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NEWS

Osteocyte supply of RANKL meets bone remodeling demand

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A recent *IBMS BoneKEy* webinar built the case that bone's most plentiful cell type has a key role in osteoclast formation

During bone remodeling, osteoclasts first remove old bone during the process of bone resorption, whereas osteoblasts then synthesize new bone during the process of bone formation. For decades, the conventional wisdom in the bone biology field has been that osteoblasts, or osteoblast progenitors, control osteoclastogenesis that is required for the initiation of bone resorption. In *Osteocyte Control of Bone Remodeling* (http://www.nature.com/bonekey/webinars/index.html?key=webinar15), a recent *IBMS BoneKEy* webinar, presenter Dr Charles O'Brien made the case that a different cell type—the osteocyte—controls osteoclast formation during bone remodeling via its production of the cytokine receptor activator of nuclear factor-κB ligand (RANKL). Focusing on research he and his colleagues published last year in *Nature Medicine*,[1] Dr O'Brien, a professor of medicine at the University of Arkansas for Medical Sciences and a VA research scientist at Central Arkansas Veterans Healthcare System in Little Rock, AR, USA, described the path that led them to the osteocyte; paving the way was a series of mouse experiments in which the researchers deleted RANKL in cell populations at different stages of differentiation, including mesenchymal progenitors, chondrocytes, osteoblast precursors, osteoblasts and osteocytes. That work reveals that osteocytes, and not osteoblasts or their progenitors, are indeed the major source of RANKL during bone remodeling. These observations represent a paradigm shift in the way the bone field thinks about bone remodeling, and continues the trend that thrusts the osteocyte, bone's most abundant but underappreciated cell type, from its tomb-like lacunae into the limelight.

A Reasonable Hypothesis with Some Chinks in its Armor

Dr O'Brien began his presentation with some history: the idea that bone-forming osteoblasts, or osteoblast progenitors, control osteoclast formation has a long pedigree, dating back to the early 1980s when Jack Martin and Gideon Rodan posed that very hypothesis.[2] '[They] postulated that osteoblasts control osteoclast formation based primarily on the observation that receptors for osteoclastogenic hormones were found on osteoblastic cells, but not on osteoclast progenitors,' Dr O'Brien explained. Co-culture studies also supported the hypothesis.[3] 'Cell preparations that are rich in osteoblast progenitors, such as calvarial digests, are good at supporting osteoclast formation in co-cultures *in vitro*,' he said.

Over the years, however, some weaknesses to the osteoblast hypothesis began to emerge. For instance, Dr O'Brien pointed to work from Gerard Karsenty and colleagues in the late 90s, before the crucial osteoclastogenic factor RANKL was discovered, showing that transgenic mice lacking osteoblasts exhibited normal bone resorption.[4] Dr O'Brien's own work, undertaken after RANKL was identified, supports a similar conclusion: he and his fellow investigators have found that transgenic mice depleted of osteoblasts have normal osteoclast gene expression, and normal RANKL levels in bone.[5] 'We can create mice in which osteoblast numbers and bone formation are greatly reduced, but consistently this has no impact on RANKL levels, confirming that the matrix-synthesizing osteoblast cannot be the essential source of RANKL,' Dr O'Brien said.

A Transgenic Approach Leads to the Source of RANKL During Bone Growth...

To identify the cell type serving as the chief source of RANKL in bone during different circumstances, such as bone growth and bone remodeling, Dr O'Brien and co-workers created mice in which RANKL can be conditionally deleted from cells at different stages along the pathway via which mesenchymal progenitors ultimately turn into mature chondrocytes, osteoblasts and osteocytes. A Cre recombinase strategy allowed the investigators to accomplish this feat, and they also made use of a reporter mouse, called R26R, to confirm that each particular Cre transgene they employed did in fact act only in specific cell populations of interest.

They turned first to Prx-Cre, a transgene that is expressed in mesenchymal condensations during embryonic development of long bones and the skull, but not the spine; the R26R reporter mice allowed confirmation that Prx-Cre had activity in hypertrophic chondrocytes of the growth plate, as well as osteoblasts and osteocytes of the femur, at 5 weeks of age. Using Prx-Cre in the experimental animals to delete RANKL, Dr O'Brien discovered increased bone mineral density in the femur,

as measured by dual x-ray absorptiometry, as well as increased cancellous bone volume, as measured by microCT, compared with control littermates. Furthermore, the mice exhibited an osteopetrotic phenotype characterized by large amounts of unresorbed calcified cartilage, along with a lack of osteoclasts in the femur as well as a lack of osteoclast-specific gene expression in the tibia. 'These results demonstrate that the RANKL required for osteoclast differentiation in long bones is produced by chondrocytes, osteoblast lineage cells, or perhaps both,' Dr O'Brien told the webinar audience. In addition, 'It's important to note that even though these bones are osteopetrotic, vascular invasion is normal, and there's at least some bone marrow. Therefore, the RANKL produced by lymphocytes or endothelial cells cannot compensate for the loss of RANKL in the mesenchyme-derived cells,' Dr O'Brien explained.

In order to pinpoint the cellular source of RANKL during bone growth, the investigators next used a number of other Cre transgenes to delete RANKL, including osterix 1 (Osx1)-Cre (active in chondrocytes, pre-osteoblasts, osteoblasts and osteocytes); osteocalcin (Ocn)-Cre and collagen X (ColX)-Cre (both active in chondrocytes, osteoblasts and osteocytes); dentin matrix protein 1 (Dmp1)-Cre (active in osteoblasts and osteocytes); and Sost-Cre (active only in osteocytes). When they observed the bones from Osx1-Cre and OCN-Cre mice (5 weeks of age) in which RANKL had been deleted, the researchers observed an osteopetrotic phenotype similar to what they observed with the Prx1-Cre transgene: a large increase in femoral bone volume resulting from a lack of osteoclasts and an inability to resorb calcified cartilage (one interesting difference they did find was that use of Osx1-Cre and OCN-Cre to delete RANKL resulted in a failure of the animals to exhibit tooth eruption, whereas use of Prx1-Cre had no effect on that process).

Meanwhile, mice in which Dmp1-Cre was used to delete RANKL did not exhibit a skeletal phenotype at 5 weeks of age. Immunohistochemical work further confirmed that the Osx1-Cre and Ocn-Cre transgenes, but not the DMP1-Cre transgene, deleted RANKL from hypertrophic chondrocytes. 'These results suggested to us that RANKL expression in hypertrophic chondrocytes drives the formation of the osteoclasts that remove the calcified cartilage during bone growth' Dr O'Brien said. However, both the Osx1-Cre and Ocn-Cre transgenes are active in committed osteoblast progenitors, so Dr O'Brien and colleagues next employed the ColX-Cre transgene that is much less active in those cells. The results—bone phenotypes similar to those observed when using the Prx1-Cre, Osx1-Cre and OCN-Cre transgenes—indicated that the investigators had indeed finally pinned down hypertrophic chondrocytes as the source of RANKL responsible for the resorption of mineralized cartilage during bone growth.

...And During Bone Remodeling

The use of additional Cre transgenes allowed Dr O'Brien to answer another question: which cell type supplied the RANKL needed for cancellous bone remodeling? Deleting RANKL in mice using the Prx1-Cre, Osx1-Cre or Ocn-Cre transgenes could not address that issue, because such animals continue to retain cartilage—they never form cancellous bone at all, even up to 6 months of age. As a result, the investigators looked to the Dmp1-Cre transgene, as use of that particular transgene to delete RANKL produces mice that exhibit normal trabecular architecture at 5 weeks of age, allowing one to then study how the bone phenotype changes over time. After 5 weeks, Dmp1-Cre mice, which lack RANKL in osteocytes, begin to exhibit high bone mass, and increased bone volume, both in the femur and in the spine, and those changes became greater over time, compared with controls.

Furthermore, the measurement of RANKL levels in the animals provided an interesting finding: a 70% decrease in RANKL genomic DNA in femoral cortical bone of 6-month-old mice, but only a small decrease of RANKL mRNA in the spine, and no change in the tibia, when measured in whole bones. 'This reveals that the contribution of osteocyte RANKL to the total amount of RANKL in whole bones is relatively small. Nonetheless—and this for us was the really surprising part—osteoclast surface on cancellous bone was reduced by $>75\%$,' Dr O'Brien said. In addition, although the group observed a significant reduction in bone resorption and formation markers in the circulation, it detected no decrease in soluble RANKL in the circulation. 'This highlights the importance of local concentrations of RANKL, in that this dramatic drop in osteoclast number is not reflected in changes in circulating RANKL levels,' Dr O'Brien said. Overall, the results from Dr O'Brien's team, together with findings along similar lines from Hiroshi Takayanagi and colleagues,[6] implicate osteocyte RANKL as the key contributor to osteoclast formation during cancellous bone remodeling.

Firming Up the Case

Yet, the argument in favor of the osteocyte was not yet cinched, Dr O'Brien explained, because osteoclast numbers, although greatly reduced in the mice when using the Dmp1-Cre transgene, were not entirely eliminated, perhaps because other cells, specifically osteoblast progenitors, were also contributing their share of RANKL. To investigate that possibility, the researchers looked back to the Osx1-Cre transgene, making use of an interesting feature of that specific transgene whereby doxycycline suppresses its expression. Their rationale was that suppressing the deletion of RANKL in hypertrophic chondrocytes in this fashion would allow normal cancellous bone to form. They would use doxycycline *in utero* in mice until the animals reached 4 months of age, at which time growth plate activity very significantly slows, such that any reduction in osteoclastogenesis they observed after that time period (when the transgene is active again) would have to result from the depletion of RANKL in osteoblast lineage cells. Using this method, the team showed that deleting RANKL from osteoblast progenitors had no effect on bone mass, osteoclast number or osteoclast expression. 'At least under basal conditions, it appears that RANKL expressed by committed osteoblast progenitors is not involved in bone remodeling,' Dr O'Brien said.

Yet another possibility still loomed: could bone-lining cells, which are derived from osteoblasts, be another source of RANKL? This seemed feasible as recent work had shown that the DMP-1 Cre transgene likely does result in deletion of RANKL from such cells.[7] To address that issue, Dr O'Brien and collaborators have worked to develop another Cre strain, called Sost-Cre, which is expressed only in osteocytes. Preliminary results using Sost-Cre to delete RANKL in mice show that as the animals age, they exhibit increased bone mineral density, both

in the femur and in the spine, up to 6 months of age, and the increase is comparable to what the group observed using the Dmp1-Cre transgene. 'If we find that osteoclast number is reduced in these animals, and we can verify the lack of Cre activity in lining cells, this should confirm that it is RANKL expression in osteocytes, but not lining cells, that controls osteoclast formation in cancellous bone,' Dr O'Brien said.

Beyond Normal Bone Remodeling

Another set of experiments using the Dmp1-Cre transgene showed that osteocyte RANKL was having a vital role not only in normal bone remodeling, but in pathological bone loss as well. Indeed, mice missing RANKL in osteocytes experienced significantly less spinal bone loss in response to secondary hyperparathyroidism (induced by a calcium-deficient diet) than did their control littermates, and although control animals lost femoral cortical bone, the experimental animals did not. In addition, circulating parathyroid hormone levels were much higher in the RANKL knockout animals than in controls, indicative of a difficulty in retrieving calcium from the skeleton in the former.

A different set of experiments next revealed a role for osteocyte RANKL in another situation of pathological bone loss: that resulting from unloading. In this instance, although control animals lost bone mass at the femur, and exhibited increased trabecular spacing and decreased cortical thickness after unloading, mice missing RANKL in osteocytes were protected from such changes (neither controls nor experimental animals exhibited a significant loss of cancellous bone volume). In addition, the osteocyte RANKL-deficient animals failed to exhibit the increase in RANKL mRNA, and in osteoclast number, seen in the cancellous bone of the control animals.

The results indicate that osteocyte RANKL contributes to the bone loss from unloading, but how unloading stimulates the expression of RANKL remains uncertain. Dr O'Brien noted that both loading and overloading stimulate osteocyte apoptosis as well as increased bone resorption, suggesting that osteocyte cell death may an important factor. Yet, as osteocytes are a source of RANKL, if those cells are dying, how can they also serve at the same time as the source? Dr O'Brien pointed to work published earlier this year from Mitch Schaffler and colleagues,[8] who studied overloading-induced fatigue damage in the rat ulna, suggesting one possible way out of that conundrum: some osteocyte populations may undergo apoptosis, whereas other populations of osteocytes surrounding the apoptotic ones may increase their expression of RANKL. 'This suggests that dying osteocytes may signal to neighboring healthy osteocytes to control bone resorption,' Dr O'Brien explained.

Although RANKL supplied by the osteocyte, then, has a key role during both normal and pathological skeletal processes, the spotlight on this historically neglected cell may continue only to grow brighter. In fact, Dr O'Brien concluded by noting that osteocytes not only provide RANKL, but they also produce sclerostin, a natural inhibitor of bone formation. 'Together these findings suggest that osteocytes have the ability to independently control both the rate of bone remodeling—via RANKL—and the magnitude of bone formation within bone remodeling units via sclerostin. This may allow osteocytes to tailor the response of the remodeling apparatus to meet specific needs,' he said. Only future research will tell...

Conflict of Interest

The author declares no conflict of interest.

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