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## *PERSPECTIVES*

# Choreography from the Tomb: An Emerging Role of Dying Osteocytes in the Purposeful, and Perhaps Not So Purposeful, Targeting of Bone Remodeling

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At any moment throughout life, from one to several millions of discrete sites in the human skeleton are undergoing a periodic turnover that lasts 6-9 months, resulting in the replacement, at each site, of a small packet (approximately 0.025 $mm^3$) of old bone with new. In healthy adults, this process is responsible for the regeneration of the skeleton at the remarkable rate of 100% every 10 years. The continual regeneration of a tissue as big, complex and hard as bone is accomplished by temporary cellular structures comprising teams of juxtaposed osteoclasts and osteoblasts that arise from the bone marrow. Elucidation of the bone regeneration process, and the origin, differentiation, function and fate of the specialized cells that accomplish it, have preoccupied bone biologists for over 50 years and form the most fundamental tenets of our current understanding of skeletal health and disease (1).

Nonetheless, the purpose of remodeling and how and why one particular skeletal site is selected at a given point of time over another has remained very poorly understood, if at all. It turns out that this poor understanding may reflect an even bigger gap in our knowledge about bone; namely, the purpose and function of osteocytes, which are the most abundant and evenly distributed, longest-lived, and best connected bone cell type that resides within the mineralized matrix. Ironically, recent insights into the death of osteocytes by apoptosis provide some of the most critical clues for how these cells function while alive. In this perspective, I will briefly review the emerging evidence for a critical role played by dying osteocytes, both in the homeostatic adaptation of bone to mechanical forces, and in the inappropriate remodeling that leads to the mechanical compromise and failure that underlie fractures in pathologic conditions.

### Numerous, Strategically Distributed, and Well-Connected Permanent Residents of Bone

Osteocytes are former osteoblasts entombed individually in lacunae of the mineralized matrix. However, unlike osteoclasts and osteoblasts which are relatively short-lived and transiently present only on a small fraction of the bone surface, osteocytes are long-lived and present throughout the skeleton. Moreover, osteocytes are far more abundant than either osteoclasts (1000 times) or osteoblasts (10 times). More importantly, each osteocyte has, on average, 50 cytoplasmic dendritic processes that radiate from the cell body— a striking morphologic feature reminiscent of neuronal cells (2). The dendritic processes run, like buried cable, along narrow canaliculi and are linked by gap junctions with the processes of neighboring osteocytes, as well as with cells present on the bone surface. These cells include the lining cells which cover quiescent surfaces, cellular elements of the bone marrow, and the endothelial cells of the bone marrow vasculature.

It has been well recognized for over a century that mechanical loading is critical for the maintenance of bone mass and that weightlessness, as with reduced physical

activity in old age, prolonged bed rest, or space flight, invariably leads to bone loss (3-6). Indeed, the concept that the skeleton adapts to meet mechanical needs was first recognized by Wolff in 1892 (7) and later expanded by Frost (8). Bone adjusts to load by changing in mass, shape, or microarchitecture (9;10), and it responds differently depending on the magnitude of the strain. Whereas levels of strain that are too high or too low induce bone loss, physiological levels of strain maintain bone mass (11). The cellular and molecular mechanisms responsible for these phenomena have remained largely unknown. Nonetheless, the strategic display of osteocytes throughout bone, and the unique ability of these cells to form a communicating syncytium extending from the mineralized bone matrix to the cellular elements on the surface of bone and the bone marrow and all the way to the blood vessels, has made these cells the most logical candidates for sensing and responding to mechanical strains (10).

## The Death of Osteocytes by Apoptosis: Influences of Hormonal and Mechanical Signals

Although osteocytes are long-lived cells, it is now clear that, similar to osteoblasts and osteoclasts, they eventually die by apoptosis. Surprisingly, the way Mother Nature usually manifests its design to mortals, the disruption of the integrity of the osteocyte network that results from apoptosis seems to serve as a quintessential signaling mechanism for bone's ability to self-repair and perhaps also to adapt to mechanical strains, in a spatially defined pattern.

The initial clues that osteocyte apoptosis influences the mechanosensory function of the osteocyte network and the mechanical competence of the skeleton were provided from several discoveries by our group and others that the increased bone fragility resulting from glucocorticoid excess or estrogen or androgen deficiency in animals and humans is indeed associated with an increased prevalence of osteocyte apoptosis (12-14). Conversely, bisphosphonates, intermittent parathyroid hormone (PTH) administration, and sex steroids were all found to prevent osteocyte apoptosis, raising the possibility that preservation of the integrity of the osteocyte network contributes to the anti-fracture efficacy of these agents (13;15;16). More to the point, studies led by O'Brien and Weinstein in our Center have provided, for the first time, compelling evidence that osteocyte viability is an independent determinant of bone strength. Indeed, it was found that blockade of glucocorticoid action on osteoblasts and osteocytes in mice by overexpression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), under the control of the murine osteocalcin promoter (OG2), preserved bone strength in spite of loss of bone mass (17).

## Survival is a Constant Strain

Extensive evidence has emerged in the last few years showing that mechanical stimuli regulate the life span of osteocytes. Indeed, studies conducted by Noble and colleagues have shown that physiological levels of load imposed on bone *in vivo* appear to decrease the number of apoptotic osteocytes (18). On the other hand, lack of mechanical stimulation induced by unloading of bone is associated with an increased number of hypoxic osteocytes. This effect is reversed by loading, suggesting that mechanical forces facilitate oxygen diffusion and osteocyte survival (19). Furthermore, studies have begun to elucidate how mechanical forces are transduced into biochemical signals that control the life-span of osteocytes. Evidently, osteocytes interact with the extracellular matrix (ECM) in the pericellular space through discrete sites in their membranes, which are enriched in integrins and vinculin (20;21), as well as through transverse elements that tether osteocytes to the canalicular wall, as elegantly demonstrated by Schaffler and co-workers (22). Thereby, fluid movement in the canaliculi resulting from mechanical loading might induce ECM deformation, shear stress, and/or tension in the tethering elements. The resulting changes in circumferential strain in osteocyte membranes might be converted into intracellular signals by integrin clustering and integrin interaction with cytoskeletal and

catalytic proteins at focal adhesions (23;24). Work led by Teresita Bellido in our Center has demonstrated that mechanical forces transduce signals through integrins and a signalsome comprising actin filaments, microtubules, the focal adhesion kinase FAK, and Src kinases, resulting in activation of the ERK pathway and attenuation of osteocyte apoptosis (25). Similarly, Bakker and colleagues have found that physiological levels of mechanical strain imparted by pulsatile fluid flow prevent apoptosis of cultured osteocytes (26).

## Osteocytes as Beacons of Targeted Remodeling

Prompted by *in vitro* evidence that physiologic levels of mechanical strain prevent apoptosis of osteocytic cells, *in vivo* studies by the Bellido laboratory have more recently examined whether, conversely, reduced mechanical forces increase the prevalence of osteocyte apoptosis (27). It was found that within 3 days of tail suspension, Swiss Webster mice exhibit an increased incidence of osteocyte apoptosis in both trabecular and cortical bone. This change was followed 2 weeks later by increased osteoclast numbers and cortical porosity, reduced trabecular and cortical width, and decreased spinal bone mineral density and vertebral strength. Importantly, whereas in ambulatory animals apoptotic osteocytes were randomly distributed, in unloaded mice apoptotic osteocytes were preferentially sequestered in endosteal cortical bone, the site that was subsequently resorbed. The effect of unloading on osteocyte apoptosis and bone resorption was reproduced in our OG2-11βHSD2 transgenic mice in which osteocytes are refractory to glucocorticoid action, indicating that stress and hypercortisolemia cannot account for these effects. The conclusion from these studies was that diminished mechanical forces eliminate signals that maintain osteocyte viability, thereby leading to apoptosis. Dying osteocytes in turn become the beacons for osteoclast recruitment to the vicinity and the resulting increase in bone resorption and bone loss. Intriguingly, we have found that a ligand-independent function of the estrogen receptor is indispensable for mechanically-induced ERK activation in both osteoblastic and osteocytic cells (28;29). This observation is consistent with reports from Lance Lanyon's group in London that mice lacking the estrogen receptors α and β exhibit poor osteogenic response to loading (30;31).

At present, the precise mechanism(s) by which reduced mechanical forces trigger osteocyte apoptosis have not been elucidated. However, a deficit in nitric oxide (NO) production has been proposed by Burger and colleagues as a potential culprit (32). Consistent with this hypothesis, mechanical stimulation increases the production of NO by osteocytes (33-35). Mechanical stimulation of chicken and canine bone also increases the production of prostaglandin E2 (PGE2) (36;37), an agent with known antiapoptotic properties (38), raising the possibility that prostaglandins and perhaps other autocrine/paracrine soluble factors are also involved in the maintenance of osteocyte viability. Signals for osteocyte survival may also be provided by the ECM itself. In agreement with this view, loss of survival signals from the ECM causes osteoblastic cell apoptosis or "anoikis" (39), and neutralizing antibodies to the ECM protein fibronectin induce osteoblast apoptosis (40). In addition, there is an increase in the prevalence of osteocyte and osteoblast apoptosis in transgenic mice expressing collagenase-resistant collagen type-I (41). Collectively, these lines of evidence suggest that exposure of cryptic sites of ECM proteins by matrix metalloproteinases is required for the maintenance of cell-ECM interactions that result in "outside-in" integrin signaling that preserves osteocyte (and osteoblast) viability. This scenario is consistent with our own finding that physiological levels of mechanical strain promote survival of osteocytic cells via a mechanism mediated by integrins (25).

## Osteocyte-derived Sclerostin and the Control of Bone Formation

Osteocytes have the unique ability, among other cells of the osteoblastic lineage, to express sclerostin, a product of the *SOST* gene that is a potent antagonist of BMP-2, -

4, -5, -6 and -7 (42-44). Sclerostin also blocks canonical Wnt signaling by binding to the Wnt receptor LRP5/LRP6 (45). Both BMPs and Wnts are, of course, critical for osteoblastogenesis as they provide the initial and essential stimuli for the commitment of multipotential mesenchymal progenitors to the osteoblastic lineage (46;47). Importantly, loss of *SOST* in humans causes the high bone mass disorders van Buchem's disease (OMIM 239100) (48) and sclerosteosis (OMIM 269500) (49), and an anti-sclerostin antibody increases bone formation in mice (50). Conversely, transgenic mice overexpressing *SOST* exhibit low bone mass (42). These observations strongly suggest that sclerostin derived from osteocytes, the ultimate progeny of the osteoblast differentiation pathway, exerts a negative feedback control at the earliest step of mesenchymal stem cell differentiation toward the osteoblast lineage (42;43).

We have found that chronic elevation of PTH in mice dramatically decreases *SOST* expression in osteocytes and that this effect results from a direct action of the hormone on this cell type (51), a finding consistent with the presence of PTH receptors in osteocytes (52). Moreover, we demonstrated the presence of sclerostin in the canalicular system, strongly indicating that this protein may function as a paracrine factor. Based on these findings, we have proposed that suppression of *SOST*/sclerostin by PTH represents a novel mechanism for hormonal control of osteoblastogenesis and that, in chronic hyperparathyroidism, the increased production of osteoblasts needed for increased bone turnover may result from such an indirect effect of PTH at the earliest stage of osteoblastogenesis.

## Aging, Osteocyte Apoptosis, and Increased Fracture Risk

Age is a far more critical determinant of fracture risk than bone mineral density (BMD) in humans. Heretofore, fracture risk was thought to be the result of changes in balance, visual acuity, and muscle mass, rather than changes in the strength of bone itself. Albeit, there is evidence that in aging bones, there is an accumulation of microdamage (53) and a decline in osteocyte density (54;55) accompanied by decreased prevalence of osteocyte-occupied lacunae (56), an index of premature osteocyte death. Furthermore, osteocyte death is associated with hip fractures in aging humans (57) and with glucocorticoid-induced osteonecrosis of the hip (58). In unpublished studies from our group (59), we have established that bone strength in the spine and hind limbs is significantly decreased in 16-month-old female C57BL/6 mice, compared to 8-month-old animals, in the absence of a detectable decrease in BMD, and that this was associated with an increased prevalence of osteocyte apoptosis. We have also found that C57BL/6 mice exhibit several additional, very intriguing age-related changes that may provide clues into the mechanisms of the age-related decline of bone strength and mass. These changes include an increase in osteoblast apoptosis, a decrease in glutathione reductase activity, a corresponding increase in the levels of reactive oxygen species (ROS) in the bone marrow, and an increase in the phosphorylation of p66shc in vertebrae, an adapter protein that is induced by oxidative stress and is an important determinant of lifespan in mammals. On the basis of this evidence, we are hypothesizing that diminished mechanical forces from reduced physical activity with aging decrease signals that maintain osteocyte viability, thereby leading to apoptosis. Dying osteocytes, in turn, recruit osteoclasts to the vicinity. Both mechanisms, disruption of the integrity of the osteocyte network and increased bone resorption, contribute to the age-related decline in bone strength.

## Concluding Thoughts

There is considerable evidence to suggest that hormonal changes, microdamage (i.e. small cracks of the mineral that disrupt the anatomical integrity of lacunae and/or canaliculi), decreased mechanical forces (unloading), and perhaps the aging process itself cause osteocyte apoptosis by severing pro-survival signals that are essential for protecting osteocytes from apoptosis and,

thereby, essential for the maintenance of an intact osteocyte network. Sex steroids prevent osteocyte apoptosis via a kinase-initiated mechanism of action that results from an extranuclear action of the classical estrogen or androgen receptors (most likely localized in caveolae), which causes activation of the cytoplasmic kinases Src and ERKs, and kinase-dependent changes in the activity of transcription factors. Intriguingly, mechanical stimuli also sustain osteocyte survival via a signalsome assembled in caveolae and comprising integrins, the focal adhesion kinase FAK, the cytoplasmic kinases Src and ERKs, as well as the estrogen receptor α and β. Remarkably, in contrast to the well-known role of estrogen receptors as ligand-dependent mediators of the effects of estrogens on reproductive and non-reproductive organs, this novel function in mechanotransduction is ligand-independent. The sharing of at least some pro-survival signaling molecules raises the possibility that hormones and mechanical forces may interact for optimal skeletal health, but this notion has not been tested directly.

Death of osteocytes by apoptosis delivers signals that can initiate microdamage repair by focal remodeling, but can also trigger bone gain or bone loss to restore local strain to the desirable range (18;60;61), by producing molecules that modulate osteoclast or osteoblast formation (42;62). Indeed, the existing evidence fully supports the notion that osteocyte apoptosis unleashes signals capable of recruiting both osteoclasts and osteoblasts to their vicinities by stimulating osteoblastogenesis and osteoclastogenesis in a spatially restricted manner. To the best of my knowledge, sclerostin, the product of the *SOST* gene, represents the first paradigm of an osteocyte-specific paracrine factor that may affect osteoblastogenesis in response to osteocyte apoptosis, by eliminating a suppressive signal on osteoblastogenesis. The nature of the osteoclastogenic signals generated by the apoptotic osteocytes are, at this stage, only a matter of conjecture. Nonetheless, it is worth noting that disuse and oxygen deprivation promote osteocyte apoptosis and also enhance the expression of the hypoxia-dependent transcription factor HIF-1α, which, itself, works in concert with the tumor suppressor protein p53 to mediate apoptosis (63), and is also a potent inducer of the angiogenic and osteoclastogenic factor VEGF (64;65).

Although the evidence I reviewed in this *Perspective* suggests that apoptotic osteocytes can stimulate both osteoblastogenesis and osteoclastogenesis, it is currently unknown what determines whether these two effects are spatially coordinated, as it will be required for the purpose of remodeling during micro-damage repair, or are spatially independent, as it will be required in order to restore strain to a desirable level, such as during the process of modeling. I suspect that many other inputs, such as the factors associated with bone growth and modeling, may largely impact the outcome.

It is also unknown, at this stage, what determines whether the response to the signals initiated by the dying osteocytes is appropriate, purposeful, and advantageous at the organismal level, as for example in the case of a micro-damage repair, or inappropriate, unpurposeful, and disadvantageous to the organism, as it happens with sex steroid deficiency or unloading. The former would, of course, maintain mechanical strength, while the latter, at least in the short-term, would lead to mechanical compromise and failure, the main underlying feature of fractures in pathologic conditions. I dare to speculate that a key determinant of whether bone undergoes a purposeful physiologic adaptation or unpurposeful and inappropriate remodeling may be the magnitude of the event, i.e., the prevalence of osteocyte apoptosis at a given point in time, most likely in combination with the extent of the anatomic distribution of the apoptotic osteocytes over a particular site, single bone, or the skeleton at large. If this turns out to be the case, both physiologic and pathologic remodeling may be, after all, targeted rather than stochastic, regardless of whether osteocyte apoptosis serves as a homeostatic or pathogenetic signaling mechanism.

Can it be that all osteocytes do is waiting to die in order to deliver a signal? I seriously doubt it. I look, therefore, with excitement to future advances in our understanding of the biology of the osteocyte mechanosensory network, a network seemingly as complex and extensive as that of the nervous system, yet, buried in its own tomb—perhaps our only excuse for ignoring it so long. The era of the osteocyte, mechanical signaling, and the qualitative, as opposed to quantitative, determinants of bone strength has dawned on our field with vengeance. We better get ready for big surprises and learning new tricks, hard as this may be for some old dogs.

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