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## MEETING REPORT

# Embedded in bone, but looking beyond: osteocalcin, epigenetics and ectopic bone formation (ASBMR 2014)

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Meeting Report from the ASBMR 2014 Annual Meeting, Houston, TX, USA, 12–15 September 2014

Following in the tradition of outstanding Plenary Lectures at the American Society for Bone and Mineral Research (ASBMR) annual meeting, Ana Maria Cuervo's introduction to the multifaceted functions of autophagy (lysosomal degradation of obsolete intracellular proteins) at the 2014 Gerald D Aurbach Lecture did not disappoint.[1] Dr Cuervo has investigated the role of autophagic signaling pathways in maintaining energy stores within cells and developed tissue-specific autophagy-deficient mouse models to investigate their multiple pathways in age-related disorders including sarcopenia and neurodegenerative diseases such as Parkinson's and Huntington's Disease. As proteins are degraded by different types of autophagic pathways depending on their conformational state, this process may be also of importance to the etiology of bone diseases such as osteoporosis and osteogenesis imperfecta. Autophagy maintains bone mass as indicated by elevated osteoclast number and bone formation rate in mice lacking an essential autophagy gene.[2,3] In abstracts presented at this year's meeting, chaperone-mediated autophagy (a highly specialized form of autophagy where the protein is directed toward the lysosome) was demonstrated to drive periosteal progenitor cells toward the chondrocytic lineage in response to nutrient deprivation, indicating the importance of this pathway in bone cell fate.[4] Autophagy was also shown to be switched on in osteoblasts during mineralization and found to inhibit their expression of RANKL,[5] suggesting direct involvement of autophagy in osteoblastic control of both bone matrix quality and bone resorption. The implication that autophagy may also have a role in pathological bone diseases, such as its potential involvement in collagen degradation in osteogenesis imperfecta, was briefly discussed and is likely to be addressed in the future, as our understanding of autophagic signaling in bone physiology and pathology increases.

Increasingly, the ASBMR meeting has highlighted the complex layers of regulation that direct bone turnover and dictate bone cell behavior by endocrine, paracrine and autocrine mechanisms. The recurring theme of this year's meeting appeared to be that we have only just begun discovering the whole-body systems that regulate and are regulated by bone-derived factors and the technologies that will enable us to investigate these interactions.

### The endocrine roles of osteocalcin

Expanding on last year's recurring theme of bone functioning as an endocrine organ, there was a surge in the number of abstracts teasing out the effect, if any, of circulating osteocalcin (OCN) on other physiological systems. There were $>15$ abstracts on the topic of OCN regulation of energy metabolism, muscle or cognitive function alone, and approximately half of these were selected for oral presentation. OCN is a bone matrix protein that acts as a marker for bone remodeling and has been reported to exert hormonal effects on beta cells of the pancreas to stimulate production of insulin and adiponectin.[6] Undercarboxylated OCN has been implicated in driving energy metabolism in mice, but its role in human glucose tolerance and insulin sensitivity remains controversial.[7]

It was proposed that OCN sustains muscle function and promotes muscle mass, an idea supported by data showing early fatigue and low muscle mass in exercising $OCN^{-/-}$ mice.[8] However, as the $OCN^{-/-}$ mice are also reported to be prone to anxiety and depression[9] and studies on isolated muscle cells have not been reported, the fatigue and subsequent low muscle mass may simply reflect a reduced interest in physical exertion in OCN-deficient mice. Preliminary evidence was also presented to suggest that neural infusion of OCN to young and old mice may improve memory.[10] The $OCN^{-/-}$ mice are not widely available, and therefore it will be some time before the hormonal effects of OCN are made clear to the field, but the effects of OCN infusion can be tested more simply to expand on these potential roles. Data were also

presented showing that AMPK/OCN-dependent insulin resistance and glucose intolerance occur in mice with low bone mass resulting from conditional deletion of the glucose transporter GLUT1 in osteoblasts.[11] Because such deletion of the major glucose transporter in osteoblasts would result in a state of perpetual starvation for those cells, it is perhaps not surprising that GLUT1-deficient osteoblasts do not survive. Conversely, GLUT1 overexpression in osteoblasts resulted in high bone mass and greater bone formation, in keeping with their greater energy supply.

OCN was also discussed as a mediator of age-related metabolic dysfunction. Transgenic mice with selectively disrupted glucocorticoid signaling in osteoblasts/osteocytes are partially protected against age-related reductions in serum OCN,[12] suggesting that OCN may mediate age-related declines in metabolic function. Data from another clinical study, this time in children, showed that normal weight children have significantly higher carboxylated OCN levels compared with overweight children. However, perhaps OCN is not the key mediator, and the low serum OCN reflects changes in an alternate pathway. For example, human data were presented showing that high serum sclerostin is associated with the metabolic syndrome in a manner independent of serum OCN levels.[13] Further to this, the effect of OCN in regulating metabolic function appeared to be dependent on sclerostin, as men with sclerostin$^{High}$/OCN$^{Low}$ serum levels had the highest prevalence of the metabolic syndrome.[13] Thus, in humans, there is still much to be learned regarding the mechanisms by which serum OCN levels associate with changes in metabolic function.

**Epigenetic regulation of osteoblasts and the physiological roles of histone deacetylases (HDACs)**

Although there is much excitement about the new frontiers of exploring the influence of bone on whole-body physiology, there is still much about the basic mechanisms of bone cell biology that remain poorly understood. For example, questions continued to be raised about sex differences in bone structure and why some gene knockouts have effects that differ depending on the sex of the animal[14–17] and how cortical and trabecular bone can be influenced differently by the same gene.[18–20]

One new mechanism of regulating chondrocyte, osteoblast and osteocyte activity that was explored at length at the meeting was epigenetics, and in particular the role of histone deacetylases (HDACs) in regulating gene expression in the cells of bone. Big-picture presentations described the transcription factor binding patterns and histone modification marks during osteoblast lineage commitment[21] and specific histone modifications that correlate with changes in osteoblast gene expression throughout differentiation.[22] DNAse hypersensitivity mapping was also presented as a tool to identify temporal non-promoter regions of transcriptional regulation in osteoblast differentiation genes,[23] suggesting that histone modification likely has an important role in mediating both osteoblast commitment and differentiation. Data were also presented identifying the miRNA families involved in osteoblast to osteocyte differentiation,[24] some of the first data to examine the molecular mechanisms underlying this late-stage transition.

HDACs are histone-modifying enzymes that remove acetyl groups from specific lysine residues, thereby allowing the histones to wrap DNA more tightly and suppress gene expression. HDACs are thought to be involved in cancer onset and progression, neurological diseases and innate immune response,[25–27] with a number of HDAC inhibitors being developed as therapies for these conditions. Eighteen mammalian HDACs have been identified to date, and these have been divided into four classes, depending on their similarity to yeast homologs;[28] the current goal is to generate class IIa-specific inhibitors (which would target HDACs 4, 5, 7 and 9). HDAC inhibitors have been reported to suppress osteoclastogenesis[29,30] and to promote osteoblast differentiation,[31] but the use of HDAC inhibitors, such as valproate, *in vivo* has detrimental effects on the skeleton in mice[32] and in humans with epilepsy.[33] At this year's meeting, much work was presented on the ability of HDACs in class I (particularly HDAC3) and IIa (mostly HDAC4 and HDAC5) to regulate osteoblast and chondrocyte differentiation.

Chondrocytic deletion of HDAC3 was shown to result in runting and embryonic lethality.[34] The use of a tamoxifen-inducible Cre to enable deletion of HDAC3 after the postnatal period indicated that HDAC3 suppresses MMP13, a gene known to regulate cartilage breakdown,[35,36] as there was elevated expression of MMP13 throughout the proliferative, pre-hypertrophic and hypertrophic zones in the inducible HDAC3-null mice.[34] A related knockout in late chondrocytes and the osteoblast lineage (using Osx1Cre)[37] led to a phenotype of osteopenia and increased marrow adiposity. BMSCs from these mice also formed more adipocytes, possibly due to an increase in lipid storage and changes in glucocorticoid metabolism. Thus, HDAC3 inhibits hypertrophic cartilage breakdown, which may impair normal endochondral ossification, and suppresses adipogenesis, although the mechanism is yet to be defined.

HDAC4 and HDAC5 are class II HDACs, and GWAS analysis revealed HDAC5 as one of a number of BMD-influencing loci.[38] PTH has also been known for some years to induce MMP13 transcription in osteoblasts by releasing HDAC4 repression.[39,40] Furthermore, HDAC4-null mice, which are runted and rarely survive weaning,[41] exhibit high MMP13 in osteoblasts and hypertrophic chondrocytes.[40] New data presented suggest that each aspect of the bone phenotype in HDAC4-null mice is partially rescued by MMP13 deletion,[39] and therefore that multiple aspects of the HDAC4-null phenotype are caused by MMP13; whether the phenotype of the HDAC3-null mice might also be rescued by MMP13 deletion is not yet known. The same group also showed that PTH induces MMP13 transcription by causing HDAC4 to dissociate from Runx2 at the MMP13 promoter.[42] It is not yet known whether HDAC3 can also bind the MMP13 promoter region, or whether HDAC3 and HDAC4 may coregulate MMP13 transcription.

HDAC4 and 5 suppress Mef2c,[41,43] a transcription factor with many roles, including the suppression of sclerostin by parathyroid hormone.[44] Both PTH and PTHrP suppress Mef2c expression via HDAC4/5 in osteocytes and chondrocytes, respectively, and in osteocytes this appears to be a mechanism by which PTH inhibits sclerostin.[45–47] HDAC5-null mice show increased SOST mRNA and low trabecular bone volume, associated with decreased Wnt activity and presumably decreased osteoblast activity.[48] This phenotype may relate to HDAC5 suppression of Mef2c, which was demonstrated *in vitro*. A requirement for HDAC4 in chondrocytes was earlier

revealed by the lethal chondrocyte phenotype in mice null for HDAC4.[41] This has now been reproduced by a chondrocyte-specific knockout,[49] which bears some similarities to the PTHrP-null mouse.[50] Further investigation revealed that crossing the PTHrP and HDAC4 hemizygous mice did not lead to a phenotype, unless they were crossed with a miR-140-null mouse;[51] these mice showed Mef2c expression released from its inhibition by HDAC4, suggesting that HDAC4 inhibition of Mef2C depends on miR-140.

HDAC4 not only regulates sclerostin expression but was also reported to stabilize ATF4 by direct interaction,[52] thereby enhancing ATF4 activation of the OCN promoter. Osteoblast-specific HDAC4-null mice showed hypoinsulinemia, infertility, decreased learning and memory, which may all relate to their low OCN expression, but no data were presented on a bone phenotype in these mice.[53] It was also reported that PTH stimulates expression of an E3 ubiquitin ligase that binds HDAC4 in the presence of PTH,[52] suggesting that this provides the negative feedback required for HDAC4 degradation.

**The problem and treatment of heterotopic ossification**

Jonathan Forsberg from Walter Reed National Military Medical Center presented an enlightening talk on the increased rates of heterotopic observation (HO) being observed in enlisted US military personnel. He discussed some of the key difficulties in managing long-term care of military personnel and civilians who have suffered amputation because of blast injury. The lack of alternatives to surgery is an area in which more research is needed, not only for military personnel and victims of land mines, but also for patients with spinal cord or traumatic brain injury, in which heterotopic ossification is a common occurrence,[54] as well as patients with genetic mutations that lead to extraskeletal ossifications, including fibrodysplasia ossificans progressiva (FOP)[55] and progressive osseous heteroplasia.[56] In that same symposium, Yingzi Yang described her recently published work in a genetic model of HO[57] that showed the formation of HO when $G_s\alpha$ (encoded by the *Gnas* gene) was deleted in the early mesenchymal lineage and their progeny using Prx1-Cre. $G_s\alpha$ links G protein-coupled receptors (such as the PTH Receptor) with cyclic AMP signaling, suggesting that the role of this protein in human biology may extend beyond hyperparathyroidism, the McCune–Albright syndrome and Albright hereditary osteodystrophy. Furthermore, reducing Hedgehog signaling inhibited the development of these *Gnas*-dependent heterotopic ossifications formed in that model, providing a possible pathway of treatment, should similar mutations be observed in humans. Maurizio Pacifici also presented the progress of his earlier work showing that RAR$\gamma$ agonists are capable of reducing heterotopic ossifications in BMP4-dependent models of HO.[58] His laboratory reported that this treatment is successful in reducing HO in a new FOP model, a Prrx1 + cell lineage gain-of-function mutation for BMP type I receptor (R206H), which results in 100% penetrance of HO.[59] Clinical trials for HO of the RAR$\gamma$ agonist Palovarotene, a treatment for emphysema,[60] are now underway. Other new genetic models of BMP-dependent HO were also presented: a Cre-inducible constitutively active ALK2 receptor (R22A mutant of BMP receptor) mouse model combined with intramuscular injection of Adeno-Cre,[61] and intramuscular injection of BMP2 that results in inflammatory-mediated endochondral bone formation regulated via the sympathetic nervous system.[62] Promising data were also presented for an OPG inhibitory antibody that increased osteoclast formation in the caALK2 receptor + Ad-Cre model of HO and resulted in higher osteoclast numbers and bone resorption at the site of HO that was greater than the general effect on the skeleton,[61] suggesting some specificity for this antibody at sites of HO, as well as an OPG-specific mechanism in ectopic bone formation. These data were from a systemic treatment with the OPG antibody, and it would be interesting to see whether these results are even more striking with intramuscular OPG treatment.

Data were also presented providing insight into the progenitor cell populations and BMP2-associated targets that contribute to HO. A specific subset of SMA ($\alpha$-smooth muscle actin) progenitor cells (SM/C2.6 + ve) differentiate into osteoblasts in the muscle in response to BMP2, indicating that muscle satellite cells are capable of osteogenic differentiation once removed from their niche.[63] Membrane-type 1-MMP (Mt1-MMP) in SM22a-positive vascular-associated cells was also found to be necessary for BMP2-induced HO. A Rosa26-LacZ reporter mouse directed by SM22a showed *in vivo* staining of osteocytes, but not osteoblasts, suggesting an additional progenitor pool capable of differentiating into osteocytes.[64] A related suggestion was made in a separate presentation where data were presented indicating that chondrocytes may directly transform into osteocytes,[65] but these data were limited to observations of related cell morphology, and lineage tracing experiments are required for validation.

The bone field will continue to evolve over the coming decades, and, although it is invigorating to witness the application of bone-derived factors in other bodily systems, there is still much to be learned about how bone cells interact with each other and the molecular mechanisms that direct bone formation in both physiological and pathological conditions. The data presented at this year's ASBMR meeting continue the theme of identifying key signaling molecules that orchestrate bone remodeling and contribute to skeletal disease etiology and provide new hope for therapeutic intervention.

## Conflict of Interest

The authors declare no conflict of interest.

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