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MEETING REPORT

New insights into osteocyte and osteoblast biology: support of osteoclast formation, PTH action and the role of Wnt16 (ASBMR 2013)

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Meeting Report from the 35th Annual Meeting of the American Society for Bone and Mineral Research, Baltimore, MD, USA, 4–7 October 2013

One of the stand-out moments at the American Society for Bone and Mineral Research (ASBMR) 2013 was Sarah Dallas' State-of-the-Art lecture on osteocyte biology.[1] Over the past several years, she has developed methods for studying live osteocytes *in situ.* Using genetically modified mice with DMP1Cre-directed green fluorescent protein (GFP) label (for osteocytes), and Col2.3Cre-directed Tomato (for osteoblasts), she showed striking live-cell movies of the calvarial surface. This showed a sub-population of highly motile GFP-labeled osteocytes, not yet embedded into the bone matrix, positioning themselves on the calvarial surface. In this process, the cells extended their dendrites in a radial manner, much as a snail would use its tentacles, to find adjacent osteocytes and locate a region evenly spaced from its neighbors. The role of osteocytes in bone remodeling and the actions of parathyroid hormone (PTH) was a recurring theme throughout the meeting.

Osteocytes as Regulators of Osteoclastic Bone Resorption

The question of which cell is the most important source of receptor activator of nuclear factor-κB ligand (RANKL) continues to generate much lively debate. Earlier work indicated that, as well as an extreme osteopetrosis in osteoblast-lineage RANKL knockouts (Osx1Cre.RANKLf/f), osteocyte-directed deletion of RANKL, using DMP1Cre, caused a more mild osteopetrosis.[2,3] This suggested that RANKL expressed by osteocytes might also play a role in bone homeostasis, a suggestion that has captured the imagination of many. However, the observation, using a Rosa26-LacZ reporter, that DMP1Cre also directed Cre expression to bone-lining osteoblasts[3] suggested this phenotype may simply have related to a loss of RANKL in osteoblasts. As an alternative, mice with RANKL deletion directed by a Sclerostin-Cre were generated.[4] Rosa26-LacZ staining clearly indicated that the Sclerostin promoter directed Cre expression to osteocytes and not osteoblasts. A mildly greater vertebral bone mineral density (BMD) was observed at 5 weeks, and at 22 weeks of age these mice showed significantly fewer osteoclasts and greater vertebral BMD and trabecular bone volume as compared with controls. However, there was prolific LacZ staining in unidentified marrow cell populations. Notably, other work indicated that direction of RANKL deletion to the CD4 positive T cell, using a CD4-Cre, also caused a mild osteopetrotic phenotype.[5,6] Whether the LacZ-positive marrow cells in which Sclerostin-Cre is expressed are T cells is not yet known.

The other key factor required for osteoclastogenesis is macrophage colony-stimulating factor (M-CSF)/colony-stimulating factor 1 (CSF-1), and *in vitro* data indicate that this, too, is expressed by osteocytes.[7] Data shown at the meeting indicated that DMP1Cre-CSF1f/f mice also exhibit high bone mass and low osteoclast formation, although not to nearly the same extent as the profoundly osteopetrotic global knockout for CSF-1.[8] Osteocytic CSF-1 may therefore also play a role, although a relatively minor one, in supporting osteoclastogenesis.

There was much discussion, as there has been for the past 3 years, about the expression of DMP1Cre in osteoblasts on trabecular bone surfaces.[3] Notably, in the videos shown by Sarah Dallas,[1] very few Col2.3Cre-Tomato cells were also positive for DMP1Cre-GFP (<5%). This suggests that DMP1Cre directs recombinase expression to a minority of osteoblasts, possibly those already programmed to become matrix-embedded osteocytes.

RANKL is membrane bound, and although both inhibitory and stimulatory secreted forms have been described,[9,10] the support of osteoclast formation by osteoblast-lineage cells *in vitro* requires direct cell–cell contact.[11] It has been difficult to understand how osteocytes, from within the matrix, could control RANKL availability to osteoclast precursors in the bloodstream.[12] Osteocytic exosomes released during apoptosis have previously been noted to stimulate osteoclastogenesis,[13] and early confocal laser scanning microscopy indicated that osteocytic processes extend to the vascular-facing surface of the osteoblast, suggesting a possible

direct interaction with the vasculature.[14] Release of osteocyte microvesicles (a type of exosome) was shown by live-cell imaging in a mouse model expressing membrane-bound GFP specifically in osteocytes.[15] Osteocyte cell processes were observed to release membrane vesicles, not only into the local bone microenvironment, but also into blood vessel channels, indicating their possible release into the circulation. RANKL-containing microvesicles were also detected in cultured osteocyte-like cells[15] and the UAMS stromal cell line.[16] In both *in vitro* models, PTH treatment increased microvesicular level of RANKL. This provides compelling new evidence for a mechanism by which the osteoblast lineage, including the matrix-embedded osteocyte, might provide RANKL to osteoclast precursors.

The contribution of osteocyte apoptosis in activating bone remodeling in disuse was addressed by caspase inhibition in a mouse hindlimb unloading model.[17] After 14 days of hindlimb unloading, the percentage of caspase-positive osteocytes was dramatically increased. This was associated with reduced trabecular bone mass and increased bone erosion, and both effects were prevented by the caspase inhibitor. This confirms that osteocyte apoptosis is a key contributor to disuse-associated bone loss, and that therapeutic targeting could be an effective preventive strategy. Notably, other work indicated that although osteocytic expression of sclerostin is increased in a sciatic neurectomy model of disuse, there is no significant alteration in osteocytic RANKL expression,[18] implying that although osteocyte apoptosis contributes to disuse-induced bone loss, osteocytic RANKL does not.

Key Players in the Anabolic and Catabolic Actions of PTH

Osteocytes are well accepted as regulators of bone formation, particularly via their expression of sclerostin, a Wnt signaling inhibitor. PTH remains the only approved anabolic therapy for osteoporosis, but its mechanism of action remains poorly understood, particularly as continuous exposure to PTH leads to bone loss. As the PTH receptor (PTH1R) is expressed by osteocytes, and PTH inhibits osteocytic expression of sclerostin,[19,20] the contribution of osteocytic PTH1R to both anabolic and catabolic actions of PTH was investigated in mice with DMP1Cre-directed deletion. Osteocytic PTH1R was shown to be required for normal bone mass in young mice, and these mice showed a blunting of the effects of both anabolic intermittent PTH injections and catabolic PTH infusion.[21,22] Surprisingly, although a second model of DMP1Cre-directed PTH1R deletion confirmed the requirement for osteocytic PTH1R in the anabolic action of PTH, bone loss due to continuously elevated serum PTH, induced by a low-calcium diet, occurred as normal in the absence of osteocytic PTH1R.[23] The mechanism for the difference remains elusive, but suggests that only the effect of exogenous PTH depends on direct actions on osteocytes.

PTH1R activates multiple G protein-dependent signaling pathways. Many of its actions are cyclic adenosine monophosphate (cAMP) dependent, therefore mediated by $G_s\alpha$ signaling, and inhibited by G_i. It was reported that PTH anabolic action was approximately double in mice with induced G_i deletion in osteoblasts,[24] supporting the role of this inhibitory G protein in suppressing PTH action. What was surprising was that a similar experiment in mice with $G_s\alpha$ deleted throughout the osteoblast lineage[25] still showed only a mild impairment in the PTH-induced increase in trabecular thickness. These mice retained a dramatic increase in osteoblast numbers and bone formation rate with PTH treatment, suggesting a pathway of PTH anabolic action that does not depend on $G_s\alpha$ action in osteoblasts and osteocytes.

Perhaps this action could be mediated by the stimulatory effects of PTH1R signaling in other cells in the bone environment, such as endothelial cells? Work from the Lafage-Proust laboratory, using their novel methods for assessing bone and vascularity simultaneously by histomorphometry,[26] showed that intermittent PTH treatment increased bone vascular perfusion by ~30%, and almost doubled microvessel size.[27] In contrast, continuous infusion of PTH did not modify vascular perfusion, but reduced microvessel size. The mechanism by which these opposing effects on microvessel size might determine the level of osteoclast formation in response to PTH remains unknown.

A number of papers used cell-specific genetic deletion and PTH treatment to provide data showing that multiple pathways are required for PTH anabolic action, in addition to many that have been identified previously.[28] The anabolic effect of PTH was blunted in mice with a tamoxifen-inducible deletion of β-catenin driven by the DMP1CreERt2 transgene.[29] However, it is not possible to determine whether it is Wnt/β-catenin signaling or β-catenin signaling activated through an alternate pathway that is involved. Notably, mice with a late osteoblast/osteocyte-specific deletion of Lrp6, driven by the osteocalcin-Cre transgene, exhibited a low level of bone formation, and increased osteoblast apoptosis, and the anabolic action of PTH was significantly blunted.[30] The parallel between these studies suggest that it is Lrp6/Wnt/β-catenin signaling that plays a role in PTH anabolic action. Of course, it is possible that both pathways downstream of β-catenin are involved.

EphrinB2, a contact-dependent protein tyrosine kinase receptor, expressed in both osteoblasts and osteoclasts has previously been reported to be stimulated in osteoblasts by PTH.[31] Mice lacking this receptor, specifically in osteoblasts, were shown to exhibit an impaired anabolic response to PTH.[32] This is consistent with a previous report using a systemic pharmacological inhibitor of the pathway[33] now indicating that it is from within the osteoblast lineage that EphrinB2 signaling supports osteoblast differentiation.

In addition, it has been known for many years that PTH induces expression of interleukin-6 (IL-6).[34] Mice with osteocytic deletion of the common IL-6 family signal transducer gp130, recently reported to have impaired bone formation,[35] were treated with anabolic PTH. In these mice, no increase in osteoblast number, mineralizing surface or P1NP levels was observed with PTH treatment.[36] This finding was partially explained by a significant reduction in PTH1R expression in femurs from the gp130-deficient mice, suggesting that IL-6 family cytokines maintain PTH1R expression in osteoblasts/osteocytes. This could have significant implications for the efficacy of anabolic PTH treatment in patients being treated with IL-6 inhibitors for inflammatory conditions such as arthritis.

One aspect of PTH anabolic action that has long been a source of puzzlement is the difficulty of reproducing the anabolic action of PTH *in vitro*. A possible explanation for this is the high level of prostaglandin E_2 expressed by osteoblasts in

culture. Cyclooxygenase-2 (COX-2) is a key enzyme in prostaglandin formation, and new data presented on the effects of PTH in COX-2-null mice go some way to resolving this question.[37] Surprisingly, when COX-2-null mice were treated with continuous PTH infusion, trabecular bone volume was significantly increased—the opposite effect to that observed in wild-type mice. Striking images showed abundant deposits of woven bone close to the endocortical surface in the PTH-infused COX-2-null mice. The influence of PTH on C-terminal telopeptide I (CTX-1) levels and on tibial RANKL/osteoprotegerin (OPG) ratio was not different between COX-2-null and wild-type mice, suggesting that COX-2 is not required for increased osteoclast formation in response to PTH infusion, but COX-2 suppression is required for anabolic PTH action.

Cellular Action of Wnt16: An Osteoblast-Derived Inhibitor of Osteoclastogenesis

Finally, data were presented that inform our understanding of the new Wnt pathway member Wnt16, recently identified as a determinant of cortical bone thickness, bone strength and fracture risk in genome-wide association studies.[38,39] Two presentations described the phenotype of mice with global deletion of Wnt16, both exhibiting spontaneous diaphyseal fractures and finding that Wnt16 is expressed in osteoblasts but not osteoclasts.[40,41] Wnt16 expression by osteoblasts was dramatically increased by treatment with oncostatin M, an IL-6 family cytokine required for both normal bone formation and resorption.[42]

As observed in humans, the mouse models exhibited thin and porous cortical bone[40,41] and reduced mineral/matrix ratio,[41] with increased osteoclasts on the endocortical surface and a marked increase in the RANKL/OPG ratio.[40] Consistent with this, recombinant Wnt16 treatment dose-dependently inhibited RANKL-induced osteoclast formation.[41] Although Wnt16-null osteoblasts showed normal activity *in vitro*,[40,41] their production of OPG was low, and treatment of wild-type calvarial osteoblasts with recombinant Wnt16 stimulated OPG production,[40] indicating that the key role of this Wnt family member is to inhibit osteoclast formation by stimulating osteoblastic OPG production. Notably, preliminary data on mice with cell-specific deletion of Wnt16 indicated that it is early osteoblasts, targeted by runx2Cre, in which the production of Wnt16 is most critical. DMP1Cre-driven deletion of Wnt16 did not recapitulate the phenotype observed in the global null mice.[41] Although both these studies suggested that the key role of Wnt16 is as an osteoblast-derived stimulus of OPG production, there was also evidence presented that Wnt16 regulates periosteal bone formation,[43] consistent with the reduced femoral cross-sectional area.

Conflict of Interest

The author declares no conflict of interest.

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