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MEETING REPORTS

Meeting Report from the 28th Annual Meeting of the American Society for Bone and Mineral Research

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CHONDROCYTES: OLD FRIENDS AND NEW ACQUAINTANCES

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This year, chondrocyte research presented at the ASBMR annual meeting was not dominated by one major theme, but was rather a collage of very interesting findings about old friends and some new acquaintances. 'In the beginning there was Soxtrio' could have been said very recently about chondrogenesis. However, it is now clear that Soxtrio (Sox9/5/6) is not alone. The transcription factor Mrf1 regulates chondrogenesis by forming a transcription factory with Soxtrio (1). The data on Mrf1 are solid, the finding is exciting, and other friends joining the Soxtrio and Mrf1 may well be on the way.

An established friend of cartilage, the transcription factor Hif-1α, has also been shown to be quite important for the early differentiation of mesenchymal cells into chondrocytes and for joint development (2). Notably, another member of the family, Hif-2α, is also expressed in chondrocytes, and particularly in hypertrophic cells, where it may regulate collagen type X expression (3). If hypoxia and Hifs are critical for cartilage, a significant role may also be played by oxidative stress through generation of reactive oxygen species (ROS) (4;5). ROS signaling appears to be required for hypertrophic chondrocyte differentiation, and Hif-2α appears necessary for maintaining the viability of hypertrophic chondrocytes by suppressing ROS production (4;5). Is it possible, therefore, that proper endochondral bone development requires a well-coordinated cycle of hypoxia/re-oxygenation? Does excess oxygen kill chondrocytes immediately before the replacement of cartilage by bone, concomitant with exposure to excess phosphate? Of note, uptake of phosphate through the sodium-dependent phosphate transporter 3, by modulating intracellular ATP levels, controls the apoptosis and mineralization of chondrocytes (6).

In the area of metabolism and survival, it is not surprising, but still very interesting, that the classical growth and survival pathway

PI3K/AKT has a leading function in cartilage growth. Lack of PTEN, a classical inhibitor of the PI3K pathway, causes disorganization of the growth plate and increased matrix accumulation (7). Consistent with an important role for PI3K in chondrocyte biology, conditional knockout of IGF-R1 impedes chondrocyte growth, survival, and differentiation (8).

Working together and being able to compensate for the loss of one retinoid receptor (RAR) gene product is something that the RAR family seems to do quite well. Cartilage knockouts of a single RAR (α, β, or γ) did not exhibit any noticeable phenotypes, whereas mice lacking RAR-γ and either RAR-α or RAR-β exhibited a postnatal delay of growth (9). Redundancy is also a common theme for the Smad family of transcription factors. When Smad7, an inhibitor of the TGF-β/BMP pathways, is knocked out (10), mice are viable but display a posterior transformation of cervical vertebral bodies, a finding similar to what has been reported for the TGF-β1 knockout (11). A lack of Smad6 produces even more serious consequences (including sternal fusion, xifoid process bifurcation, and extra ribs), and when both Smad6 and Smad7 are absent, the result is embryonic lethality (10). Curiously enough, an excess of inhibitory Smads can lead to hypoplastic limbs with just a trace of cartilage anlagen (12).

A new relationship has been unveiled between the Wnt canonical pathway and the C-type natriuretic peptide (CNP)/GC-B pathway (13). Researchers have now provided evidence that cyclic GMP-dependent protein kinase II (cGKII), a kinase that lies downstream of CNP, controls the hypertrophic differentiation of chondrocytes through phosphorylation and inactivation of glycogen synthase kinase-3β, leading to increased canonical Wnt signaling.

Two important follow-up studies of TRPS1 and Indian hedgehog (Ihh) have also been presented this year. The transcriptional repressor (TRPS1) that is associated with tricho-rhino-phalangeal syndrome (TRPS) is involved in chondrocyte terminal differentiation by regulating Runx2 expression and activity (14). A clever conditional knockout of Ihh, an old friend of cartilage, exclusively in postnatal cartilage has shown that Ihh is a critical gatekeeper of the hypertrophic zone and regulates the modeling of trabecular bone (15). Notably, activation of the canonical Wnt pathway is also essential for the postnatal growth plate, as transgenic overexpression in cartilage of ICAT, an inhibitor of β-catenin and TCF, resulted in delayed replacement of cartilage by bone and abnormal vascularization and formation of the secondary ossification center (16). More importantly, VEGF appears to be a target for β-catenin signaling in chondrocytes (16). In studies on blood vessel invasion and the replacement of cartilage by bone, a new transcription factor, the Kruppel-like zinc-finger transcription factor 5 (KLF5), has been identified as a positive regulator of MMP9 in chondrocytes (17). This is a novel and provocative finding, since the current model proposes that MMP9 produced by osteoclasts affects chondrocyte biology. In turn, chondrocytes appear to modulate osteoclast activation at the border between cartilage and bone, as BMP-2 treatment clearly upregulates RANKL expression in chondrocytes (18).

It has been further confirmed that the matrix has not only a structural role, but also a function in the regulation of signaling in cartilage, as perlecan, a large heparan sulphate proteoglycan, critically modulates FGFR3c (a chondrocyte-specific FGF receptor) activity (19). Finally, investigators have postulated an endocrine role for the growth plate, which for years has been considered the prototype of an autocrine-paracrine circuit, as vitamin D in chondrocytes may regulate production of FGF23 by osteoblasts in an “endocrine” fashion (20).

In the year when the Nobel Prize in Physiology or Medicine has been awarded to the discovery of siRNAs, it is rewarding to see that the biological role of siRNAs in cartilage is under investigation (21). The field is truly looking forward to learning more about this new area of research.

Finally, one study started from a very simple anatomical observation: most cells of the body have cilia, including chondrocytes (22). The use of a genetic tool —the conditional knockout— has demonstrated that a lack of Kif3a, a protein required for cilia formation, results in severe dwarfism. Notably, chondrocytes without a functional cilium lose their orientation and are round, rather than flat, in the columnar region. All in all, this demonstrates that a basic anatomical observation, together with the tools provided by modern genetics, can still go a long way in enhancing our understanding of chondrocyte biology.

Conflict of Interest: The author reports that no conflict of interest exists.

GENETICS OF OSTEOPOROSIS: OF MICE AND MEN

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Mice and humans got together to reveal a new molecular mechanism for hereditary rickets. A few years ago, Ye *et al*. (23) reported that mice lacking dentin matrix protein 1 (DMP1), a non-collagenous acidic phosphoprotein expressed by odontoblasts and present in the mineralized matrix, develop a severe tooth defect after birth (reminiscent of human dentinogenesis imperfecta Type III), due to partial failure of dentin maturation. DMP1 has also been shown to be selectively expressed in osteocytes (24;25) and was detected by immunostaining in tumors causing oncogenic osteomalacia (TIO) (26). The latter observation raised the intriguing hypothesis that DMP1 could be involved in the regulation of phosphate homeostasis. *Dmp1* KO mice indeed develop hypophosphatemia and osteomalacia-like features, by 5 months of age, that were rescued by a high phosphate diet (27). In human studies, sequencing of the *DMP1* gene in two siblings with hereditary rickets whose parents were apparently unaffected, a disease that would qualify as autosomal recessive hypophosphatemic rickets (ARHR), identified 2 loss-of-function mutations (27). Using a completely different approach in three families with ADHR, a study (28) mapped the disease to chromosome 4q21 using a genome-wide linkage analysis with very high density SNP markers (100 k). Upon sequencing a few genes in the region that includes several genes involved in bone and mineral metabolism (*DMP1*, *DSSP*, *BSP*, *OPN*, *MEPE*), loss-of-function mutations were identified that were homozygous in the affected siblings and heterozygous in their parents, as predicted by the recessive pattern of inheritance of the disease. Interestingly, one of the cases exhibited high levels of circulating FGF23, a phosphaturic hormone. The question of whether DMP-1 could be a substrate for PHEX was then raised, but no evidence was found for this hypothesis. This work has now been published (29). In addition, another study (30), also now published (31), reported that serum levels of FGF23 were nearly as high in *Dmp1* KO mice as in *Hyp* mice. Targeted re-expression of DMP1 in bone by the Col 3.6 promoter normalized FGF23 levels and rescued hypophosphatemia in these mice. How DMP1 regulates serum phosphate homeostasis remains to be elucidated.

Continuing seminal work aiming to identify the cause of fibrodysplasia ossificans progressiva (FOP) (32), a devastating genetic disorder of ectopic and exuberant ossification in soft tissues, a study (33) confirmed the presence of a c.617G>A mutation in *ACVR1*, a gene encoding an activin A/BMP type 1 receptor, in all of 60 sporadic cases with the classical features of the disease. Furthermore, additional mutations were identified in the ACVR1 protein kinase domain that were present in FOP cases with some unusual features, such as severe digit reductions. Like the *ACVR1* KO mouse, mice lacking a closely related BMP receptor, BMPR1a, that has a high affinity for BMP-2 and -4, never develop because of gastrulation failure. To circumvent this problem and analyze the implications of BMP1Ra signaling after birth, a young investigator award study (34) used a conditional *BMP1Ra* KO targeted to osteoblasts/osteocytes by the Col I 3.2kb promoter. Most interestingly, these mice

develop a high trabecular bone volume phenotype.

Mice have also been extensively used for linkage analysis and gene identification related to bone mass and structure. Congenic mice (6T) carrying a 30 cM region of Chr 6 from C3H onto B6 are known to have low BMD, increased marrow adipocytes and low IGF-I (35). Two genes that map in that region, *Pparγ* and *Alox5*, are directly involved in the differentiation of mesenchymal stem cells towards the osteogenic or adipogenic lineage and therefore represent good candidates for this phenotype. Looking at the level of *Ppar* and *Alox5* transcripts and protein in bone from 6T, a study found, surprisingly, that expression levels were decreased, whereas expression of pre-adipocytic genes was up-regulated (36). To reconcile these apparently discordant findings, the genomic 3' region of *Alox5* was examined. Not only was a possible regulon in this region discovered, but, most incredibly, *Pparγ* and *Alox5* were found split from their regulatory elements by a 25 cM paracentric inversion on Chr 6 that may have appeared in the C3H mouse strain in the '70s! The congenic mouse in a strain that did not carry this inversion was then re-created, resulting in a phenotype opposite to the original 6T mouse, *i.e*., a high bone mass phenotype (similar to *Pparγ* heterozygous mice). These experiments not only confirm the role of *Pparγ* (and possibly *Alox5*) in the regulation of bone mass (*i.e*., less Ppar is associated with an increase in bone mass), but they are also of crucial importance for all those who use C3H mice for linkage analyses.

A number of linkage and association studies in humans were reported, highlighting how the rapid technological improvements with regard to genotyping now allow examination of a very large number of genes, and SNPs therein, at once (37-40). As an example, one study (38) examined association of femur neck aBMD with nearly 3000 SNPs in 242 candidate genes in 882 Caucasian-American men (MrOS Study) using the Illumina Bead Station. Thirty-three SNPs in 24 genes were significantly associated with FN BMD, including several markers near/in the gene for sclerostin (*SOST*). As seen before with LRP5, this approach implicates *SOST* in the regulation of bone mass in the male population. This observation was replicated in a smaller cohort of osteoporotic patients and controls (41). Vitamin D not only regulates bone and mineral metabolism, but also influences the risk of fractures by its effects on muscle and the risk of falls. Although association between VDR alleles and BMD/fractures has been studied extensively, association studies with falls are rare. A study reported that 3'-UTR *VDR* gene polymorphisms are associated with the (self-reported) incidence of falls in a large cohort of women (n=3200) (42). Interestingly, carriers of the famous "B" (bigB) allele were at significantly decreased risk (-39%) of falls, without evidence for an interaction with $25(OH)_2D_3$ levels. This may aid in explaining some previously discordant findings about the "dissociation" of *VDR* genotypes with BMD, compared to fracture risk.

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HOW MANY OSTEOS DOES IT TAKE TO HAVE A BLAST?

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The development and regeneration of bone involve a complex interplay of cells and growth factors. Cells of the osteoblast lineage, orchestrated by their proliferation, differentiation, and lifespan largely dictate bone formation. At the 2006 ASBMR meeting, both *in vivo* and *in vitro* approaches delineated various pathways involved in osteoblast proliferation and differentiation, and the growth factors and hormones that drive this activity.

Several novel strategies to investigate osteoprogenitor cell proliferation and osteoblast activity were introduced and further characterized. In a transgenic model where the Col2.3 promoter drives the thymidine kinase gene, osteoblasts can be

eliminated with ganciclovir (43). Following ablation, there was a large increase in trabecular bone and a dramatic increase in a population of cells expressing the smooth muscle actin gene that typifies the myofibroblastic phenotype. These cells congregated in endocortical niches where bone formation occurs, and flow cytometric analysis correlated smooth muscle actin with osteoblastic phenotypic characteristics, suggesting that myofibroblastic cells are a trackable precursor population of the osteoblast lineage. To define the function of osteoblasts during osteoclast differentiation, the Col3.6 promoter was used to drive thymidine kinase (44). Following ganciclovir treatment, there was no alteration in transcript levels of RANKL, M-CSF, or OPG, and osteoclasts remained attached to the bone. There were also no changes in vertebral bone volume or in osteocytes. These findings suggest that the source of RANKL, M-CSF, and OPG in adult murine bone is a mesenchymal ancestor prior to Col3.6 expression, or a long-lived cell resistant to acute ganciclovir effects. Furthermore, the lack of bone loss in the absence of osteoblasts suggested that osteoblast support is required for osteoclastic bone resorption in adult mice. Another novel approach is lineage tracing of osteoblasts, accomplished by using tamoxifen-inducible osterix-cre-ER^{T2} in the Rosa26R reporter background (45). A brief exposure to tamoxifen on embryonic day 12.5 turned osteoblast precursors blue by removing a sequence that blocks expression of the *lacZ* gene. This system allows "pulse-labeling" of osterix-positive osteoblast precursors, which could then be followed from the bone collar to perivascular regions and the mineralized bone surface. The system has great potential: blue cells and their progeny can be followed to determine the fate, life-span and self-renewal potential of osteoblasts.

Osteoblast proliferation is an integral and early component of bone accrual. The Pten phosphatase, which downregulates the pro-survival Akt kinase, was examined for its role in proliferation and bone formation by a conditional deletion via an osteocalcin cre recombinase promoter in mice (46). Pten mutant mice had increased whole body BMD by six weeks of age, and a 250% increase in cortical bone thickness by twelve months of age. Significant increases in trabecular thickness and number, osteoid volume and bone formation rate were also found. *In vitro*, there was an increase in osteoblast proliferation and a reduction of apoptosis.

Runx2 and *osterix* are essential for osteoblast differentiation. The transcription factor Runx2 directs multipotent mesenchymal cells to an osteoblastic lineage, and *osterix* is a downstream gene of Runx2. *Osterix* transgenic mice under the control of the mouse 2.3 kb Col1a1 promoter had enhanced osteoblast proliferation, but there was a lack of maturation that led to osteopenia (47). Interestingly, there was increased angiogenesis in bone with upregulated VEGF expression in osteoblasts and increased numbers of osteocytes. *Runx2/Osterix* double transgenic mice showed reduced osteocyte numbers, and the mice were extremely fragile. These findings suggest that while *osterix* plays a role in bone angiogenesis and inhibits osteoblast maturation, Runx2 negatively regulates the osteoblast transition to osteocytes. The Runx2 territory is complex, and the role of Runx2 in osteoblast growth and differentiation needs to be fully elucidated. Detailed studies on the convergence of the Runx2 and BMP-2 signaling pathways identified specific residues at the C-terminus of Runx2 required for Smad association and osteogenic differentiation, and provided evidence that Runx2 acts as an endpoint for BMP-2 response (48). Runx2 was also implicated in the retention of the osteoblast phenotype during cell division to support lineage-specific control of gene expression, by binding to genes encoding osteoblast growth and differentiation signaling molecules, and by controlling histone modifications that influence gene activation or suppression (49). Osteocalcin-driven dominant-negative and constitutively active forms of MAPK (TgMek-dn and TgMek-sp) in osteoblasts were evaluated in novel transgenic models that facilitated the delineation of MAPK function in post-proliferative osteoblasts. These models, and their combination with Runx2 mutant mice,

established a role for ERK/MAPK *in vivo* via phosphorylation of Runx2 (50). New information has also emerged on the role of the cell cycle protein cyclin D1, a suggested regulator of osteoblast growth and differentiation, in Runx2 expression, phosphorylation and function (51).

Several studies focused on the osteoblast as a target of PTH and PTHrP action during bone remodeling. Gsα is a ubiquitously expressed G-protein α subunit of G protein-coupled receptors, including parathyroid hormone/parathyroid hormone-related peptide receptor (PPR). Mice with conditional deletion of Gsα in osteoblasts, OsxCre:Gsα(fl/fl), showed dramatic decreases in bone mass and bone mineral density, and in trabecular and cortical bone. Expression patterns of early and late osteoblast differentiation markers, and TRAP staining, showed a defect in bone formation, rather than excessive resorption, suggesting a role for Gsα in early osteoblasts (52). Studies using osteoblast-specific constitutively active PTH/PTHrP receptor transgenic mice (Colla1-caPPR, Tg) demonstrated that PTH receptor signaling supports osteoprogenitors *in vivo*, as evidenced by bone apposition on the endosteal surface (53). Ectopic bone generated from BMSCs of luciferase-expressing mice demonstrated that the anabolic action of PTH involves proliferation of early osteoblastic cells *in vivo*, resulting in increased bone (54). PTHrP also regulated cyclin D1, a cell cycle protein, both *in vivo* and *in vitro*, and increased osteoblastic cell proliferation *in vitro* (55). That PTH might act on preosteoblasts and increase their proliferation was also demonstrated in experiments employing a murine model of intermittent PTH administration (56).

Studies relevant to osteoblast growth suggest an inhibitory role of *Hey1*, a Notch target gene, in the process of osteoblast maturation (57). Overexpression of Hey1 resulted in decreased bone mass via the loss of trabecular number, thickness, and decreased mineral apposition rate. Sox4, a BMP-2 and PTH-regulated transcription factor, is also expressed by osteoblasts. *Sox4*(+/-) osteoblasts exhibited lower mRNA expression of *osterix* and *osteocalcin*, while transcription of *Runx2* and *osteopontin* were unaffected. Knockdown and silencing experiments revealed *Runx2*-independent osteoblast failure in *Sox4*(+/-) osteoblasts (58). Inhibition of osteoblastic activity and bone formation by matrix metalloproteinase-13 (MMP-13) was suggested in experiments with calvarial osteoblasts overexpressing MMP-13 (59). The role of Pyk2 kinase, a stimulator of osteoclastic bone resorption, was also examined. Pyk2 deletion in *Pyk2*(-/-) mice was shown to induce osteoblast growth and differentiation *in vivo* and *in vitro* (60).

An important mechanism regulating skeletal development involves the Wnt/LRP5 pathway. One study reported that Wnt3a suppressed vitamin D3- and PTH-induced RANKL and OPG mRNA in osteoblast-like cell cultures (61). Another study using OPG reporter constructs revealed that both Wnt/β-catenin and BMP-2 signaling regulate Wnt responsive gene expression during osteoblast differentiation (62). Overexpression and siRNA studies demonstrated control of osteoblast differentiation through coupling of Runx2 and Wnt signaling in FGF18 regulation of bone development (63). Mice with ablation of the Wnt co-receptor Lrp5 are osteopenic due to reduced osteoblastic activity. GFP transgenes were introduced in early (Col3.6-GFP) or late (Col2.3-GFP) osteoblasts in the Lrp5 mutant background, and mutant mice had greater numbers of early osteoblasts compared to wild-type mice (64). Col3.6-GFP expression also persisted in Lrp-deficient osteocytes, compared to wild-type, with no change in Col2.3-GFP expression between mutant and wild-type mice. Alkaline phosphatase expression was higher in calvarial cells from mutant mice. This study suggested that Lrp signaling regulates early stage osteoblastic genes. The regulation of Lrp5 in bone formation was also suggested in a study where Dkk3, a member of the Dickkopf family of secreted molecules, was overexpressed. Dkk3 treatment inhibited formation of mineralized nodules in wild-type cultures, but had no effect on Lrp-5 deficient osteoblasts, suggesting that Dkk3 may be a ligand for Lrp5 (65).

Integrin-dependent mechanisms in osteoblasts were also described at the meeting. Skeletal unloading induced by tail suspension in rats resulted in 50% decreased integrin (α5β1) levels in osteoblasts, compared to loaded bones (66). Reduced trabecular number and thickness and diminished osteoblast surface in the long bone metaphysis, with induced metaphyseal bone loss, was subsequently noted. Diminished Bcl2 levels and increased osteoblast apoptosis in this study suggested that the loss of integrin was responsible for lowered Bcl2 and thus led to osteoblast cell death. Osteoblast differentiation by BSP, an extracellular matrix integrin-binding molecule, occurred independent of RGD mediation, suggesting an integrin-independent action of BSP in human mesenchymal stem cells (67).

The analysis of genetically modified mice and cells has provided new insights into the biological functions and roles of many players in osteoblastic signaling, proliferation and bone formation. The emphasis in the osteoblast presentations suggests that the impact of various factors depends intimately on the osteoblast stage of proliferation and differentiation and on osteoblast interactions with other cellular players in the bone microenvironment.

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OSTEOCLASTS: BEFORE OR AFTER FUSION?

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Fusion of mononucleate precursor cells is an essential event in the differentiation of osteoclasts, since multinucleation is required for adequate resorption of bone. Nonetheless, in spite of the importance of such fusion in osteoclastogenesis, the molecular machinery responsible for the fusion of macrophages remains poorly understood. Indeed, cell-cell fusion is in general a poorly understood biological phenomenon that will be the focus of a new Gordon Research Conference next year (http://www.grc.uri.edu/programs/2007/cellcell.htm). The participants in the 28th annual meeting of the ASBMR did not unveil major advances in the characterization of the mechanism of macrophages fusion *per se*, but they did introduce a large number of novel molecules that play a key role in macrophages, both before fusion and after. Close to 200 abstracts and more than 20 oral presentations focused on the differentiation and activation of osteoclasts. This report summarizes some of the presentations selected on the basis of the novelty of the approach and the exceptionally high quality of the work presented.

DAP12 and FcRγ Work in Tandem

Questions have been raised about the role of DAP12 in osteoclastogenesis (68). DAP12 is an immunotyrosine-based activation motif (ITAM)-containing adapter that associates with receptors in myeloid and NK cells. DAP12-associated receptors can produce signals upon activation that lead to the production of cytokines. However, in some situations, DAP12 inhibits cytokine production that has been stimulated via toll-like receptors (TLRs) and FcRs. DAP12-deficient mice develop osteopetrosis, despite the presence of normal numbers of osteoclasts, indicating that bone resorption is impaired in these mice (69). Mice in which DAP12 lacks the ITAM domain (DAP12 KI mice) lose bone after ovariectomy (OVX), suggesting that the osteoclast defect is not cell-autonomous but is rather a consequence of an abnormal environment. Confirmation of this observation (70) in DAP12-deficient mice was extended in studies of mice deficient in FcRγ, another ITAM-containing adapter, the deletion of which also induces osteopetrosis. To examine the role of lymphocytes in the restoration of bone resorption when levels of estrogen are low, DAP12 KI mice were crossed with mice that lack the *RAG1* gene and, hence, both T and B lymphocytes. While RAG*1*(-/-) mice did not show any bone abnormalities, mice that lacked both DAP12 and RAG1 developed severe osteopetrosis.

However, these mice also lost bone after OVX. When lymphocytes were added to DAP12 KI spleen cells *in vitro*, they restored osteoclastogenesis to some extent via a process that was independent of RANKL. These observations suggest that lymphocytes promote the differentiation and/or activation of osteoclasts via alternative receptors, such as TREM2 or OSCAR, whose ligands remain to be identified.

A New Twist for TRAF6

Research focused on RANK intracellular signaling provided clues to the mechanism by which the receptor-adaptor TRAF6 activates the stress kinase pathways downstream of RANK (71). TRAF6, whose amino-terminal RING domain has ubiquitin (Ub) ligase activity, in combination with the Ub-conjugating enzyme complex Ubc13/Uev1A, catalyzed the synthesis of a unique polyUb chain with linkages through Lys-63 (K63). The auto-ubiquitination of TRAF6 is a proteasome-independent process that is central to activation of TAK1. Activated TAK1 phosphorylates IKK and MKK6, leading to activation of NFκB and of JNK plus p38, respectively. Bone marrow cells infected with a retrovirus that encoded TRAF6 underwent RANKL-independent osteoclastogenesis, while mutant TRAF6 that lacked the RING domain inhibited RANK-mediated downstream signaling and osteoclastogenesis. Thus, the TRAF6 Ub ligase appears to play a critical role in regulating the "non-traditional" role of Ub in RANK signaling.

Cbl and Cbl-b: Yin-Yang?

A new role was reported for Cbl-b, namely the ability to antagonize Cbl (72). Cbl proteins are adaptors and E3 ligases, and both Cbl and Cbl-b, which have strongly homologous sequences, are present in cells that belong to the myeloid lineage. While it had previously been reported that Cbl plays a role in osteoclast migration, and that Cbl-b inhibits the osteoclastic resorption of bone, it now appears that Cbl-b increases, while Cbl decreases, the expression of RANK in HEK cells transfected with vectors that encode RANK and Cbl or Cbl-b. It seems likely that the increased bone-resorbing activity of osteoclasts that lack Cbl-b might be due, in part, to an increase in the surface expression of RANK via Cbl, when the action of Cbl is not antagonized by Cbl-b, which promotes the degradation of RANK via the ubiquitin-proteasome pathway. Moreover, mice in which the expression of Cbl is "knocked-in" with a null mutation in the RING domain (73) have a lower bone-mineral density than heterozygous mice, indicating that ubiquitination of Cbl inhibits the differentiation of osteoclasts. It is, therefore, possible that the mechanisms that regulate the expression of Cbl and Cbl-b might be critical to RANK signaling and bone resorption.

Is CD47 a Fusion Partner?

Some participants at the ASBMR meeting examined whether CD47-deficient mice display a defect in their osteoclasts that is associated with a specific bone phenotype (74). CD47 (an integrin-associated protein) is a five-transmembrane domain protein that is known to interact with β3 integrins and is a ligand for SIRPα, which is also called macrophage fusion receptor (75;76). Thus, it has been suggested that CD47 is part of the fusion machinery for macrophages, which is involved in the differentiation of osteoclasts and giant cells (77). Bone marrow macrophages from mice that lack CD47 had a defect in osteoclast differentiation in response to M-CSF and RANKL. However, the frequency of apoptosis in mature CD47(-/-) osteoclasts was reduced. While NF-κB signaling was normal in CD47(-/-) macrophages, the extent of activation of p38 was reduced as compared to that in wild-type osteoclasts. The goal of the above mentioned study was to investigate whether CD47(-/-) mice, like β*3*(-/-) mice, are protected from bone metastasis. Since fusion of macrophages with tumor cells has been proposed as a possible mechanism that promotes metastasis (78), it is of great interest that there was decreased tumor-associated destruction of bone by osteolytic melanoma cells in mice that lack CD47, as was the case in mice that lack β3. Together, these observations implicate CD47-SIRPα

in the recognition and attachment of macrophages that leads to cell fusion.

Can Osteoclasts be Giants?

A summary was presented of recent work on signaling of Fos proteins in osteoclasts (see http://www.imp.ac.at/wagner/wag_pro1.html) Fra-2, a Fos-related protein, was analyzed using analogous loss- and gain-of-function approaches. Fra-2 was essential for the postnatal development of mice and for maintenance of proper bone mass; mutant newborns died with severe osteopenia. Conversely, transgenic mice that overexpressed Fra-2 had increased bone mass and developed pulmonary fibrosis. In a study of the underlying molecular mechanisms that lead to bone disease, it was found that Fra-2-deficient mice develop giant osteoclasts. This was the first example of a genetically modified mouse that develops osteoporosis as a result of excessively large osteoclasts, which might result from an excessive rate of fusion and/or an excessive number of fusogenic macrophages. In humans, only the osteoclasts in patients with Paget's disease are excessively large. Fra-2-deficient osteoclasts might, therefore, offer an ideal model for studies designed to identify the molecular players that mediate the fusion of macrophages, with subsequent formation of osteoclasts and giant cells.

Cathepsin K is Making Its Way Up

Cathepsin K (CK) is a cysteine protease, secreted by osteoclasts, that plays a central role in bone resorption. Pycnodysostosis, in humans, is a rare inherited osteochondrodysplasia caused by mutations in the gene for cathepsin-K, characterized by osteosclerosis, short stature, and acroosteolysis of the distal phalanges. Mice that lack CK survive and are fertile but have an osteopetrotic phenotype with excessive trabeculation of the bone-marrow space (79;80). As reported at the ASBMR meeting, a new function for CK has been identified (81). Mice that lack CK were generated with a 129/Sv background (in contrast to mice generated previously with a C57BL/6 background). Osteoclasts from calvariae, not from long bones, appeared to function almost normally in these novel mice, but they developed defects in apoptosis and senescence. While the numbers of osteoclasts from these mice were elevated, the levels of expression of p19, p53 and p21 were reduced. Thus, it was proposed that CK might participate in bone resorption and mediate apoptosis and senescence in osteoclasts. Other experimenters (82) confirmed these observations and found that the mutant calvarial osteoclasts expressed lower CK activity than did osteoclasts from the mutant long bones and that inhibitors of CK increased TRACP activity. In parallel, a number of inhibitors of CK have been developed and tested in mice (83) and monkeys (84;85), and CK has become a marker of bone turnover in women with postmenopausal osteoporosis (86) and in Paget's disease (87).

Conflict of Interest: The author reports that no conflict of interest exists.

THE OSTEOCYTE: THE "THIRD" CELL OF BONE GETS TO CENTER STAGE

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It was a big year for the osteocyte field at the 2006 ASBMR meeting. Three main developments have contributed to the osteocyte boom. First, *in vitro* mechanistic studies using established osteocytic cell lines (88) continue to provide insights into osteocyte biology and the mechanisms mediating osteocyte responses to hormonal and mechanical stimuli. Second, novel approaches are taking advantage of the development of promoters that allow transgene expression exclusively in osteocytes *in vivo* (25). Lastly, *SOST* and its product sclerostin (89-91), expressed exclusively in osteocytes in bone, are being used as molecular tools to study the mechanisms of osteocyte communication with other bone cells and to target osteocytes with therapeutic purposes.

"Green" Osteocytes

The use of bones from dentin matrix protein 1 (Dmp1)-GFP transgenic mice, in which osteocytes are labeled with green fluorescent protein, allowed live imaging of osteocytes for the first time (92). These studies showed that osteocytes and the connections among them are highly dynamic, as evidenced by constant contraction and expansion of the osteocyte bodies within the lacunae and by the extension and retraction of the dendrites. This is consistent with the gene expression signature of authentic osteocytes (GFP-positive cells isolated by sorting from the same transgenic mice (93)) and of MLO-Y4 osteocytic cells (94), which revealed high expression of genes involved in muscular contraction, axon guidance, and cytoskeleton rearrangement.

Osteocyte Apoptosis: A Beacon for Bone Remodeling and More

Through their network, osteocytes might continually compare present mechanical strains to usual levels of strain (the set point of the mechanostat) and trigger signals to osteoclasts or osteoblasts resulting in bone loss or gain, as needed (95;96). Whereas physiological levels of strain maintain bone mass, levels of strain that are too high or too low induce bone resorption (97). One mechanism by which osteocytes may trigger bone resorption is by undergoing premature apoptosis. Indeed, osteocyte apoptosis is elevated in bones exposed to high levels of mechanical stimulation (98) or in unloaded bones (99). Notably, in both cases, osteocyte apoptosis precedes temporally, and is spatially associated with, increased resorption and subsequent bone loss (98;100-102). Apoptosis of osteocytes may therefore initiate a cascade of events to replace bone in the same location, namely targeted remodeling. Evidence was presented that supports a cause and effect relationship between osteocyte death and osteoclast resorption (103). Administration of diphtheria toxin (DT) to transgenic mice expressing the DT receptor exclusively in osteocytes (by virtue of the DMP1 promoter) induced a rapid increase in the number of empty osteocyte lacunae, which was followed by elevated bone resorption and bone loss. Although the actual number of osteocytes, or indeed whether there was an increase in osteocyte apoptosis, was not evaluated, this evidence adds strength to the hypothesis that osteocyte apoptosis is a signal for osteoclast recruitment.

Osteocyte apoptosis may also contribute to bone strength by mechanisms independent of the amount of mineral. For example, decreased bone strength induced by glucocorticoid (GC) administration was reversed by blocking the proapoptotic effects of GC in cells of the osteoblastic lineage, even when bone mass was lost (104). Bone strength also decreased faster than any detectable change in bone mineral density in aging bones (105;106). Old mice also exhibited increased GC production as well as elevated osteocyte apoptosis and decreased osteocyte lacunar-canalicular circulation (cardinal features of GC-induced bone disease) (105). This evidence suggests that elevated endogenous GC action may be one of the pathogenic causes of involutional osteoporosis.

Mechanotransduction: The Primary Cilium and Connexin 43

Integrins, focal adhesion kinase, connexin 43, P2X7 receptors, caveolin, and unligated estrogen receptors, among other molecules, have been shown to be required for eliciting particular responses of osteocytes to mechanical stimulation. This year a new player has been proposed: the primary cilium. Present in most vertebrate cells, this organelle is a cell-surface projection with well-established roles in smell, sight and mechanosensation (107). Defects in mechanoreception mediated by cilia are linked to polycystic kidney disease. The transmembrane proteins polycystin (PC) 1 and PC2 translate fluid flow-induced deflection of the cilium into cell cycle regulation; their absence, or the absence of cilia, results in unrestrained proliferation of renal epithelial cells (mediated by Wnt/β-catenin and Stat6) and formation of cysts. Evidence was presented indicating that primary cilia have a crucial function in bone cells as well. Thus, mice lacking primary cilia in chondrocytes exhibit postnatal dwarfism

due to defects in the development of the growth plate (22). Primary cilia and the PC1/PC2 mechanosensing complex are present in osteoblasts and osteocytes, and mice expressing an inactivating mutation of the gene encoding for PC1 exhibit delayed bone formation and osteopenia (108;109). Moreover, cilia depletion – by pharmacological means or by knocking-down the intraflagellar transport protein polaris required for cilia formation – abolished ERK activation and osteopontin expression, but not the increase in intracellular calcium, induced by fluid flow in osteoblastic cells (110).

Mechanical stimulation of osteocytic cells leads to opening of connexin 43 hemichannels, which in turn allows prostaglandin release (111). Evidence was presented suggesting that integrin α5 co-localizes with connexin 43 and that a blocking integrin α5 antibody decreases hemichannel opening (112). β-arrestin – a molecule involved in internalization of G protein-coupled receptors, such as PTHR, and their cross-talk with MAP kinases – was also shown to interact with connexin 43, and this interaction is required for anchoring bisphosphonate-activated ERKs outside the nucleus and for osteocyte survival (113). β-arrestin and connexin 43 both appear to be required for a normal anabolic response of bone to intermittent PTH administration (114;115). Therefore, the interaction of connexin 43 – the main connexin protein expressed in osteocytes – with signaling and structural proteins might be responsible for modulating signaling pathways activated by mechanical, hormonal and pharmaco-therapeutic stimuli in osteocytes.

Sclerostin Regulation by PTHR Signaling and Loading

A potential mediator of the communication between osteocytes and the cells of the bone surface is *SOST* and its product, the protein sclerostin. Sclerostin produced by osteocytes acts as a paracrine factor to inhibit osteoblast production and bone formation by antagonizing the actions of Wnts on cells of the osteoblastic lineage (91;116-118).

SOST expression is potently inhibited by parathyroid hormone (PTH) (119;120), raising the possibility that PTH actions on the skeleton are due, at least in part, to a direct effect of the hormone on osteocytes, leading to decreased *SOST*, increased Wnt signaling, and increased bone mass. This hypothesis was supported by the phenotype of recently developed transgenic mice in which a constitutively active PTHR was expressed exclusively in osteocytes (121). The transgenic mice exhibited reduced *SOST* and sclerostin expression and increased expression of Wnt and BMP target genes, as well as elevated expression of osteoblast marker genes, compared with wild type littermates. These biochemical changes were associated with a remarkable increase in bone mass and strength in the axial and appendicular skeleton, abundant tetracycline labels, the presence of cartilage remnants, and abnormal modeling, suggestive of a combination of increased bone formation and decreased bone resorption. These findings are consistent with the recognized pro-differentiating actions of Wnts on cells of the osteoblastic lineage, as well as with recent evidence indicating that activation of the Wnt/β-catenin signaling pathway inhibits osteoclast differentiation (61;122;123).

Remarkably, bone loading – a stimulus known to require the Wnt co-receptor LRP5 to induce bone formation (124) – also reduced sclerostin expression (125), suggesting that sclerostin is the molecular signal by which mechanical forces sensed by the osteocyte network are transduced into an anabolic response.

Osteocytes as Therapeutic Targets

The potential of osteocytes as targets for the treatment of osteoporosis was demonstrated by animal experimentation. Thus, administration of a sclerostin neutralizing antibody increased bone mass, bone formation rate, and strength in aged ovariectomized rats (126) as well as in intact female cynomolgus monkeys (127). These findings suggest that inhibition of sclerostin may be useful in humans for building bone,

even in clinical conditions where significant bone loss has already occurred.

Conclusions

The osteocyte reached center stage at the ASMBR meeting in 2006, and, most likely, it is here to stay. The high relevance and increased number of abstracts call for a separate section on osteocytes in the meeting's oral program, instead of relegating the osteocyte-related abstracts to "Osteoblasts-other." Proof that osteocytes orchestrate the function of osteoblasts and osteoclasts, that they indeed sense and transmit mechanical and hormonal signals, and that they are ideal targets for therapies aiming to improve bone mass and strength are slowly but surely accumulating. It therefore may not be wise to keep calling the osteocyte the "third" cell of bone.

Conflict of Interest: The author reports that no conflict of interest exists.

FGF23: THE MATRIX RELOADED

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It seems that each year the ASBMR meeting brings us a new insight into the physiology of the phosphatonin FGF23. Last year's meeting featured the insight that FGF23 and Klotho act as an obligatory pair to induce renal phosphate reabsorption and to inhibit renal synthesis of $1,25(OH)_2D$. This year, major advances were presented on the factors controlling the secretion of FGF23.

Three abstracts at this year's meeting disclosed that inactivation of the gene for dentin matrix protein 1 (*Dmp1*) produces a hypophosphatemic disorder. Removal of *Dmp1* was previously reported to produce a mineralization disorder characterized by rickets, osteomalacia, and hypophosphatemia (128;129). *Dmp1* null mice have markedly increased FGF23 gene expression in osteocytes and high serum levels of FGF23 (30); the results have recently been published (31). Their entire phenotype can be rescued by expression of *Dmp1* in bone, using the col 3.4 promoter. Two groups (27;28) reported the presence of inactivating mutations in *DMP1* in autosomal recessive hypophosphatemic rickets, a rare disorder with a phenotype similar to autosomal dominant hypophosphatemic rickets; both groups have now published these findings (29;31). Serum FGF23 levels are increased in most patients with the syndrome, as in the *Dmp1* knockout mouse, placing DMP1 upstream of FGF23.

It seems likely that DMP1 also plays a role in the most common form of hypophosphatemic rickets, X-linked hypophosphatemic rickets (XLH). FGF23 gene transcription in the osteocyte is markedly increased in the *Hyp* mouse, an animal model of XLH (130). The mutation responsible for XLH inactivates the *PHEX* gene, which encodes a zinc metalloprotease that is displayed on the plasma membrane of osteoblasts and osteocytes. Hence, the key event in XLH involves loss of a protease cleavage event that normally suppresses the transcription of the *FGF23* gene in the osteocyte. Could DMP1 be the substrate for Phex cleavage? This looks like a good bet, though the pathway is unlikely to be so simple. Lorenz-Depiereux *et al*. report that soluble Phex does not cleave recombinant DMP1 (29), a result consistent with the general preference of Phex-like proteases for smaller substrates. Perhaps another protease cleaves DMP1 to a form that is amenable to Phex cleavage.

DMP1 belongs to the same SIBLING family as the phosphatonin MEPE, suggesting a complex relationship of these proteins to mineralization. In an elegant study, the phenotype of mice with a global *Phex* knockout was compared to mice with targeted inactivation of osteoblast *Phex* (131). As previously reported, both knockout models develop the phenotype of the *Hyp* mouse, including markedly increased FGF23 levels. Expression of both MEPE and sFRP-4 is increased in bones of the global knockout; their serum levels are likewise increased. Neither expression nor serum levels of these phosphatonins is increased in the osteoblast-specific *Phex* knockout mouse, however. Thus, increased

levels of MEPE and sFRP-4 are not required for the *Hyp* phenotype.

At last year's ASBMR meeting, FGF23 action was shown to require an association with the Klotho protein; consistent with this, deletion of the genes encoding FGF23 and Klotho produces an identical phenotype. To determine which FGF receptor (FGFR) is involved in the interaction of this complex, several soluble FGFRs were tested for inhibition of FGF23 signaling and sFGFR1(IIIc)-Fc was found to be active. Coexpression of this receptor isoform with Klotho enabled FGF23 to activate Egr-1 in L6 myoblasts (132). Klotho levels in a distal convoluted tubule cell line are regulated by calcium and phosphate in a fashion that enhances FGF23 signaling (133).

Klotho is expressed as a transmembrane protein mainly in the distal nephron but soluble form also circulates. Can soluble Klotho interact with circulating FGF23 to produce diverse tissue effects? Addition of soluble Klotho (1μg/ml) to MC3T3-E1 cells enabled FGF23 to signal in the cells; addition of both FGF23 and Klotho inhibited their differentiation (134). The authors are not persuaded, however, that soluble Klotho would be active at the levels present *in vivo*.

Although the tissue distribution of Klotho is restricted, removal of the *klotho* gene affects diverse tissues, as does removal of *FGF23*. At last year's ASBMR meeting, ablation of either the vitamin D receptor (VDR) or the vitamin D 1α-hydroxylase were shown to rescue many features of the *FGF23*(-/-) phenotype, suggesting that high $1,25(OH)_2D$ levels are responsible for most of the effects of FGF23 deficiency outside the kidney. At this year's meeting, further studies showed that metabolic effects of FGF23 deficiency such as hypoglycemia and increased insulin sensitivity, as well as hypercalcemia, hyperphosphatemia, growth retardation, skin atrophy, ectopic calcification and renal dysfunction, could be rescued by removing the *Vdr* gene and are thus consequences of high $1,25(OH)_2D$ levels (135). The authors conclude that the physiological role of FGF23 is to provide negative feedback from bone to kidney to control renal synthesis of $1,25(OH)_2D$.

FGF23 is a young hormone, and at this point there are still three competing hypotheses (which are not mutually exclusive) about its physiological role. It was first thought that FGF23 would be primarily a serum phosphate-regulating hormone that is secreted in response to high phosphate intake or release of endogenous phosphate and designed to dump phosphate into the urine, block phosphate absorption (136) and reduce vitamin D activation, thereby preventing a high calcium-phosphate product. Serum phosphate levels are not tightly regulated, however, and changes in serum FGF23 levels in response to altered phosphate intake are notably small and slow (137).

Second, FGF23 could be primarily a vitamin D-regulating hormone, designed to assure that vitamin D levels are adequate for bone and to prevent them from becoming excessive (135). The view that FGF23 is so closely linked to vitamin D is supported by findings that all the effects of FGF23 deficiency seem to require vitamin D (135). The VDR/FGF23 double knockout mouse is not a perfect model to address this issue, however: for example, the normal serum phosphate level in double knockout mice is probably a complex consequence of low FGF23 and high PTH levels. It seems likely that FGF23 is both a phosphate and vitamin D-regulating hormone.

A third hypothesis that is suggested by this year's new results is that FGF23 connects tissue needs for phosphate with the renal transport system that sets the serum level of phosphate and thereby determines the availability of phosphate to tissues. In this view, the osteocyte (newly recognized as the spokesperson of the extracellular matrix of bone) talks to the kidney, relaying news of changes in the mineralization state of the extracellular matrix of bone in order to regulate phosphate supply by setting the levels of phosphate transport in kidney (and potentially in the gut (136)). The model is made attractive by the identification of the osteocyte as the site of excess FGF23 secretion in XLH and a bone matrix protein,

DMP1, as crucial to regulation of FGF23 secretion. In such a model, FGF23 might also regulate phosphate transport in bone cells and chondrocytes. Phosphate uptake regulates both matrix mineralization by hypertrophic chondrocytes and their apoptotic demise (6;138;139), but we know little of what controls phosphate uptake in chondrocytes. For FGF23 to affect phosphate uptake in cartilage or bone cells, it would be necessary to postulate that circulating Klotho levels are sufficient to support local FGF23 effects on phosphate transport in bone and cartilage. It will be important to determine whether FGF23 can act directly in cartilage or bone.

Conflict of Interest: The author reports that no conflict of interest exists.

OSTEOCYTES COME TO THE FOREFRONT

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It has long been thought that osteocytes are important for controlling plasma mineral homeostasis under the regulation of calcium-regulating hormones like PTH. Thirty years ago, Parfitt argued that osteocytes can cause rapid mobilization of calcium and phosphate from a hypomineralized region of bone directly adjacent to osteocyte lacunae (140). Recent findings are helping to clarify the role of osteocytes in mineral homeostasis. Several studies reported at the 2006 ASBMR meeting point to the important roles of dentin matrix protein 1 (DMP1), a matrix protein produced almost exclusively by osteocytes. DMP1 promotes mineralization of dentin and bone. Mice with a null mutation of the *Dmp1* gene have enlarged osteocyte lacunae and a disorganized lacuno-canalicular network due to loss of the lamina limitans, a structure that creates a boundary between the mineralized matrix and canalicular space. New findings reported at the meeting demonstrated that loss of function mutations in DMP1 are responsible for autosomal recessive hypophosphatemic rickets (ARHR) (27;28). The clinical phenotype of ARHR patients is very similar to autosomal dominant hypophosphatemic rickets (ADHR), which results from over-production of fibroblast growth factor 23 (FGF23). In addition, *Dmp1* null mice have phenotypic similarities to *Hyp* mice, which are a model for X-linked hypophosphatemic rickets due to an inactivating mutation in *Phex* (30). Like *Dmp1*, *Phex* expression is much higher in osteocytes compared to other bone cells (93). These two mouse models have in common highly elevated production of FGF23 from their osteocytes (30;141). FGF23 is known to act as an endocrine and cause phosphate dumping by the kidneys that can result in hypophosphatemic rickets when serum levels are too high (142). Consequently the apparent common feature of ADHR, ARHR, and X-linked hypophosphatemic rickets is elevated FGF23 production by osteocytes, thus illuminating a role for osteocytes not anticipated by Parfitt – endocrine production.

Osteocytes are also thought to be the mechanosensors in bone tissue. Studies conducted almost 20 years ago demonstrated changes in nucleoside levels by osteocytes in loaded bones (143). Since then, numerous experiments have demonstrated specific osteocyte responses to mechanical loading. At the ASBMR meeting, a protein called sclerostin, produced almost exclusively by osteocytes, was shown to be mechanically regulated. Sclerostin is a negative regulator of bone formation that acts as an inhibitor of Wnt co-receptors LRP5 and LRP6. Loading rapidly decreased sclerostin levels in osteocyte cell bodies and canaliculi (125). These changes in sclerostin are greatest near bone surfaces and bone formation is increased the most after loading, suggesting a link between local sclerostin suppression and increased osteoblastic activity. These findings suggest that sclerostin is an osteocyte-specific mediator of the osteogenesis following mechanical loading.

The mechanism(s) by which osteocytes detect mechanical deformation or strain is still somewhat mysterious. One possible mechanosensor is an organelle called a primary cilium. Kidney epithelial cells use their cilia as sensors to monitor fluid flow

within renal tubules. These cilia contain polycystin-1 (PC1) and polycystin-2 (PC2), transmembrane molecules that serve as mechanosensors. At the meeting, the presence of PC1 and PC2 was demonstrated in cilia on osteocytes and osteoblasts (109). Mice deficient in the mouse homolog of PC1 demonstrated delayed bone formation and impaired osteoblast function, suggesting a role of PC1 in bone formation. Another group tested the role of cilia in bone cells by ablating cilia with siRNA against the ciliary protein polaris (110). Bone cells treated with siRNA were less responsive to fluid shear stress, suggesting a role for primary cilia in bone cell mechanotransduction.

With emerging evidence for so many roles of osteocytes, one might ask what would happen to bone if the osteocytes were removed. A mouse model was reported that allows targeted ablation of osteocytes *in vivo* (103). Transgenic mice expressing the diphtheria toxin (DT) receptor specifically in osteocytes were generated. Although DT is highly cytotoxic in many animals, mice and rats are insensitive to it. Consequently, transgenic mice can be injected with DT and suffer no ill effects other than high toxicity in the specific cells expressing the DT receptor. The *Dmp1* gene was used to target the DT receptor to osteocytes in transgenic mice. When these mice were injected with DT, empty osteocyte lacunae were observed within 24 hours. After osteocyte ablation with DT, aggressive bone resorption was observed, followed by bone formation and replenishing of osteocyte populations within several weeks. These findings have interesting implications for osteocyte biology. For instance, previous studies have observed osteocyte apoptosis preceding bone remodeling and repair of microdamage (98). It was previously proposed that, while undergoing apoptosis, osteocytes send recruiting signals to osteoclasts to initiate repair. However, in the DT model, osteocytes die without undergoing apoptosis and osteoclasts are recruited nevertheless. This finding suggests that osteocytes provide a constitutive antiresorption signal, which is removed when the osteocytes die. We can look forward to gaining more insights about osteocytes from this interesting mouse model.

Conflict of Interest: The author reports that no conflict of interest exists.

IMAGING AND FINITE ELEMENT ANALYSIS OF BONE

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Finite element analysis (FEA) derived from QCT scans was again a hot topic this year, as in the last three years. FEA mechanically integrates all the geometrical and material property data within CT scans to provide measures and predictions of bone mechanical strength. QCT BMD values of each bone voxel are converted into elastic modulus values using pre-determined correlations between the elastic modulus and QCT-derived BMD.

Finite Element Analysis with PTH Treatment.

A finite element analysis study of *in vivo* clinical QCT images from 100 randomly selected subjects from the PTH and alendronate (PaTH) trial was presented (144). The PaTH trial randomized postmenopausal osteoporotic women to PTH(1-84) for 1 year followed by either a placebo (PLB) or Alendronate (ALN) for year 2; the combination of PTH and ALN (PTH+ALN) for 1 year followed by ALN; or ALN continuously for 2 years (n=25/group). Using non-linear FEA, the predicted percent change in the femoral strength for a sideways fall showed a significant increase from baseline at two years (+6%) in the PTH-ALN arm (see Figure 1). The only significant difference among treatment arms was between PTH-ALN vs. PTH-placebo, which indicates the biomechanical advantage of PTH followed by ALN. The correlations between FE strength with QCT/DXA BMD were modest, with large variations in change of strength within each treatment group. Data on vertebral body were reported at last year's ASBMR meeting (145).

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Re-analysis of QCT images from a study presented at last year's ASBMR meeting showed that treatment with PTH(1-84) for 18 months increased femoral neck bone strength estimates, as reflected by torsional and compressive strength indices (146). In another study, high resolution CT scans of T12 were performed at baseline and after 6, 12, and 24 months in 17 subjects with PTH (1-34) treatment. For FEA, the entire segmented vertebrae were converted into finite elements of 700 µm isotropic resolution. Two-year treatment was associated with an 18% increase in FE-derived stiffness, with an increased sensitivity compared to DXA (147).

Finite Element Analysis and Fractures

With fast technological development in QCT, scanning resolution and speed are constantly improving. Extensive studies investigated QCT-based FEA and its prediction of fracture.

In one investigation, mean fracture load was determined, after QCT examination, on 40 excised femora tested in stance (lateral) configuration. The following CT protocol represents a compromise between radiation exposure and image quality: 120 kV, 170 mAs, pitch 1, slice thickness 1 mm, range from above the femoral head to 1 cm below the lesser trochanter. QCT BMD values-based FE-computed fracture loads were significantly correlated with experimental results (slope 1.006, r^2=0.87) (148).

Another study imaged 7 human calcanei at 34 µm using µCT, and voxel meshes were generated using the pixel-to-voxel

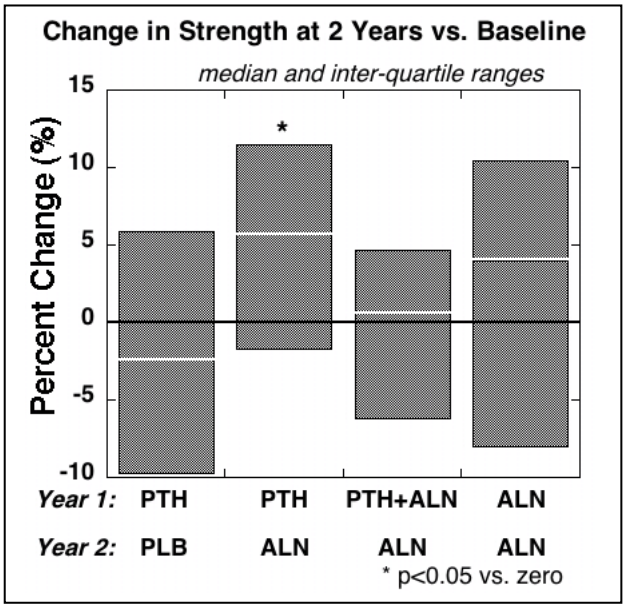

Figure 1. The predicted percent change in femur strength for a sideways fall, for each of the four treatment groups from the PaTH study. Figure reproduced from *J Bone Miner Res*. 2006 Sep;21(Suppl 1):S43 with permission of the American Society for Bone and Mineral Research.

technique. Smoothing was applied to virtually extracted cubic volumes. Improved prediction of local trabecular strain enhanced non-invasive assessment of micro bone strength using a surface smoothing technique in FEA (149). Previous linear FEA of simulated trabecular networks has shown that bone loss via trabecular perforation is more detrimental to bone strength than loss via trabecular thinning. Consequently, FEA was performed on human vertebral trabecular bone, from the cadaver of an 84 year old female, scanned at 30 µm voxel size (150). Perforation effects were

simulated by removal of the transverse elements. Perforation of the trabecular cross-struts in the FEA model resulted in a large decrease in buckling load. Thus, preventing perforation of trabecular cross struts is critical to maintaining bone strength.

High resolution peripheral Xtreme pQCT has recently been developed for measurements of the distal radii and tibiae of patients, with nominal isotropic resolution of 82 µm. This device has not yet been approved by the FDA. A study in Canada using 10 cadaver forearms and 15 human subjects, with voxels converted to finite elements, found that trabecular bone carried on average 73% of the load at the distal end of the sample (27% carried by the cortical bone), and the cortical bone carried on average 74% of the load at the proximal end of the sample (26% carried by the trabecular bone) (151). There was a range of 48-82% correlation between morphological indices and force to induce 1% strain. The highest correlation was found between load carried by the cortical bone and mean cortical thickness (90-92%). The variation in relative load carried by trabecular and cortical bone regions from the distal to proximal ends was likely due to the changing cross-sectional geometry of the cortical and trabecular compartments.

Twelve cadaveric vertebral bodies from 4 male human lumbar spines (L1-L5, age 47 to 83) with known DXA BMD were scanned with an Xtreme CT system at 82 µm isotropic resolution, and then tested mechanically in axial compression (152). The QCT voxels were degraded at a 656-µm resolution and directly converted to finite elements with linear elastic transverse isotropic material properties. Numerical FE predictions of vertebral stiffness correlated well (r^2=0.68) with the experimental stiffness. Voxel models built from QCT images were significantly better at predicting *in vitro* compressive vertebral strength (r^2=0.82) than DXA BMD (r^2=0.62).

Forty women with a clinical vertebral fracture due to moderate trauma (median age 78 years) were compared to 40 control women with no osteoporotic fracture (median age 70 years) (153). Lumbar spine QCT volumetric density, cortical thickness, FE compressive strength, FE AP bending stiffness, and 90° forward bend load were lower in fractured than in control subjects, while ultradistal radius microstructure by high resolution Xtreme pQCT and DXA BMD were about the same. These diverse measures of bone density, structure, and strength provide a basis for a better understanding of vertebral fracture pathogenesis. In another study, seventeen subjects with Colles' fracture (median age, 78 years) and 17 control women with no osteoporotic fracture (76 years) were examined using high-resolution Xtreme pQCT (154). Bone strength was estimated from µFE models. The subjects with fractures generally had lower bone density, structural parameters, and bone strength measurements. µFE bone strength was predicted by combined cortical and trabecular bone volume (r^2=0.80), with correlations that increased to 0.94 when cortical thickness was added. By multivariable logistic regression, the best predictors were vBMD, cortical thickness, BV/TV, and axial rigidity.

A total of 162 human cadaveric forearms were imaged with a pQCT scanner with an isotropic nominal resolution of 93 µm (155). Mechanical tests were performed. The best prediction of failure load using a simple microarchitectural bone parameter was obtained from a model with bone volume from only the most distal region (r =0.75). Other structural parameters also showed good correlation (r=0.51-0.73). Multiple linear regression analysis showed that only slightly better correlation could be obtained by including trabecular number (r=0.78). Ten specimens were selected for FE analysis, with no significant correlation between bone volume and experimental failure load. Using the FE-calculated failure load, the prediction could be increased (r=0.87).

Controversies in Hip Structural Analysis

DXA Hip Structural Analysis (HSA) calculates geometrical and mechanical parameters from DXA scan data and is widely used in osteoporosis trials and research. Although many papers use HSAs, the calculation remains controversial because of limited DXA resolution and limited signal/noise ratio, and because of

debatable, simple two-dimensional projectional assumptions for the complicated three-dimensional anatomic structure of the hip.

Results from two implementations of HSA were compared to QCT volumetric measurements as a gold standard in the same population of 121 women, with 3 mm slice thickness, 0.94 mm pixel size for QCT, and DXA scans of the left hip with standard hip mode (156). DXA HSA was performed using two methods: John Hopkins HSA and GE/Lunar HSA. Pairwise linear regression of HSA variables was conducted by method-to-site-matched QCT variables for BMD, cross-sectional area, and cross-sectional moment of inertia of the femur neck. The Hopkins HSA correlated weakly with QCT, whereas GE/Lunar HSA correlated modestly, and the 2 DXA HSA methods correlated only weakly with each other. As the different implementations of DXA HSA do not produce equivalent results and correlate differently to QCT, the Hopkins HSA for the femoral neck, as well as the Prodigy device HSA, should be used with caution.

Hip Fracture

QCT and DXA scans of the hip were analyzed in 3357 men aged over 65 years (mean age 74 and 87% Caucasian) for the MrOS study (157). Men were followed for a mean of 4 years, with 36 hip fractures during observation. It was found that low femoral neck DXA BMD, low percent cortical volume, and small cross-sectional area from the femoral neck QCT scan all contributed to higher hip fracture risk in older men.

In another investigation, unembalmed and previously frozen human cadaveric femora (N=34; ages 36-92 years) were scanned with QCT, and then loaded to failure in an apparatus designed to simulate a sideways fall on the greater trochanter (158). Cortical area was most strongly associated with failure load in the femoral neck fracture, while trabecular BMD had the strongest association in the trochanteric fracture.

Falling is an important risk factor for hip fracture. Thigh muscle size and attenuation measures from CT are correlated with frailty-related risk factors for falling, such as reduced muscle strength and slow gait. DXA measurements of total hip BMD were made in 2941 subjects, aged 70-79 years, enrolled in the Health ABC study (159). Over a 7-year follow-up, 55 hip fractures occurred. CT cross-sectional area and mean attenuation of the thigh muscle bundle of the middle thigh contributed to increased hip fracture risk independently of BMD.

Micro CT

µCT continues to have extensive applications. Strontium ranelate has been shown to stimulate bone formation and decrease bone resorption in preclinical studies, and to reduce vertebral and hip fracture risks in postmenopausal women with osteoporosis. A total of 41 biopsies of the iliac crests from 2 clinical trials were examined with µCT at an isotropic resolution of 20 µm (160). Compared with placebo, strontium ranelate treatment significantly improved trabecular structural model index, shifting trabeculae from rod-like structures to a plate-like pattern. Treatment with strontium ranelate also decreased trabecular separation and increased trabecular bone number, based on a plate model assumption, and increased cortical thickness. In a retrospective study, µCT analysis was performed on biopsies from 23 osteoporotic patients (mean age 53 years; 18 male, 5 female) before and after 38 months of bisphosphonate treatment (161). The analysis showed a mean increase in BV/TV from baseline by 1.7% per year in the treatment group. After 38 months, BV/TV had increased by 5.2%. This was accompanied by an improvement in other structural parameters. µCT and pQCT were also used to study various animal models, including the osteoporotic monkey (162), as well as mice lacking the novel transmembrane protein Osteopotentia and thus exhibiting catastrophic defects in bone modeling (163).

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TREATMENT OF OSTEOPOROSIS

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Anti-Resorptive Agents

Zoledronic Acid

The newest clinical data presented at the ASBMR annual meeting were from the HORIZON Pivotal Fracture Trial of zoledronic acid (ZA) (164). For this trial, 5 mg of ZA was administered intravenously on an annual basis for 3 years, and compared to placebo, in 7736 postmenopausal women with osteoporosis at the femoral neck or osteopenia with a prevalent fracture. There was an 84% retention of subjects. Risk reductions were 70% for vertebral fractures, 40% for hip fractures, and 25% for non-vertebral fractures. A flu-like illness within the first 3 days following the infusion was more common with treatment. Increased incidence of cardiac arrhythmias was reported, but whether this adverse event was causally related to treatment is not established. Another study (165) reported that ZA prevented ovariectomized (OVX)-induced microarchitectural decay in 35-week-old retired breeder rats, while late treatment partly restored structural changes, consistent with slowed remodeling, allowing partial refilling of remodeling cavities created by the OVX-induced high remodeling.

Over-Suppression of Remodeling – Myth or Reality?

Although concerns have been raised about over-suppression of remodeling and potential adverse events with bisphosphonates, evidence for an increase in fracture rates was not forthcoming. No increase in the incidence of osteonecrosis of the jaw with ZA was found in the HORIZON study (164). Another study found that women receiving alendronate who were switched to one 15-minute IV infusion of ZA at 5 mg maintained BMD (166). Biomarkers decreased, and then increased to remain within the premenopausal range, suggesting that remodeling suppression was reversible. In yet another study, histomorphometry was reported in 10 subjects treated with ZA, and 13 subjects treated with alendronate (167). Almost identical effects were described, with suppression of remodeling to levels no lower than those in premenopausal women.

It was also reported, from histomorphometric assessment in 89 biopsies, that IV ibandronate, after 2 years, lowered activation frequency and mineralizing surface to premenopausal ranges, with no evidence of adverse effects on mineralization (168). In a study in men, risedronate, given at 35 mg once weekly for 2 years, reduced activation frequency (biopsies in 6 placebo and 11 risedronate-treated), but continued remodel-ing with normal appositional rates and confirmed normal osteoblast function (169). Results were also reported from iliac crest biopsies in 45 postmenopausal women with osteoporosis who received pamidronate, alendronate, or risedronate for at least 4 years (170). Microcrack frequency was low: 0.15 microcracks/mm^2 per BV, and no higher than controls (0.07 microcracks/mm^2 per BV). There were no microcracks in 54% of treated women, and no association between microcrack frequency and the duration of therapy or fracture prevalence.

Anabolic Agents

Parathyroid Hormone(1-34)

OVX rats treated with daily subcutaneous PTH(1-34) (20 μg/kg/d for 16 weeks) showed increases in BV/TV, Conn.D, Tb.N, trabecular thickness (Tb.Th), Ct.Th, maximum load, stiffness, and energy (171). The anabolic activity of PTH was examined in *Lrp5* knockout mice (*Lrp5* KO) (172). hPTH (20 μg/kg), a glycogen synthase kinase β inhibitor (GSKi), or vehicle was administered on calvaria of 5-week-old male *Lrp5* KO mice for 7 days. Mineral apposition rate (MAR) increased in GSKi-treated WT and *Lrp5* KO mice; PTH increased MAR of WT but not *Lrp5* KO mice. Daily hPTH produced osteogenic effects in mature male Swiss Webster and female C57BL6 and LEX129 mice. A similar study in 12-week-old male mice revealed that 30 days of hPTH treatment increased femoral cortical density in both *Lrp5* KO and WT mice, and

trabecular density in WT but not *Lrp5* KO mice. This suggests that the osteogenic effects of hPTH on calvaria and trabecular bone, but not on cortical bone, are mediated by the Lrp/Wnt pathway.

Most data suggest that anti-resorptive therapy impairs the response to PTH, at least in terms of BMD and remodeling markers. A study found that PTH augmented trabecular bone mass in rats, but rats receiving risedronate for 18 weeks had an attenuated onset of effects of PTH (173). Ten weeks washout restored PTH stimulation of osteoblast activity. In another study, weanling female rats were treated with hPTH(1-34) for 3 weeks with or without calcitonin (174). PTH increased femoral trabecular BMD by 32%. This was prevented or attenuated when calcitonin was administered at the same time as PTH. Calcitonin given 1 hour before, or 5 hours after, PTH did not block the anabolic effect, indicating that active osteoclasts are required within one hour of PTH for the full anabolic effect of PTH to be achieved.

Strontium Ranelate – Is It An Anabolic Agent?

There is compelling evidence supporting the anti-fracture efficacy of strontium ranelate (SR). Whether the drug reduces bone resorption and increases bone formation remains uncertain. It was reported that SR increased osteoblast replication, increased OPG mRNA expression, and decreased RANKL expression (175). The authors suggest that SR promotes primary human osteoblast replication, increases OPG expression, and decreases RANKL expression, thereby decreasing the ability to promote osteoclastogenesis. However, in OVX rats treated with a low or normal calcium diet, periosteal bone formation indices were lower in animals treated with SR (176). There were no differences among groups for endocortical bone formation. In another study, SR given to OVX rats 4-weeks post-OVX produced a higher vertebral BMD but reduced mineralizing surface, with no effect on mineral apposition rate. Bone formation rate (BFR/BS) was reduced (177).

Steps in Remodeling as Therapeutic Windows

Osteocytes, Sclerostin Inhibition, and Bone Formation

Osteocytes define the size and location of a region of damage, such as a microcrack. This is achieved at a microcrack when the canaliculi are severed, causing death by apoptosis of osteocytes surrounding the damaged region and thus defining the demographics of the damage. Focal remodeling is targeted to remove this damage and restore bone strength. The osteocyte also detects stress on bone locally and signals where new bone formation is needed, so that bone can adapt its material and structural properties to the load.

Osteocytes synthesize sclerostin, a protein that inhibits bone formation by binding to LRP5/6, antagonizing Wnt signaling, and promoting osteoblast and osteocyte apoptosis. When sclerostin is inhibited, bone formation results. In rats given monoclonal antibodies to sclerostin, femur cortical area and vertebral trabecular bone volume increased (126). Whether these changes were the result of inhibition of bone resorption with completion of bone remodeling by bone formation, or whether this was an anabolic effect, remains uncertain. Similar benefits were reported in studies in monkeys (127).

RANKL and Bone Resorption

Receptor activator of NFκB ligand (RANKL) from osteoblast precursors mediates osteoclast formation by binding to its receptor, RANK, on hematopoietic lineage cells. This is a process inhibited by the decoy receptor, OPG, which binds RANKL to prevent osteoclast formation. OVX in rats at 3 months of age increased serum RANKL (178). In OVX rats receiving 6 weeks human OPG-Fc, OPG suppressed TRAP-5b and increased BMD at the distal femur and LV5. Adult monkeys treated with the humanized RANKL antibody denosumab for 16 months had reduced bone resorption markers and increased BMD, compared with OVX-Vehicle or SHAM controls (162). vBMD

increased in the metaphyses of the distal radius and proximal tibia. Trends toward increased periosteal and decreased endocortical circumference resulted in a greater cross sectional moment of inertia at the tibia diaphysis.

Acidification and Bone Resorption

Bone resorption requires acidification of the resorption space through the active transport of protons driven by a V-type H+ATPase. Passive transport of chloride preserves electroneutrality and is mediated through the chloride channel, ClC-7. The chloride channel inhibitor, PB5430, inhibited osteoclastic lysosomal acidification and bone resorption *in vitro* and reduced bone resorption in OVX rats (179). Bone formation was comparable to vehicle-treated animals, suggesting a possible dissociation between resorption inhibition with continued formation.

Collagen Degradation and Bone Resorption

Dissolution of mineral exposes the organic matrix. Cathepsin K, a cysteine protease expressed in osteoclasts, degrades type I collagen matrix. Cathepsin K inhibitors may also reduce bone resorption but not bone formation, perhaps in part because inhibition of osteoclastic resorption in this manner does not impair osteoblastogenesis, and in part because there may be transitory increases in PTH. The cathepsin K inhibitor, balicatib, reduced bone resorption markers, but not bone formation markers, in postmenopausal women (180). Balicatib inhibited collagen degradation but not earlier osteoclast function, which is important in osteoblastogenesis (181). In 675 women, treatment increased BMD, while remodeling markers decreased, but formation markers remained unchanged or increased. Similar dissociation in markers of formation and resorption were reported in a 12-week trial in 140 postmenopausal women treated with different doses of balicatib (182).

Compound A, another cathepsin K inhibitor, inhibited resorption markers in OVX monkeys without affecting formation markers, and increased BMD (84). The effect appeared to be greater than that produced by alendronate. In OVX adult rabbits, the cathepsin K inhibitor Compound J or ALN prevented bone loss (183). ALN reduced MS/BS surfaces and Haversian labeling, but neither dose of Compound J affected MS/BS. Administration of the cathepsin K inhibitor, relacatib, to monkeys (10 mg/kg for 5 days) inhibited NTx and increased (2-3 fold) plasma PTH temporarily (85).

Wnt and Bone Formation

Dickkopf-1 (Dkk1) inhibits Wnt signaling in osteoblasts and so reduces bone formation. Anti-Dkk1 antibodies increased BMD by increasing bone volume and osteoblast surfaces with minimal increase in osteoclast surfaces (184). Bone formation rate was elevated, increasing femur midshaft cortical area, vertebral trabecular bone volume and trabecular number in the distal femur. Glucocorticoids suppress bone formation by increasing Dkk1. CD40 ligand reversed suppression of bone formation and decreased osteocyte apoptosis, and reversed the increased expression of SOST and Dkk1 produced by prednisolone treatment. This reversed glucocorticoid-induced suppression of bone formation and osteocyte apoptosis by reducing sclerostin and Dkk-1, which reversed the suppression in Wnt signaling and restored bone formation (185).

Glycogen synthase kinase 3-β (Gsk3) in osteoblastic lineage cells phosphorylates and destabilizes β-catenin, which, if stabilized, increases in the cytoplasm, translocates into the nucleus, and plays a role in osteoblastogenesis. AR28, an inhibitor of Gsk3, increased β-catenin in human osteoblast-like cells and decreased proliferation, osteoprotegerin secretion, and *in vitro* mineralization (186). When administered orally to C57BL/6J mice for 14 days, trabecular bone density increased in the proximal tibiae. No change was seen in cortical BMD. BIO (6-biomoindirubin-3'-oxime) inhibits Gsk3 in primary mouse calvarial osteoblasts (187). Non-phosphorylated β-catenin increased and localized to the nucleus. BIO-induced alkaline phosphatase activity increased 5-

fold and promoted osteoblast differentiation. When injected over calvaria in 4-week-old male mice, new woven bone was formed, so selective activation of β-catenin signaling in osteoblasts may have potential as a clinical intervention for the treatment of bone loss-associated diseases.

RAP Dancing

Activin is a member of the BMP superfamily and regulates bone metabolism. RAP-011, a soluble form of the extracellular domain of the type 2 activin receptor fused to a IgG-Fc fragment, was administered for 12 weeks in mice 2 months after OVX and increased trabecular density in the proximal tibia in both SHAM and OVX mice (188). In vertebrae, the drug increased trabecular density by about 50% in OVX and SHAM, increased trabecular number, and decreased trabecular separation at the spine and distal femur. In the mid-femoral shaft, CSA increased, as did cortical area and thickness in SHAM and OVX animals.

NO Means YES?

Nitric oxide (NO) regulates bone remodeling, and NO donors have stimulatory effects on bone mass in rats and humans. NO donor-bisphosphonate (NO-BP) conjugates stimulated bone formation and increased alkaline phosphatase activity (189). Alendronate inhibited interleukin-1 (IL-1)-induced bone resorption. The alendronate-NO conjugates also inhibited bone resorption and stimulated new bone formation and osteoblast differentiation. *In vivo*, the compounds increased local bone formation when injected into subcutaneous tissue over calvaria of normal mice (alendronate alone had no effect). The effect in these mice was also present in the proximal tibiae, with a 100% increase in trabecular bone volume at the 10 mg/kg/day dose. When administered by subcutaneous injection to mature rats, the compounds caused a 77% increase in trabecular bone volume.

Conflict of Interest: Dr. Seeman reports that he is an advisory committee member for Sanofi-Aventis, Eli Lilly, Merck Sharp & Dohme, Novartis, and Servier, and that he lectures occasionally at conference symposia for those companies.

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