VDR gene in a large population of whites, Asians, and African Americans and found 62 polymorphisms (*i.e.*, single nucleotide polymorphisms [SNPs]), several of which could alter potential regulatory elements in the promoter or destabilize 3'-UTR. Examining several SNPs in a large Rotterdam study population of more than 6000 elderly whites, the authors found associations between SNPs in the locus containing *cdx-2* or in the 3'-UTR and fractures, although no effect on BMD could be demonstrated. In contrast, Quesada-Gomez *et al.* (193) found an association between the *cdx-2* polymorphism and BMD in a Spanish population.

Target Tissue Response

Bone

The surprising observation several years ago that VDR null mice fed a high calcium/lactose diet had essentially normal bone was further explored by Yamamoto *et al.* (194), who found that when VDR knockout was confined to bone, such that all general metabolic parameters of calcium metabolism were otherwise normal, the bones had increased BMD, with decreased trabecular bone turnover. In contrast, ovariectomized monkeys and rats showed increased BMD, if treated with $1,25(\text{OH})_2\text{D}$ or an analog (*e.g.*, ED-71 or ED-120) (160,161,165,195). Effect on bone formation rate (BFR) depended on the site examined. Unlike $1,25(\text{OH})_2\text{D}$, EB1089 prevented bone loss during hindlimb suspension (163). Thus, whether VDR is good or bad for bone is unclear and may depend on where one looks, the presence or absence of estrogen, or the vitamin D analog used. The long-held view that $1,25(\text{OH})_2\text{D}$ is required for a normal skeletal response to PTH received confirmation when Miao *et al.* (196) showed that PTH was more effective in increasing BMD in normal than 1α -hydroxylase null animals, although PTHrP restored a number of skeletal abnormalities in 1α -hydroxylase null animals that were also null for PTH (197).

Intestine

TRPV6 (CaT1) is a calcium channel in the intestinal brush border that seems to be the rate-limiting step in intestinal calcium transport. This channel is induced by

1,25(OH)₂D, but the mechanism is unclear. Watanuki *et al.* (198) identified several potential sites in the TRPV6 promoter at which such induction could occur. Mice heterozygous for VDR showed partial resistance to 1,25(OH)₂D, with respect to calcium absorption and induction of TRPV6 and calbindin 9k (199).

Kidney

The direct actions of 1,25(OH)₂D on renal function have received little study. One report indicated that 1,25(OH)₂D induces a splice variant of Npt2 (200) that could conceivably increase phosphate reabsorption. PTH, which increases 1,25(OH)₂D production by the kidney, may also impact the actions of 1,25(OH)₂D on the kidney by increasing the VDR in the distal tubule, but decreasing VDR in the proximal tubule (201).

Cancer

Valrance *et al.* (202) developed mammary tumor cell lines from VDR null mice and showed resistance to the antiproliferative actions of 1,25(OH)₂D. These cells may serve as a model for exploring the role of nVDR in mediating various effects of 1,25(OH)₂D. Polymorphisms in VDR at the Fok and Taq loci did not correlate with prostate cancer incidence (203), suggesting that to the degree these polymorphisms alter tissue response to 1,25(OH)₂D, they are not influential in the effect of 1,25(OH)₂D on tumor development. A number of analogs of 1,25(OH)₂D are more potent than 1,25(OH)₂D in suppressing tumor growth; Tu *et al.* (204) noted that the same is true for PTHrP suppression. The difficulty in showing clinical effectiveness of these analogs in human malignancies, however, is disappointing.

GROWTH PLATE DEVELOPMENT: NEW PIECES ADDED TO THE PUZZLE

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Endochondral bone development is a complex process in which undifferentiated mesenchymal cells differentiate into chondrocytes, which then undergo well-ordered and controlled phases of proliferation, hypertrophic differentiation, death, blood vessel invasion, and finally, replacement of cartilage with bone. The process recapitulates basic and fundamental mechanisms of cell biology in a highly specific spatial and temporal pattern and thus constitutes an excellent model for the analysis of such mechanisms. In recent years, the tools provided by modern genetics have been instrumental in the process of identifying and dissecting basic molecular mechanisms of endochondral bone formation both in mice and men.

This year's American Society for Bone and Mineral Research meeting has definitively added more critical pieces to the puzzle.

In Search of the "Right" Couple

Bone morphogenetic proteins (BMPs) play a variety of crucial roles during animal development (205). These cytokines were first recognized for their bone-inducing abilities. Surprisingly, because of the lethality of universal knockouts before skeleton formation, there is currently no *in vivo* evidence of a general requirement for BMP signaling during skeleton development. Conditional knockout experiments, and eventually the appropriate combination of some of them, will provide the key to a complete understanding of the role of BMPs in chondrogenesis. The tour de force started at this year's meeting. Researchers generated "floxed" BMP2 and BMP4 mice and bred them with mutant animals carrying Cre recombinase under the control of the PRX1 promoter, which is specifically expressed in limb bud mesenchyme. Surprisingly, lack of both BMP2 and BMP4 seems to have an effect on osteoblast (but not chondrocyte) differentiation (206). Conversely, mice that are universally null for the BMP6 gene and heterozygous for the BMP2 null allele do display abnormalities of hypertrophic chondrocytes (*i.e.*, the cells that express both BMPs at very high levels) (207). Is BMP2/BMP6 then the "right" couple? Many more mice to come will eventually provide us with the answer.

A Cascade of Transcription Factors Turns a Mesenchymal Cell Into a Chondrocyte

There are three main phases of skeletogenesis: migration of cells to the site of future skeletogenesis; tissue interactions (epithelial-mesenchymal) that result in mesenchyme condensations; and differentiation of chondrocytes and osteoblasts from mesenchymal cells. Each phase involves specific genetic controls. The transcription factor Sox9 is critical for commitment of mesenchymal cells toward chondrocytes. But, how is Sox9 expression regulated? It has previously been reported that Nkx3.2, a transcriptional repressor, promotes axial chondrogenesis by derepressing the expression of Sox9 in somitic mesoderm (208). Evidence has now been presented that a negative feedback loop between two transcription factors (Nkx3.2 and Runx2) could be upstream of Sox9 during chondrogenesis (209). Briefly, to express Sox9 and thus become chondrocytes, condensed mesenchymal cells would need to repress Runx2 expression; Nkx3.2 would play the "repressor." The experiments supporting this model have been carried out *in vitro* in the pluripotent mesenchymal cell line C3H10T1/2. Therefore, we are still a bit far from the *in vivo* reality, but the beginning is promising.

New Evidence: Members of the Canonical Wnt Pathway Are Critical for Endochondral Bone Development

The best characterized roles of Wnt signaling use the so-called canonical pathway to regulate cell differentiation (210). Upon the binding of Wnt proteins to their receptor, disheveled (Dvl) is activated, and this in turn, induces dissociation of the GSK3/axin/ β -catenin complex, which normally leads to phosphorylation of β -catenin and its degradation by the proteasome. Activation of the canonical pathway thus increases the level of β -catenin in cells and moves β -catenin into the nucleus, where β -catenin binds to members of the T-cell factor/lymphoid enhancer factor family of DNA binding proteins to regulate gene transcription. β -catenin also has distinct functions, as part of a complex that binds to cadherins at the cell surface.

At this year's meeting, evidence was presented that β -catenin is critical for skeletogenesis, in general, and for chondrocyte proliferation and differentiation, in particular. Conditional knockout of β -catenin in mesenchymal condensations using a Cre recombinase driven by the Dermo-1 promoter causes perinatal lethality and severe skeletal malformations, secondary to impairment of chondrocyte and osteoblast function (106). In particular, lack of β -catenin impairs both chondrocyte proliferation and hypertrophic differentiation (106). Consistent with these data, *in vitro* "knockout" of Dvl, using small interfering RNA in the chondrocytic cell line RCJ3.1, also affects proliferation and hypertrophic differentiation (211).

It will be interesting to study how the canonical Wnt pathway interacts and cooperates with all of the other different pathways that regulate cell proliferation and differentiation in the developing growth plate.

More About Nkx3.2 and Chondrocytes

Nature apparently likes to "recycle" molecules for numerous and various functions. It is not unusual to find the same transcription factor critically involved in different phases of organogenesis. This is the case for Sox9 and probably also for Nkx3.2 and Runx2. We discussed (above) that these two transcription factors could be part of a negative feedback loop during early stages of chondrogenesis. Strong evidence supporting a role for Nkx3.2 at later stages of endochondral bone development has now also been presented (212). It is well established that parathyroid hormone-related protein (PTHrP) signaling negatively regulates the switch from proliferative chondrocytes to postproliferative hypertrophic chondrocytes. Indian hedgehog (Ihh) inhibits hypertrophic chondrocyte differentiation, and this effect is mediated by increasing PTHrP synthesis at the growth plate periarticular region. Conversely, Runx2 positively regulates chondrocyte hypertrophic differentiation (213). Nkx3.2 dramatically inhibits chondrocyte hypertrophy, and concomitantly, represses Runx2 expression in a chick limb system. Furthermore, Nkx3.2 seems to lie downstream of PTHrP, in that its expression is lost in growth plates isolated

from PTHrP null embryos. Another interesting loop has been added to the already complex network of molecules that tightly regulates chondrocyte proliferation and differentiation. Of interest, mice lacking Nkx3.2 as a result of homologous recombination have severe axial deformities, but no obvious limb abnormalities (214). Some pieces of this nice tale are clearly still missing.

About “Uncoupling” Proliferation and Hypertrophy

Proliferation and hypertrophic differentiation are two tightly coupled events during growth plate development. At this year's meeting, a very interesting and novel mouse model was presented, in which the impairment of cyclic guanosine monophosphate (cGMP)-dependent protein kinase II (cGKII) function led to the unique expansion of a layer of chondrocytes that stopped proliferating, but were not yet classical hypertrophic cells. Mice deficient in cGKII as a result of homologue recombination have been previously reported, and they develop impaired endochondral ossification (215). A naturally occurring mutant rat (KMI) with a 5-kb deletion in the cGKII gene has now been presented (216). The deletion results in a truncated product that lacks the kinase domain. The KMI rats show an abnormal accumulation of postmitotic (but not yet hypertrophic) chondrocytes. cGKII dysfunction seems to impair the synchronized switching from proliferation to hypertrophic differentiation of chondrocyte by prolonging Sox9 nuclear localization through mechanisms that could be independent of Sox9 phosphorylation. These undoubtedly interesting and provocative findings were published in the October issue of *Genes and Development* (217).

Snapshots on Matrix and Its Degradation

It is well established that Ihh is important for endochondral bone development. However, it is still unknown how such a hydrophobic morphogen can travel in the highly charged environment of the cartilaginous matrix. Interesting data were discussed at the meeting supporting the notion that lipid modifications of sonic hedgehog (a member of the hedgehog family that is highly homologous to Ihh)

could make a difference in modulating its function, at least *in vitro* (218). What we still do not know is how the lipidic modifications affect the journey of the molecule through the growth plate.

Everybody agrees that “the” critical fundamental function of both chondrocytes and osteoblasts is to make “matrix,” so it is always exciting when a new molecule that regulates matrix production is discovered and studied. The novel collagen triple helix repeat-containing 1 (*Cthrc1*) gene seems to be critically important for both bone and cartilage formation via regulation of collagen matrix production (219). Numerous pieces of *in vitro* and *in vivo* evidence have supported this conclusion. As is often the case, we are now left with the challenge of unveiling the molecular mechanisms of *Cthrc1* action.

Collagen genes have been instrumental in the identification of transcription factors that turn mesenchymal cells into chondrocytes and/or osteoblasts. Collagen type X is in many respects a mysterious molecule (220). It is tightly coupled to hypertrophic differentiation, even if chondrocytes do not need it to become “hypertrophic.” Significant progress has been made this year by the identification of a specific cartilage-specific enhancer of this gene (221). Is the discovery of a novel transcription factor in the air?

Having only one Runx2 allele seems adequate for protecting articular cartilage from osteoarthritic changes (222), at least in mice. Conversely, too much Smurf2, an E3 ubiquitin ligase that targets Smad2 to the proteasome, is not good news for the health of mouse joints (223). It is likely that two new targets have been identified for the treatment of a painful and deforming disease.

One More Arrow and a “BIG” Conclusion

Normally, bone collars are formed in the perichondrium abutting prehypertrophic and hypertrophic chondrocytes. Ihh is produced by prehypertrophic cells and is required for differentiation of the surrounding perichondrium into bone collar (213). Presented evidence now shows that in cells of the osteoblast lineage, specific overexpression of the BMP2-induced molecule BIG3 leads to premature

formation of the bone collar and concomitant upregulation of *Ihh* activity in the growth plate, as shown by upregulation of *Patched* expression in proliferating chondrocytes (224). The upregulation of *Patched* expression is secondary to a specific action of bone collar cells on adjacent chondrocytes and not to ectopic expression of *BIG3* in chondrocytes. Of interest, it has been previously reported that overexpression of *BIG3* in chondrocytes can indeed lead to accelerated hypertrophy, at least *in vitro* (225). In the cross-talk between *Ihh* and the perichondrium, is the arrow truly going in both directions? The model is interesting and provocative.

PHOSPHATE HOMEOSTASIS AND FGF-23

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Since its discovery as a phosphatonin, fibroblast growth factor 23 (FGF-23) has assumed an emerging physiological role as a regulator of both phosphate and vitamin D metabolism (226). The physiological importance of FGF-23 has been shown most decisively by the finding that removal of the gene from mice leads to hyperphosphatemia and a high 1,25(OH)₂D level (227). Work presented at this meeting has advanced our understanding of the physiology of FGF-23 and its pathophysiological role in both phosphate-wasting and hyperphosphatemic disorders.

In both mice and humans, the serum level of FGF-23 is regulated by phosphate intake. In normal mice, a low-phosphate diet decreases the serum level of FGF-23 (181,228) and a high-phosphate diet increases the level (181). The same is true in humans consuming a low- or high-phosphate diet (229), but the excursions in FGF-23 serum level are much lower in humans (30%) than in mice (700%). Some had previously failed to find a relationship between the FGF-23 level and phosphate intake in humans (230). Mice in whom the vitamin D receptor (VDR) has been ablated have a low or undetectable serum

FGF-23 level (228,231), but feeding a rescue diet to normalize serum calcium and phosphate concentration restores FGF-23 in *VDR(-/-)* mice to the level seen in wild-type mice (228), indicating that phosphate (or possibly calcium or PTH), not vitamin D, is the primary regulator of FGF-23 in *VDR* knockout mice.

Vitamin D also regulates *FGF23* gene transcription, and administration of 1,25(OH)₂D increases the serum FGF-23 level (232), but not the serum phosphate level. This finding led the authors to propose that one of the physiological roles of FGF-23 is counterregulatory (*i.e.*, to prevent postprandial hyperphosphatemia in the face of intestinal phosphate absorption from a meal). It is now clear that FGF-23, in turn, controls the renal synthesis of 1,25(OH)₂D. It seems plausible that in early renal insufficiency, an increase in FGF-23 level (owing to phosphate retention) is primarily responsible for the impairment in vitamin D activation that is the genesis of secondary hyperparathyroidism (226).

It has not been determined whether FGF-23 plays a role downstream of either the *PHEX* gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) in X-linked hypophosphatemia or the corresponding mouse *Phex* gene in the *Hyp* model of phosphate wasting (226). The serum level of FGF-23 is high in *Hyp*, and neutralizing FGF-23 antibodies reverse phosphate wasting (233), suggesting that *Phex* mutations cause phosphate wasting by somehow increasing the level of this phosphatonin. Two laboratories provided genetic evidence that FGF-23 is downstream of *Phex* (234,235). Both groups removed FGF-23 from *Hyp* mice by crossing *Hyp* and *Fgf23(-/-)* mice and showed that in the absence of FGF-23, phosphate wasting and rickets do not occur.

A patient with tumoral calcinosis was reported (236). This young man, the product of a consanguineous marriage, was found to have a very high FGF-23 level in an assay directed toward the carboxyl-terminus, but no increase in full-length or amino-terminal FGF-23, suggesting that the processing of FGF-23 was abnormal. Sequencing revealed a homozygous serine mutation, S129F, in

the *FGF23* gene. The predominant form of familial tumoral calcinosis was recently shown to result from inactivation of the *GALNT3* gene (237), which encodes an enzyme responsible for O-linked glycosylation. Patients with this form of tumoral calcinosis also had high FGF-23 levels, which could be either an inability to produce a biologically active form of FGF-23 or simply a response to hyperphosphatemia. If serine 129 is a site for O-linked glycosylation, as predicted, it seems more likely that both forms of tumoral calcinosis are probably attributable to abnormal metabolism of FGF-23 molecules that are not properly glycosylated. FGF-23 may thus link hyperphosphatemic disorders together in much the way that it has proven to be the common factor in multiple phosphate-wasting disorders.

PAGET'S DISEASE

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Paget's disease, a focal disorder of aging bone, is characterized by abnormal bone turnover, resulting in the deposition of thickened structurally unsound bone. Called osteitis deformans by Sir James Paget in 1876, the disease was initially postulated to be "a form of chronic inflammation of bones" (238). The geographic and familial clustering of Paget's disease, and observations that environment (e.g., rural vs. urban, pet ownership, and unpasteurized milk ingestion) may play a role (239,240), have led to investigations into both the genetic and environmental causes of the disease.

Genetic Causes

In familial Paget's disease of bone (PDB), inheritance seems to be autosomal dominant, with variable penetrance and late disease onset. Seven susceptibility loci are now identified with PDB, most through linkage studies. The most prevalent, PDB3, was identified in 2002, in a large Canadian kindred with PDB, as well as in some sporadic PDB patients. The common mutation was in the *SQSTM1* gene, which encodes the ubiquitin-binding protein p62, also called

sequestosome 1. This lesion produced an amino acid substitution (P392L) in the ubiquitin-binding domain of p62. In the Canadian study, the P392L lesion was found in 46% of families with PDB and 16% of sporadic PDB cases (241). Subsequent studies in other cohorts, in other countries, have found the P392L lesion to be less prevalent, but have described other mutations clustering in the ubiquitin-binding domain of p62 (242-245).

If receptor activator of NF- κ B ligand (RANKL), interleukin 1 (IL-1), or tumor necrosis factor α (TNF α) stimulates an osteoclast, p62 serves as a scaffold protein that links atypical protein kinases, segregating them into different signaling cascades (246). The mechanism of NF- κ B activation involves inactivation of I κ B through phosphorylation, resulting in the translocation of NF- κ B to the nucleus, where it mediates transcription of genes critical to osteoclast differentiation and function. The genetic inactivation of p62 in mice results in an inhibition of this pathway; I κ B is not phosphorylated, and there is no nuclear translocation of NF- κ B. p62 may interact directly with TNF receptor-associated factor 6 (TRAF6), a mediator essential in the classic RANK signaling pathway. But unlike TRAF6 knockout mice, which have an osteopetrotic phenotype, p62-deficient mice have normal bone at birth; the litters are viable and fertile. Subtle differences are noted in a diminished osteoclast resorptive response to cytokines or parathyroid hormone-related protein. In the Paget Symposium that preceded the ASBMR meeting, J. Windle discussed the effect of p62 mutations on osteoclast function, noting that in p62 knockout mice, the osteoclast response to RANKL and TNF α was blunted, but that in transgenic mice expressing the P392L mutation, the findings were reversed; NF- κ B signaling was enhanced in response to RANKL or TNF α (247). This preliminary work suggests that mutations in *SQSTM1* may result in enhanced osteoclastogenesis, polarization of the cell, and resorptive activity at physiological levels of osteoclast stimulation.

The segregation of p62 lesions in families with clinical PDB, and their absence in control populations, argues that the ubiquitin-binding domain of p62 is critical in the expression of PDB, but it is clearly

not sufficient. Although some patients with extensive lesions in this ubiquitin-associated domain may express more severe disease (248,249), other patients with a mutation in the gene show no evidence of clinical PDB (250). This variable penetrance was noted in the first report on the Canadian cohort (251). Functional binding studies have failed to consistently demonstrate impaired binding with the P392L mutation (252), thus the mechanism by which this lesion predisposes to PDB remains unclear (253). Mutations in the *SQSTM1* gene have not been reported to date in persons with osteosarcoma arising from pagetic bone.

What is fascinating about the p62 mutation is the identification of dysfunctional ubiquitination pathways involved in other degenerative diseases of aging, including Alzheimer's, Pick's, and Parkinson's disease (254). Watts *et al.* (255) recently describes mutations in the valosin-containing protein -- a protein involved in the ubiquitination-proteasome degradation pathway -- as causative in inclusion body myopathy associated with frontotemporal dementia and early PDB.

Environmental Causes

Although p62 does not seem to be causative in PDB, it may predispose to the disorder in some families in the context of a second environmental or genetic determinant of disease. The family of paramyxoviruses (e.g., canine distemper virus [CDV], respiratory syncytial virus, measles, and others) has long been implicated in the pathogenesis of PDB, although a pivotal role is controversial (256-259). The debate was enlivened this year by work showing that infection of human osteoclasts by CDV increased their resorptive activity (260) and intriguing preliminary work on the development of a transgenic mouse model of PDB, in which osteoclasts constitutively expressed measles virus nucleocapsid protein (MVNP). Mice that expressed MVNP in osteoclasts had modest increases in osteoclast perimeter, increased woven bone, and an approximately 50% increase in alkaline phosphatase (261). Pagetic osteoclasts have an abnormal phenotype, with increases in the number of nuclei, resorptive activity, sensitivity to physiological levels of 1,25-

dihydroxyvitamin D, and expression of TAF_{II}-17, a putative coactivator of the vitamin D receptor (262). Expression of MVNP in normal osteoclast precursors mimics these findings, whereas the expression of the p62 mutation does not (247,258,263). The reason for this discrepancy is not yet known.

In this preliminary work, the amount of MVNP expressed in cells, and its correlation to true infection, remains unclear. Measles virus genomic sequences have been reported in 46% of the peripheral blood mononuclear cells of individuals with prior infection, and in another study, in up to 20% of the tissues of 51 persons who underwent autopsy (264). Earlier work by has demonstrated transcripts of MVNP in circulating mononuclear precursors of patients with PDB and in some pagetic osteoclasts by *in situ* hybridization and reverse transcriptase (259,265). The role of these sequences in human disease remains unclear. If the pagetic lesion is focal and not apparent until middle age or older, it is appealing to think of the seeding of osteoclast precursors with measles virus during acute infection as permissive to later disease. If measles does have a role in PDB, the prevalence of measles infections internationally contrasts strikingly with the geographic clustering of PDB.

The CD46 receptor, a type I glycoprotein member of the regulator of complement activation, was identified as a measles virus receptor in 1994. It is widely expressed on many cell types, including monocytes, endothelial cells, neuronal cells, and respiratory epithelial cells, where the infection first occurs in humans (264). Binding of CD46 by measles virus permits entry into the cell and triggers nitric acid production in macrophages. Normal mice lack CD46 and are hence resistant to measles virus infection. The transgenic expression of the CD46 receptor in the osteoclasts of mice is an appealing target, permitting measles virus infection of the osteoclast with the Edmonston strain (266). The critical role of CD46 in measles virus infection became controversial in 2002, at which time CD150 (or signaling lymphocyte activation molecule [SLAM]) was identified as a measles wild-type receptor. SLAM is a member of the immunoglobulin

superfamily and is found on different cells than CD46 (mostly dendritic cells and lymphocytes); it triggers interferon γ production on viral engagement. The mechanism by which binding to SLAM mediates clinical measles infection, or whether binding to both CD46 and CD150 receptors is needed to cause measles, remains to be defined. The virology of acute and chronic measles infections will have implications in interpreting this CD46 mouse model of bone disease. (267)

Putting it all together, the work ahead will be to understand the following: what underlying lesions the osteoclast abnormalities reflect (238); the role of ubiquitination pathways in the expression of PDB and their interaction with environmental determinants of disease (239); the regional differences in the disease and variable penetrance in a

family (240); and the focal disorder of bone that occurs only with aging, with no tendency to occur in new bones over the lifetime of an individual (241). A form of chronic inflammation of bone? Stay tuned.

Treatment

Treatment for PDB with zoledronate (5 mg intravenously over 15 minutes) promises to be safe and effective. Zoledronate is more potent than the older bisphosphonates, inhibiting resorptive activity of the osteoclast at lower doses. Clinical trials suggest that it is also more potent in normalizing biochemical parameters of bone turnover in patients with PDB, compared with risedronate (268). In extension studies, patients treated with zoledronate may prove to have a more durable remission.

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