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MEETING REPORTS

The Rise of Osteocyte Biology Continues: Meeting Report from the 32nd Annual Meeting of the American Society for Bone and Mineral Research

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Traditionally, osteocytes have not been amenable to extensive experimental interrogation due to their localization within the mineralized bone matrix. Within this matrix, they form a complex cellular network interconnecting with each other and cells on the bone surface via an extensive canalicular network within which their dendritic processes are located. Based primarily on observational science, this most abundant bone cell type was either judged to be quiescent or suspected of being implicated in skeletal responses to mechanical strain. With the emergence of mouse genetics and *in vitro* tools in concert with novel imaging techniques, it has rapidly become clear that these cells are indeed not only playing a role in mechanosensation, but also in mineral homeostasis and bone mass regulation. Accordingly, the number of presentations on osteocyte function has grown steadily during recent ASBMR annual meetings, and this year was no exception. Findings were presented that both increase our knowledge of "classical functions" of osteocytes and hint at previously unanticipated roles such as involvement in lipid metabolism.

The Osteocyte's Role in Mechanotransduction

Several lines of evidence suggest that connexin 43 (Cx43), which is the most abundant gap junction protein in the skeleton, plays a role in osteocyte-to-osteocyte and osteocyte-to-osteoblast communication in response to mechanical loading. For example, it has been demonstrated recently that Cx43 is induced following shear stress-mediated prostaglandin release stimulating gap junction formation at the tip of dendritic processes (1). It was shown, at the molecular level, that prostaglandin inactivates GSK3 in a PI3K/Akt- and cAMP-PKA-dependent manner, thus facilitating β-catenin translocation into the nucleus and binding to the *Cx43* promoter. Likewise, it has been described recently that Cx43 hemichannels open upon mechanotransduction of their dendritic processes (2). At this year's meeting, a mouse model was introduced in which the animals expressed a dominant negatively-acting mutant form of *Cx43* in osteocytes (10kb *Dmp1-Cre*); this model lacks gap junction function but not hemichannel function, with the aim to dissect their relative role (3). Mice heterozygous for loss of *Cx43* gap junction function presented with decreased bone mass and increased cortical porosity, suggesting a requirement of osteocyte Cx43 gap junction function for normal bone mass accrual. The potential contribution of elevated Cx43 hemichannel activity to the skeletal phenotype cannot be ruled out yet in this transgenic model. A gap junction-independent role of *Cx43* is the prevention of osteocyte apoptosis via src-ERK-dependent opening of *Cx43* hemichannels. One study (4) consistently demonstrated that the deletion of osteocytic *Cx43* (8kb *Dmp1-Cre*) induces osteocyte apoptosis. In addition, this study found the animals to have wider, yet thinner cortices with inferior material stiffness, thus recapitulating the phenotype of animals

lacking *Cx43* in osteoblasts and osteocytes and confirming a role for Cx43 in osteocytes in adult skeletal homeostasis.

In addition to *Cx43*, the primary cilia has been hypothesized to be a mechanosensor in bone. To date we know that partial loss of cilia function in the osteoblastic lineage results in osteopenia. At last year's meeting it was shown that disruption of cilia function in osteocytes results in decreased bone mechanosensitivity to loading (5). In this context, it is worthwhile mentioning a preliminary analysis of mice lacking the essential primary cilia component *Ift88* in mature osteoblasts and osteocytes (*OC-Cre*) (6). Though the data need to be taken with caution due to the preliminary nature of the work, they indicate age-dependent, diverse bone mass phenotypes consistent with a role of the primary cilia in osteoblasts and/or osteocytes in skeletal homeostasis.

Several lines of circumstantial evidence suggest that the osteocyte-secreted bone formation inhibitor sclerostin, encoded by the *Sost* gene, is one of the downstream mediators of skeletal responses to the mechanical environment. At this year's meeting it was shown that mice expressing *Sost* under the control of the 8kb *Dmp1* promoter displayed a decreased anabolic response to loading, suggesting that reduction of sclerostin levels is obligatory for osteogenic responses to occur following loading (7). Conversely, *in vitro* murine osteocytic cell and organ culture data (8) indicated that *Sost*/sclerostin is up-regulated in microgravity, consistent with observations in unloading/disuse situations in rodents and humans (9). Finally, sclerostin inhibition through use of a neutralizing antibody was shown to overcome disuse-induced bone loss (10). The question as to how exactly osteocyte-expressed sclerostin exerts its action was also not resolved at this year's meeting. It is currently hypothesized to travel through the osteocytic network to the bone surface where it inhibits Wnt/β-catenin signaling in osteoblasts upon binding to Wnt co-receptors LRP5 and/or LRP6, although the local role of LRP5 in bone has been challenged recently. Two groups presented data from independently-generated *Sost/Lrp5* double knockout mice (11;12). Both data sets consistently demonstrated that the *Sost* loss-of-function-induced bone gain relies in part on *Lrp5*-dependent pathways, but also on other pathways. The protection of knock-in mice expressing mutated forms of *LRP5* associated with high bone mass (HBM) from *Sost* overexpression-induced osteopenia provided further support for a partial dependence of sclerostin effects on this receptor (13). Whether the other pathways involve LRP6 and/or other players remains to be elucidated. The description of two *LRP4* HBM mutations, which result in decreased binding to sclerostin and loss of LRP4's ability to facilitate sclerostin action (14), indicates that much remains to be learned concerning how this osteocyte-expressed glycoprotein exerts its action, in line with recent publications (15).

The Osteocyte Is a Dynamic Cell: Regulation of Bone Mineralization, Bone Mineral Homeostasis, and Bone Resorption

Besides the role of osteocytes in skeletal responses to mechanical input, probably the most highly scrutinized role for these cells is in mineral metabolism. Osteocyte-expressed PHEX, MEPE and DMP1 have been implicated in mineral homeostasis. Interestingly, mice that lack *Phex* in osteocytes (10 kb *Dmp1-Cre*) present with distinct bone mineralization defects, while they do not present with elevated Fgf23 or hypophosphatemia, in contrast to *hyp* mice carrying loss-of-function mutations in *Phex* or mice lacking *Phex* from the mature osteoblast stage onwards (*OC-Cre*) (16). In *in vitro* studies, two groups independently identified sclerostin as the candidate mineralization inhibitor, which might give rise to mineralization defects in these mice (16;17). One group observed an enhanced inhibition of bone mineralization in primary osteoblasts from *hyp* mice, compared to wild-type animals (16). The other group concluded, based on work with primary human osteoblasts, that sclerostin targets cells at the late osteoblast/early osteocyte stage and acts as a negative regulator of bone mineralization through the PHEX/MEPE axis (17). These data provide

supportive evidence for the postulated role of early osteocytes in bone matrix mineralization (18). Likewise, the change in material stiffness found in mice with osteocyte-specific ablation of *Cx43* (4) might hint at such a role. Moreover, live cell imaging revealed that mineralization occurs exclusively in the proximity of *Dmp1/E11*-expressing osteocytic cells shedding vesicles, which could be implicated in the process (19). Time-lapse imaging data presented by this group over recent years indicate that osteocytes are far from being quiescent. Their observations also question whether osteocyte differentiation is indeed irreversible as is assumed. Work using mouse genetic tools (20) allowing for the tracking of past states of osteocyte differentiation now indicates that osteocytes emerging from *ex vivo* bone chip culture can de-differentiate and, post-transplantation, re-differentiate.

One of the long-standing debates in the field is whether osteocytes can remove and replace mineral matrix in the perilacunar space surrounding them and thus contribute to bone mineral homeostasis (21). While this debate has not been settled to date, observational evidence establishes that osteocyte lacunar size varies with different skeletal challenges, not all of which can be readily explained by differences in osteocyte age or technical artifacts, though such contaminating factors cannot be totally excluded either. The increasing accessibility of computed tomography and electron microscopy techniques that can provide osteocyte lacunar imaging now allows for at least more systematic mapping of differences in osteocyte lacunar size under various challenges (22-25) including drug treatment (23;26). Interestingly, it was reported that mice lacking *Pthr1* in osteocytes (10kb *Dmp1-Cre*) fail to display increases in average osteocyte lacunar size during lactation (27) as opposed to wild-type mice (25;27), implicating PTH/PTHrP action in mediation of these effects. Moreover, this group identified genes associated with osteoclast function that are up-regulated in osteocytes of lactating animals, providing clues as to what mechanisms might be involved in such a process similar but not identical to osteoclast function.

A substantial amount of evidence has accumulated during recent years showing that osteocytes impact osteoclastic bone resorption. *In vitro* and *in vivo* data demonstrate that osteocyte death/apoptosis triggers bone resorption. Mouse genetic data presented in a study (using the 10kb *Dmp1-Cre*) (28) revealed that osteocytes also produce substantial amounts of the osteoclast differentiation factor RANKL. These findings complement published *in vitro* and *in vivo* findings suggesting that both RANKL and the RANKL decoy receptor OPG are robustly expressed by these cells and differentially regulated upon challenges (29). Overall, the data suggest that osteocytes might modulate the RANKL/OPG ratio, providing an additional level of osteocyte regulation of osteoclastic bone resorption.

The Osteocyte's Role in the Response to Hormonal Bone Mass Regulators: The Relevance of Osteocytic PTH Signaling, and Osteocyte Defense Against Glucocorticoid-Induced Stress

Previous work demonstrated that expression of constitutively active *Pthr1* in osteocytes (8kb *Dmp1-Cre*) gives rise to increased bone modeling and remodeling. A role for osteocytic PTH/PTHRP-dependent signaling for induction of sub-periosteal bone modeling was revealed at last year's ASBMR meeting (30). This year, this same group crossed the same animal model with mice overexpressing *Sost* in osteocytes followed by treatment with a bisphosphonate, showing that resorption is essential for increased endosteal bone formation driven by Pthr1 signaling (31). Together, these data imply *Sost* down-regulation during bone modeling consistent with the observation that *Sost*/sclerostin exerts its action more during modeling than during remodeling. Not only was this shown to be the case for rodents, but also in non-human primates (32), where PTH-induced bone anabolism is more bone remodeling-dependent. Taken together, the data indicate once more that osteocytic

PTH/PTHRP signaling is implied in PTH bone anabolism and that *Sost* suppression, which was also observed in humans following PTH treatment (33), is only one of several putative mechanisms by which this is accomplished.

Recently, autophagy has been identified as a mechanism utilized by osteocytes to defend themselves against glucocorticoid-induced stress (34), and data regarding time and dose of glucocorticoid treatment were presented at this year's meeting showing that shorter times and lower doses induced autophagy, whereas longer treatments with higher doses caused apoptosis (35). Further support for the relevance of autophagy in osteocytes in response to reactive oxygen species and hypoxia was also shown (36). Moreover, expression of autophagy-related genes apparently declines with aging, providing a putative mechanism that contributes to the increase in osteocyte apoptosis with age.

Emerging Novel Roles of Osteocytes: Osteocytes and Muscle Cells – Analogies and Cross-talk, and Putative Roles of Osteocytes in Lipid and Energy Metabolism

Myocyte enhancer factors (Mef) 2 are best known for their crucial role in muscle. Recently it was discovered that they are also expressed in osteocytes and that *Sost* expression and its PTH-induced inhibition are mediated via Mef2 binding to the *Sost* bone enhancer (37). Data now reveal (38) that PTH-mediated *Sost* inhibition involves nuclear import of a class IIa histone deacetylase and subsequent Mef2 repression – analogous to Mef2 repression in muscle. Two groups reported increases in bone mass when deleting Mef2c, which is the most strongly osteocyte-expressed Mef2, from the mature osteoblast stage onwards (*OC-Cre*) (39) or in osteocytes (10kb *Dmp1-Cre*) (40). While the data demonstrated the expected suppression of *Sost* and loss of bone enhancer-dependent *Sost* transcription (39), it also indicated Mef2c roles beyond *Sost* regulation (40). Moreover, *Mef2c* was demonstrated at this year's meeting to be differentially regulated in osteocytes downstream of Akt/GSK3 signaling, which is implied in the osteocyte response to mechanical loading (41) and following unloading in microgravity (8). In sum, these findings identify a role for osteocyte-expressed Mef2c in bone mass regulation in line with the recently described association of *MEF2C* with bone mineral density in meta-analysis of genome-wide association studies (42).

Two data sets were presented at this year's meeting investigating putative cross-talk between muscle and osteocytic cells. This is of interest since sarcopenia and osteoporosis frequently manifest together and interdependence cannot be excluded. One group demonstrated an activation of the PI3K/Akt pathway in osteocytic MLO-Y4 cells upon addition of conditioned media collected from *in vitro* muscle cell and *ex vivo* muscle organ culture systems (43). The second group took the reverse approach and added conditioned media from MLO-Y4 cells to different *in vitro* muscle cell culture systems, demonstrating promotion of myoblast-to-myotube formation (20). Further work is now required to identify the responsible secreted factors and to translate their relevance *in vivo*.

While it was proposed (44) that osteocytes do not impact only kidney function in an endocrine fashion, but also muscle function, a presentation (45) revealed yet another putative, novel endocrine function. The authors discovered that mice heterozygous for loss of G protein subunit Gsα in osteocytes (10kb *Dmp1-Cre*) do not develop an osteopenic phenotype, in contrast to homozygous mice. Yet, interestingly, both homozygous and heterozygous mice presented with a reduction in peripheral fat. Adipocytes were found to be of reduced size and altered morphology, hinting at disturbed lipid metabolism, while fasting insulin and glucose levels were unchanged. Intriguingly, the data thus suggest that osteocyte Gsα signaling might regulate peripheral adiposity. Finally, it was reported (46) that osteocyte ablation (*Dmp1*-DTR) resulted in elevated fasting insulin and glucose levels, while osteocalcin and adiponectin levels were unaffected, hinting at a putative endocrine

impact of osteocytes on energy metabolism independent of the described osteocalcin-mediated one in bone.

Tools to Study Osteocytes: Limitations and Novelties

Data utilizing reporter mouse lines were presented suggesting a lack of selectivity of 10kb *Dmp1-Cre* transgenic mice for pre-osteocytes and osteocytes. Reporter signal was found in osteoblasts during bone growth and in muscle (20;28) in addition to osteocytes and the brain, where reporter expression has been described previously (47). The argument was made by other groups, which have established and used this Cre driver line (*e.g.*, (3;45)), that during early growth, a rapid transition from osteoblast-to-osteocyte occurs, therefore, reporter signal may be observed in some osteoblastic cells, but in the adult skeleton this is not the case. Overall, these findings highlight the fact that the 10kb *Dmp1-Cre* transgenic mouse – like any Cre transgenic mouse model – is likely to have limitations. Generation of novel models for osteocyte targeting will be useful for cross-validation of findings made using this transgenic model. However, it must be kept in mind that it is unlikely for any of them to be a perfect tool. In support of this notion, two groups demonstrated that transgenic *Sost-GFP* mice (48) and mice generated by crossing *Sost-Cre* mice with Rosa 26-lacZ reporter mice (41) not only display reporter signal in osteocytes, but also in an unknown bone marrow cell population.

Finally, the vast majority of groups addressing osteocyte function *in vitro* utilize MLO-Y4 cells, which reflect some aspects of osteocyte biology (*e.g.*, morphology), but not all (*e.g.*, mature osteocyte marker gene expression). A novel, interesting, mouse osteoblast/osteocyte cell line – IDG-SW3 – was presented (49), which embeds in mineralized matrix, is able to integrate into 3-D matrices and in which the complete osteoblast-to-late osteocytic marker gene expression profile, including *E11*, *Phex*, *Dmp1*, *Mepe*, *Sost*, and *Fgf23*, can be induced.

Conflict of Interest: Dr. Kneissel reports that she is an employee of the Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel.

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